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# Effect of Different Formulations of Polyphenolic Compounds Obtained from OMWW on the Growth of Several Fungal Plant and Food Borne Pathogens. Studies *in vitro* and *in vivo*.

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**Abstract**

The assessment of different concentrations and forms of polyphenols (free and encapsulated) from olive mill waste (OMWW) as plant protection materials against economically important plant pathogenic fungi in *in vitro* and *in vivo* tests is the main research area of this work. In the first stage of the experimental process, it was assessed the zone of inhibition in mm of several fungal pathogens using the methods of disk diffusion assay, and well diffusion assay. In the second stage of the evaluation, the effect of polyphenols against 14 fungal microorganisms was examined in order to determine the MIC / microbicidal concentration (MIC / MFC). In a later stage *in vivo* evaluation of liquid polyphenols (LFP) obtained from OMWW as natural bio-chemicals against several fungal pathogens on tomato plants was carried. The evaluation of the results obtained by determining the MIC and MLC, demonstrated that the fungus *Aspergillus flavus* appeared highly resistant to the LFP concentration required minimum sample rate > 35-40 % for inhibition and killing effects respectively. In order of major protection resulting from the use of polyphenolic compound against major diseases, *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Ascochyta lentis* gave the most promising results. Moreover, the use of low concentration of LFP at 5 and 10 % could control in some cases fungal pathogens. However, higher concentration of LFP (20 and 30%) appeared possible phytotoxic effects.



*Keywords:* Polyphenols, Biocontrol, Plant diseases, OMWW.

## 1. Introduction

Olive mill waste water (OMWW) is the result of the production of olive oil. It has been calculated that a medium-sized mill produces about 1.000 tn waste per harvest olives with organic load equivalent to the annual waste of a city of 30,000 inhabitants. The components of "OMWW", of particular interest are the phenols, which as antioxidants prevent the degradation of fatty acids to glycerides and help maintain the olive oil [1, 2]. However, it is the main pollutant parameter, which is responsible for the severe environmental impact of wastewater mills. By the term polyphenols characterized a large heterogeneous group of compounds with a common feature that carry one or more hydroxyl groups bonded directly to one or more aromatic or heterocyclic nuclei. So far, are known most of 8000

polyphenols and more than 4000 different phenolic compounds in plant tissues [3]. The concentration of polyphenols in the olive oil ranges from 50 to 1000 mg / g oil, depending on the variety of olives and the extraction technique. However, according to Rodis et al. [4] the total amount of the antioxidants in olive oil is only 1-2% while the rest of them are lost as waste (about 53%). Low molecular weight phenolic compounds, appears to be the main determinants of the anti-microbial action of the smashed olive core [5, 6], while high molecular mass polyphenols, organic acids, lipids, oligosaccharides and glycoproteins can contribute to the OMWW phytotoxic effects when applied in high concentrations in plants [7]. Nowadays, although they have been made several attempts to solve the environmental problem of disposal of OMWW, their elimination remains one of the main environmental problems associated with the production of olive oil in Mediterranean countries, where Greece, Spain and Italy are the major producers [8].

### *1.1 Olive mill wastes as ecological phytoprotective products*

Plants affected by many pests and pathogenic microorganisms. For their protection they produce natural metabolites which influence these pests and microorganisms. Many phenolic compounds, free fatty acids and aromatic compounds have been detected to consist as waste in the production process of olive oil [9, 10] and linked phytotoxic and antimicrobial properties inhibited microbial growth [11, 10, 12, 13]. More specific, studies done by Xia et al. [14] showed that plant polyphenols can be used as natural plant protection agent exhibiting antimicrobial activity to control infestations of bacteria [15, 16], fungi [17] and viruses [18]. Also, it is known that OMWW can be used also as herbicides since in high concentrations have phytotoxic effects [19].

Also, OMWW act as phytoprotective compound on fruits and vegetables during the growing season and after harvest during storage offering a promising solution for preventing losses of fruits and vegetables from post-harvest attacks such as those from the fungus *Botrytis cinerea*. Similar results have been reported by Bonanomi et al. [20] where the use of OMWW can affect the growth of saprophytic fungi, the incidence of foliar diseases, and the growth of soil-borne pathogens before and after harvest.

Thus, in recent years distinguished growing interest in isolation, testing and utilization of agricultural waste or other sources of plant tissues rich in polyphenols, such as tissues of species *Olea europaea*, *Prunus amygdalus*, *Stevia rebaudiana*, etc., and their waste, such as OMWW has been arised. Therefore, the use of these by-products which are deal as wastes may offer new products with high added value, reducing also environmental risks and pollution levels if they were discharged into the environment.

Moreover, the excessive use of synthetic pesticides and fungicides in modern agriculture has not only led to the development of resistant strains of pathogens, but also affect the environment and human health remained as toxic residues in crops and soil. Therefore, alternative, environmentally friendly methods, of combating pests and diseases of plants are required [21] to reduce the impact of specialty chemicals to human health and the environment.

### *1.2 Purpose of the research work*

Recently, the interest in the effects of polyphenols from OMWW against important phytopathogenic microorganisms, especially fungi, has been arised. The assessment of different concentrations and forms of polyphenols (free and encapsulated) as plant protection materials against economically important plant pathogenic fungi in *in vitro* tests is the main research area of this scientific work. In the first stage of the experimental process, it was assessed the zone of inhibition in mm of several fungal pathogens using the methods of disk diffusion assay, and well diffusion assay. In the second stage of the evaluation, the effect of polyphenols against 14 fungal microorganisms was examined in order to determine the MIC / microbicidal concentration (MIC / MFC).

In a later stage *in vivo* evaluation of liquid polyphenols obtained from OMWW as natural bio-chemicals against several fungal pathogens on tomato plants was carried. For the evaluation of the antimicrobial activity of the examined polyphenolic compound against ten (10) fungal pathogens, different concentrations of liquid formed polyphenol (LFP) at 5, 10, 20, and 30% were applied in 40 days old tomato plants, variety Majeo S1.

## 2. Materials and Methods

### *2.1 In vitro evaluation of polyphenolic compounds*

To determine the antimicrobial activity of OMWW polyphenols against fungal phytopathogenic species were followed the techniques of *Well diffusion assay*, *paper disk diffusion assay* and the rate of spread of the mycelium in

Petri dishes with purpose to determine the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC). Before the application of the above described methods, a preliminary test examined the inhibition zone and the number of colonies in medium agar.

Each individual method aims to study the antimicrobial activity of polyphenols obtained from OMWW against plant pathogens. From the results of these methods applied, was observed the potency of different forms and concentrations of the examined polyphenols forms (encapsulated, liquid) against important phytopathogenic fungi. It was also evaluated and determined the major effect in inhibiting the mycelium growth and the fungicidal concentration of this polyphenol's formulation.

However, these methods differ in their results and in terms of how they are implemented well. In the method of measuring zones of inhibition, the concentrations of formulated polyphenols that they were studied were 10%, 20 % and 30% while in the method of determining the MIC / MFC the studied concentrations of polyphenolic compounds were 3%, 5 %, 10 %, 15 %, 20 %, 25 %, 30 %, 35 %, 40 % and 50 %.

The majority of fungal microorganisms used in this study were obtained from the Benaki Phytopathological Institute (B.F.I.) in the medium PDA in Petri dish, while *Aspergillus fluvus* was obtained from microbiology laboratory of Annex Food Technology, of Technological Educational Institute of Thessaly, Karditsa Branch. Parts of hyphae of plant pathogens from the initial medium were placed in test tubes containing nutrient medium agar (PDA) and stored in the refrigerator at 3 C to be preserved for longer time. Conducting experiments to evaluate the efficacy of OMWW polyphenols treatment was conducted using sections and spores of this stored material. For the development of phytopathogenic fungi used nutrient substrate and MRD PDA. The forms of polyphenol derived from OMWW were a) Liquid polyphenol substance (LFP), b) polyphenols encapsulated in protein, c) polyphenols encapsulated in maltodextrin, d) polyphenols encapsulated in protein and maltodextrin.

*2.1.1 Disk diffusion and well diffusion assays*

These two methods were applied to compare the antimicrobial activity of various forms of encapsulation and concentration of the polyphenol substances obtained from OMWW against 20 phytopathogenic fungi. Three concentrations (10%, 20% and 30%) of each type of formulation (encapsulated or not) of olive mill wastes was tested. The treatments were a) liquid – polyphenol at 10, 20 and 30% of the initial volume, b) polyphenols encapsulated in protein at 10 and 20 % of the initial volume, c) polyphenols encapsulated in maltodextrin at 10 % of the initial volume, d) polyphenols encapsulated in a mixture of maltodextrin and protein at concentration of 10 and 20 % of the initial volume and e) control (no polyphenols applied). The treated petri dishes were incubated at 28 $^{o}$C for up to 7 days. The number of replicates per treatment was 3 and the experiments were repeated twice. Measurements were taken on the last day of incubation ($7^{th}$) by means of zones of inhibition in mm which was formed around the area of the applied immersed paper disk.

In the method of the well diffusion assay after stabilization of the PDA nutrient agar the medium surface was coated with 0,5 ml of spore and mycelium suspension of each of the twenty (20) fungal pathogens. Then, a small well in the center of agar in petri dish was created. This well was then poured with 10 μl of each examined polyphenols' treatment. After incubation at 28 $^{o}$C for up to 7 days zone of inhibition around the area of the well was measured.

*2.1.2 Mycelium growth in PDA medium*

To examine the activity of different forms of encapsulated OMWW polyphenols (protein, maltodextrin, protein+ maltodextrin and liquid polyphenol) three concentrations of 10, 20 and 30% of the solutions mixed with nutrient substrate (PDA) were used. The treatments were a) liquid – polyphenol at 10, 20 and 30% of the original volume, b) polyphenols encapsulated in protein at 10 and 20 % of the initial volume, c) polyphenols encapsulated in maltodextrin at 10 % of the initial volume, d) polyphenols encapsulated in a mixture of maltodextrin and protein at concentration of 10 and 20 % of the initial volume and e) control (no polyphenols applied).

After stabilization of PDA, sterilized paper discs of 5 mm in diameter were located in the middle of the petri dish. The sterilized paper disks were previously dipped in hyphae and spore suspension of the examined phytopathogenic fungi for about one minute. The treated petri dishes then incubated at 27 $^{o}$C for up to 16 days. Mycelium growth measurements were taken after 2, 4, 8, 13 and 16 days from the time of infection. The number of replicates per treatment was 3 and the experiment was repeated twice.

*2.1.3 Determination of MIC / MFC*

Sterile test glass tubes containing 10 ml of Malt Extract (ME) were heated at 60 $^{o}$C until the medium agar starts to dissolve. Then in the dissolved substrate volume of liquid-form polyphenolic (LFP) compound was added as shown in Table 1.

Table 1. Volume of LFP used in ml for the creation of the different concentrations

| 0% | 1% | 5% | 10% | 20% | 30% | 40% | 50% |
|---|---|---|---|---|---|---|---|
| Control | 0,05 ml (LFP) | 0,26 ml (LFP) | 0,55 ml (LFP) | 1,25 ml (LFP) | 2,14 ml (LFP) | 3,33 ml (LFP) | 5 ml (LFP) |

Then, the tubes were shaken in vortex in order to homogenize the LFP solution with the dissolved ME. Each tube then was inoculated with a streak of the plant pathogenic fungus and was incubated at 27 $^{o}$C for 24-48 hours in the incubator. By the end of the time of incubation the development of a mycelium in the surface or in the bottom of the tubes was recorded.

The test tubes which had developed mycelium of the pathogenic fungi showed that the concentration-quantity of LFP was not sufficient to inhibit the growth of the microorganism, for this reason the sample was characterized as positive. However, tubes which did not appear any mycelial growth, they were determined as "negative". To determine whether these amounts of LFP were capable to inhibit mycelium growth or to cause lethal effect, a further experiment was conducted. In glass tubes containing 5ml of MRD, 0,1 ml of the sample in the test tube in which there had not developed any fungus mycelium was added. The amount of each test tube was stirred well at Vortex and incubated at 27 °C for 48 hours. After incubation time it was observed the mycelium growth in the tube as sediment or/and turbidity. If there were any of these characteristics it was agreed that the tested concentration-quantity of the LFP was only inhibit the growth of the fungus without killing it. In contrast, it the test tubes which no growth or sediment and/or turbidity was recorded, the amount of LFP concentration applied appointed as the lethal one.

*2.2 In vivo* evaluation of LFP

For the *in vivo* evaluation of the effectiveness of LFP against 10 fungal pathogens of tomato plants and the determination of the possible phytotoxic effect on their growth the following techniques were applied.

*2.2.1 Preparation of the fungal pathogens*

The microorganisms used in this scientific work grown on medium PDA and were obtained from the Benaki Phytopathological Institute (B.F.I.). However, *Aspergillus fluvus* was obtained from Department of Food Production of TEI of Thessaly, laboratory of food microbiology. Parts of mycelium hyphae and spores were washed-off from the initial medium with sterile water and placed in a sterile glass test tube. In order to ensure that the final inoculum contains similar number of spores an haemocytometer was used for the determination of the number of spores. A dilution applied where was necessary. The inoculum of each pathogen was placed in the refrigerator at 3 °C for about a week before conducting the *in vivo* experiments.

*2.2.2 Preparation of plants-inoculums application*

As mentioned above tomato hybrids 40 days old, variety Majeo S1 were used to ascertain the effectiveness of the antimicrobial activity of different concentrations of LFP against 10 important fungal pathogens in tomato plants. The possible phytotoxic effects of the LFP were also evaluated. The plants were transplanted in disposable plastic pots which were filled with a mixture of high peat Potgrad P, suitable for propagation of horticultural seedling obtained from Company Klassman-Dolmann GmbH Germany as imported in Greek market from AGROCHOUM SA. Then, the transplanted plants were watered in order to avoid dryness. The application of fungal suspension and different concentrations of LFP were applied after two days of transplanting. A hole, near the root system was made in order to apply 1ml of spore suspension for the plant infection. Similar amount (1ml) of LFP was also applied in order to evaluate the effectiveness of LFP in plant growth and fungus action. Then, the plants were placed in the greenhouse and were irrigated daily with automatic flushing for 30 minutes. Because of its short photoperiod special artificial lighting lamps were used extending daytime for 4 hours. Five plants were used in each treatment. After 40 days of application, plants were harvested and dry plant weight and dry root weight were evaluated. For the

determination of dry plant weigh and dry root weigh the examined plants were placed on paper bags and drying in a drying oven at 60 °C for 24 hours.

## 3. Results

### *3.1 Results in vitro*

In the experiment using the method of disk diffusion assay the liquid formed polyphenol in a concentration 30% of the initial one significantly reduced the growth of phytopathogenic fungi examined, creating zones of inhibition around the disk-paper that was previously immersed in the LFP. In contrast, no other form of encapsulated polyphenol showed encouraging results probably because of the slow release of them in the nutrient medium (PDA). It was also reported that the zone of inhibition was greater in fungi such as *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Ascochyta lentis* while more limited action appeared in fungi *Armillaria mellea*, *Alternaria alternata*, *Eutypa lata*, *Fusarium oxysporum* and *Verticillium dahliae*. The possible reasons of the limited inhibition zone were generating in the morphological characteristics of the pathogen (e.g. rhizomorphs etc.) the rapid spread of the mycelium, the production of numerous conidia and the production of mycotoxins. In the case of the fungus *Aspergillus flavus* it was recorded zone of inhibition of growth only at high concentration of LFP. However, it is important to test the effectiveness of various types of formed polyphenols of OMWW *in vivo* in various plants since the use of high concentration of polyphenolic compounds (30%) it is likely to affect plant growth, thereby causing phytotoxicity .

In the experiment using the method of well diffusion assay the liquid formed polyphenol in the 30% concentration of the initial one, significantly reduced the growth of the examined phytopathogenic fungi, creating zones of inhibition around the treated medium growth. However, the inhibition zones using this method were more extended than those observed with the method of disk diffusion assay, probably because of the easier diffusion of polyphenol in the nutrient agar. The use of LFP in 30% concentration cause zones of inhibition in all examined fungi. However, the zone of inhibition was more efficient in fungal species such as *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Ascochyta lentis.* The inhibition zone was also significant in case of fungus such as *Cercospora beticola* and the two species of *Penicillium*, the *P. expansum* and the *P. itallicum*. Contrarily, the less efficient zone of inhibition with this method was recorded in the treatments with *Armillaria mellea*, *Monillia laxa*, *Monillia fructigena* and *Phytopthora nicotiana*. It is worthily to be mentioned that the fungus *Aspergillus flavus* showed inhibition zone only in high concentration of LFP (30 %).

Also, important observation in this experimental work was the creation of zones of inhibition in a smaller concentration of LFP (20 and 10%). However, there was no appearance of zones of inhibition using other encapsulated forms of polyphenols such as protein, maltodextrin or combination of both, except the cases of *Botrytis cinerea* and *Gauemanomyces graminis* where small zones of inhibition were appeared while the fungi were treated with polyphenols encapsulated in a mixture of protein and maltodextrin.

From the results obtained for the mycelium growth in nutrient agar (PDA) it was demonstrated that the use of LFP inhibited the growth of several important plant pathogenic fungi (Table 1). More specific, mycelium of fungal species of *Ascochyta lentis*, *Verticillium dahliae*, Sclerotinia sclerotiorum, *Botrytis cinerea*, *Pythium ultimum*, *Aspergillus flavus*, *Rhizoctonia solani*, and *Alternaria alternata* was not developed when the medium agar contained LFP at concentration of 30%. However, the mycelium of fungal pathogens such as *Monilia laxa*, *Gaeumanomyces graminis*, *Armillaria mellea*, *Penicillium italicum*, *Aspergillus niger*, *Cercospora beticola*, *Eutypa lata*, *Phytopthora nicotiana*, *Monillia fructigena* and *Fusarium oxysporum* was developed even at the concentration of LFP of 30%. In the other forms of encapsulated polyphenols of OMWW the mycelium of the examined phytopathogenic fungi was not affected at all in comparison with that of the control.

Table 2. Effect of different forms of polyphenols obtained from OMWW on several plant and food borne pathogens after 16 days of incubation at 27 °C.

| | LFP | | | Encapsulated polyphenols in protein | | Encapsulated polyphenol in maltodextrin | Encapsulated polyphenol in protein + maltodextrin | | Control |
|---|---|---|---|---|---|---|---|---|---|
| *Concentration* | 30% | 20% | 10% | 10% | 20% | 10% | 10% | 20% | |
| *Ascochyta lentis* | - [a] | - [a] | - [a] | +++ [d] | +++ [d] | +++ [d] | +++ [d] | +++ [d] | +++ [d] |
| *Gaeumanomyces graminis* | ++ [c] | +++ [d] | +++ [d] | +++ [d] | +++ [d] | +++ [d] | +++ [d] | +++ [d] | +++ [d] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Aspergillus flavus* | -[a] | -[a] | +[b] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Monillia laxa* | ++[c] | ++[c] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Armillaria mellea* | ++[c] | ++[c] | +++[d] | +++[d] | +++[d] | +[b] | +++[d] | +++[d] | +++[d] |
| *Verticillium dahliae* (tomato) | -[a] | -[a] | -[a] | +++[d] | +++[d] | ++[c] | +++[d] | +++[d] | +++[d] |
| *Sclerotinia sclerotiorum* | -[a] | -[a] | +[b] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Penicillium italicum* | ++[c] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Aspergillus niger* | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Botrytis cinerea* | -[a] | +[b] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Pythium ultimum* | -[a] | -[a] | -[a] | +++[d] | +++[d] | +[b] | +++[d] | +++[d] | +++[d] |
| *Cercospora beticola* | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Penicillium expansum* | -[a] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Eutypa lata* | ++[c] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Rhizoctonia solani* | -[a] | -[a] | -[a] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Phytopthora nicotiana* | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Monillia fructigena* | ++[c] | ++[c] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Alternaria alternata* | -[a] | -[a] | ++[c] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Verticillium dahliae* (olive tree) | -[a] | -[a] | -[a] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |
| *Fusarium oxysporum* | ++[c] | ++[c] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] | +++[d] |

+++ Strong-normal mycelium growth ++ Medium mycelium growth + Weak mycelium growth - No mycelium growth

The evaluation of the results obtained by determining the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) (Tables 3 and 4), demonstrated that the fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum* showed high growth sensitivity at the very low concentration of LFP (<3 %) applied in the medium agar. Based on these results it is understood that the use of LFP might be an alternative, bio-control method of plant diseases caused by these two pathogens. Disease control of *Botrytis cinerea* considered easier since the phytopathogenic fungus mainly affects stored products such as strawberries and aerial fruiting bodies of plants such as grapes, where spraying application straight to the target-plant tissue is more sufficient. Likely, the application of the LFP, even in a low concentration directly on the fruits and tissues of plants, may contribute to the prevention and suppression of growth of the pathogen. However, the control of the disease caused by fungus *Sclerotinia sclerotiorum* theoretically should be harder since the phytopathogenic fungus is a soil borne pathogen. Moreover, the appearance of the symptoms perceived after stem tissues infection e.g. in tomato plants, makes the implementation of the LFP difficult. Finally, the appearance of morphological characteristics such as sclerotia which are resistant to adverse environmental conditions and applications of plant protective materials resulting control of the fungus less effective. Moreover, results obtained by determination of the MIC and MLC demonstrated that the fungus *Ascochyta lentis* was sensitive in expression of very low concentration of LFP (> 5 and <10 %). Thus, the LFP could be used as an alternative biocontrol agent against Ascochyta blight disease damaged any leguminous plants. It is believed that the application of the LFP, even in a low concentration, directly on leaves and infected stems of leguminous crops will be able to prevent and to suppress the development of the pathogen.

Furthermore, MIC and MLC results on fungus *Alternaria alternata* shown moderate sensitivity to LFP while at least 10% of the LFP was needed to be applied in order to inhibit mycelium growth and spore germination. Based on these results it is demonstrated that the application of LFP can be decisive for the pathogen growth.

The application of LFP could be considered as a possible alternative biological agent to control *Alternaria* disease which affects different parts of host plants such as leaves and roots. Clearly the use of LFP for controlling pathogenic fungus foliage infections is easier than that of infestation in the root system.

The evaluation of the results obtained by determining the MIC and MLC, demonstrated that the mycelium growth of the fungus *Cercospora beticila* shown moderate sensitivity to 10% concentration of LFP. However, to achieve killing effect of the LFP this concentration could be arised to 15%. Clearly, the use of the LFP in plant foliage is easier than in root system although, the beet leaf surface requires a large amount of active substance or very strong action. The results of these experiments demonstrate that the effect of LFO was not the most efficient against *C. beticola* and possibly this can be a limiting factor in extensive use.

The evaluation of the results obtained by determining the MIC and MLC, demonstrated that the species of the fungus *Penicillium expansum*, *P. itallicum*, and *Aspergillus niger* shown moderate sensitivity in LFP application, required at least 15% of LFP concentration. The use of LFP against infections of stored agricultural products can be considered significant if it does not affect the taste of the product.

The evaluation of the results obtained by determining the MIC and MLC, demonstrated that the fungi *Verticillium dahliae* and *Fusarium oxysporum*, shown moderate sensitivity to LFP while at 15% of their suspension is needed for the expression of this effect. However, for the *Verticillium dahliae* isolated from tomato plant the LFP concentration needed to MLC effect arises to 20%. Moreover, the implementation and therefore combating phytopathogenic causes the disease control is difficult since morphological parts of the fungus grown on the ground and within the vascular tissues of the plant. Nevertheless, possible use of the LFP in soil could show promising results for preventing the initial infection.

The evaluation of the results obtained by determining the MIC, demonstrated that the fungus *Rhizoctonia solani* causing root rots and seedling rots, shown moderate sensitivity to LFP while at least 15% of the substance is needed for mycelium inhibition. However, MLC effect appeared above 20% concentration of LFP. Nevertheless, possible use of the LFP in soil could show promising results for preventing the initial infection.

The evaluation of the results obtained by determining the MIC demonstrated that the fungus *Eutypa lata* shows moderate sensitivity to LFP, while a concentration of 20% of LFP is need as MLC and fungus suppression. The growth of the pathogen in vascular tissues of plants limits the use and application of the LFP. Furthermore, the disease symptoms appeared after several years of infection when very little control methods can be applied. It is considered that simultaneous use of LFP with irrigation could be helpful to prevent the disease growth at early stages. However, it could be useful to examine if the LFP could be applied by spraying immediately after pruning cuts provided protection from airborne ascospores of the fungus produced in other aboveground plant parts such as stems and infected adjacent plants through these incisions.

Finally, the evaluation of the results obtained by determining the MIC and MLC, demonstrated that the fungus *Aspergillus flavus* appeared highly resistant to the LFP concentration required minimum sample rate > 35-40 % for inhibition and killing effects respectively causing also, possible phytotoxic effects and taste denaturation.

Table 3. MIC of different concentrations of LFP against 14 fungal pathogens

| Pathogens | Concentration of LFP | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3% | 5% | 10% | 15% | 20% | 25% | 30% | 35% | 40% | 50% |
| *Botrytis cinerea* | - | - | - | - | - | - | - | - | - | - |
| *Alternaria alternata* | + | + | + | - | - | - | - | - | - | - |
| *Ascochyta lentis* | + | + | - | - | - | - | - | - | - | - |
| *Penicillium italicum* | + | + | + | + | - | - | - | - | - | - |
| *P. expansum* | + | + | + | + | - | - | - | - | - | - |
| *Eutypa lata* | + | + | + | + | + | - | - | - | - | - |
| *Fusarium oxysporum* | + | + | + | + | - | - | - | - | - | - |
| *V. dahliae* (ελιας) | + | + | + | + | - | - | - | - | - | - |
| *Rhizoctonia solani* | + | + | + | + | - | - | - | - | - | - |
| *Sclerotinia sclerotiorum* | - | - | - | - | - | - | - | - | - | - |
| *V. dahliae* (τοματας) | + | + | + | + | - | - | - | - | - | - |
| *Aspergillus flavus* | + | + | + | + | + | + | + | + | - | - |
| *Aspergillus niger* | + | + | + | + | - | - | - | - | - | - |
| *Cercospora beticola* | + | + | + | - | - | - | - | - | - | - |

\+ Mycelium growth or sediment - No mycelium or sediment on the tested tubes

Table 4. MLC of different concentrations of LFP against 14 fungal pathogens

| Pathogen | Concentration of LFP | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3% | 5% | 10% | 15% | 20% | 30% | 40% | 50% |
| *Botrytis cinerea* | - | - | - | - | - | - | - | - |
| *Alternaria alternata* | + | + | + | + | - | - | - | - |
| *Ascochyta lentis* | + | + | - | - | - | - | - | - |
| *Penicillium italicum* | + | + | + | + | - | - | - | - |
| *P. expansum* | + | + | + | + | - | - | - | - |
| *Eutypa lata* | + | + | + | + | + | - | - | - |
| *Fusarium oxysporum* | + | + | + | + | - | - | - | - |
| *V. dahliae* (olive) | + | + | + | + | - | - | - | - |
| *Rhizoctonia solani* | + | + | + | + | + | - | - | - |
| *Sclerotinia sclerotiorum* | - | - | - | - | - | - | - | - |
| *V. dahliae* (tomato) | + | + | + | + | + | - | - | - |
| *Aspergillus flavus* | + | + | + | + | + | + | - | - |
| *Aspergillus niger* | + | + | + | + | - | - | - | - |
| *Cercospora beticola* | + | + | + | + | - | - | - | - |

\+ Mycelium growth or sediment - No mycelium or sediment on the tested tubes

Therefore, in order to classify the effectiveness of LFP against the tested fungal pathogens it was created the following list with the most sensitive and the most resistant fungal species: *Botrytis cinerea, Sclerotinia sclerotiorum, Ascochyta lentis, Alternaria alternate, Cercospora beticila, Penicillium expansum, Penicillium itallicum, Aspergillus nigre, Verticillium dahliae* (isolate stem from olive) *Fusarium oxysporum, Verticillium dahliae (isolate from tomato stems), Rhizoctonia solani, Eutypa lata* and *Aspergillus flavus.*

*3.2 Results in vivo*

In this experimental work the activity of various concentrations of LFP from olive mill wastes, against important phytopathogenic fungi and observation considering possible phytotoxicity on tomato plants were evaluated *in vivo*. However, *in vivo* results could differ from cultivation reality in both in terms of effectiveness as well as on application form, since it is still difficult to implement LFP as it is, in large scale in field or in greenhouse cultivations.

From the results obtained about the effect different concentrations of LFP on dry plant weigh of tomato plants infected with different fungal pathogens it was observed that the use of LFP at 5% concentration prevented the appearance of symptoms of infection in some of the treatments such as *Botrytis cinerea*, *Alternaria alternata, Sclerotinia sclerotiorum,* and *Penicillium italicum.* However, there was statistically important difference between control and treated plants infected with *Penicillium expansum, Aspergillus niger, Verticillium dahliae, Fusarium oxysporum, Rhizoctonia solani* and *Ascochyta lentis*. Furthermore, it was observed that the use of LFP at 10% concentration did not prevent the appearance of symptoms of infection in some of the treatments such as *Penicillium expansum, Aspergillus niger, Verticillium dahliae, Sclerotinia sclerotiorum,* and *Rhizoctonia solani.* However, there was no statistically important difference between control and treatments infected with *Fusarium oxysporum, Botrytis cinerea, Alternaria alternata, Penicillium italicum* και *Ascochyta lentis.* Moreover, it was observed that the use of LFP at 20% concentration did not prevent the appearance of symptoms of infection in some of the treatments such as *Penicillium expansum, Aspergillus niger, Verticillium dahliae, Botrytis cinerea, Penicillium italicum, Rhizoctonia solani* and *Ascocyta lentis.* However, there was no statistically important difference between control and treatments infected with *Fusarium oxysporum, Alternaria alternata* and *Sclerotinia sclerotiorum.* Also, it was observed that the use of LFP at 30% concentration did not prevent the appearance of symptoms of infection all treatments because of the possible phytotoxic effect. Finally, it was observed that when LFP applied at 20 and 30 % concentration, potential phytotoxical symptoms were appeared while concentrations of 5 and 10 % of LFP did not differ statistically from control.

From the results obtained about the effect different concentrations of LFP on dry root weigh of tomato plants infected with different fungal pathogens it was observed that the use of LFP at 5% concentration prevented the appearance of symptoms of infection from *Sclerotinia sclerotiorum.* However, there was statistically important difference between control and treated plants infected with *Botrytis cinerea, Alternaria alternata, Penicillium italicum, Penicillium expansum, Aspergillus niger, Verticillium dahliae, Fusarium oxysporum, Rhizoctonia solani* and *Ascochyta lentis.* The lower dry root weight was observed in the treatment inoculated with the fungus *Rhizoctonia solani.* The use of LFP at 10% concentration did not prevent the appearance of symptoms of infection in treated plants while control differs statistically from all the other treatments having the heavier dry root weight. The lower dry root weight was observed in the treatments inoculated with the fungi *Penicillium expansum* and *Alternaria alternata.* Furthermore, the use of LFP at 20% concentration did not prevent the appearance of symptoms of infection in treated plants while control differs statistically from all the other beside the treatment inoculated with *Verticillium dahliae*. The lower dry root weight was observed in the treatments inoculated with the fungi *Penicillium expansum, Penicillium italicum* and *Aspergillus niger.* Also, the use of LFP at 30% concentration did not prevent the appearance of symptoms of infection in treated plants while control differs statistically from all the other treatments. The lower dry root weight was observed in the treatments inoculated with the fungi *Penicillium expansum* and *Aspergillus niger.* Finally, it was observed that when LFP applied at 20 and 30 % concentration, potential phytotoxical symptoms were appeared in plant growth.

## 4. Discussion

The effect on the growth of fungi and bacteria of natural polyphenols derived from different parts of plant species such as almond nuts [22], tea leaves [23, 24], leaf thyme (*Thymus vulgaris*) [25], mango fruit [26], seeds of black cumin (*Cuminum nigrum*) [16], citrus fruits [14], nutsedge rhizomes [27], olive mill wastes [8] has been studied by many researchers. Furthermore, according to Mahanil et al. [28], polyphenol oxidases (PPOs) which catalyze the oxidation of phenols to quinones has reported as natural compounds which contribute to the defense mechanisms of plants [29], enhancing the defense against attacks by bacteria of the genus *Pseudomonas syringae* and various insects such as Spodoptera species, *Helicoverpa armigera* and whitefly (*Bemissia tabaci*) in cotton.

According Ilova et al. [26], antifungal activity of mangiferin was recorded for the mango fungal pathogens of *Thermoascus aurantiacus*, while weaker effect presented in fungus *Saccharomyces cerevisiae*, *Trichoderma reesei*, *Aspergillus flavus*, and *Aspergillus fumigatus*. However, there were no zones of growth inhibition in fungi such as *Candida albicans*, *Aspergillus niger*, *Fusarium moniliforme* and *Fusarium oxysporum* suggesting that with the effect of mangiferin polyphenols were similar to those obtained from OMWW.

Also, according to the Yangui et al. [30] the use of 20 μl of the substance extracted form OMWW inhibited the growth of mycelium of fungi that cause root rots such as *Fusarium solani* and *Rhizoctonia solani* noting that there is probably a synergistic effect of the volatile polyphenols nucleotides and derivatives of proteins [31]. Similar results against fungus *Rhizoctonia solani* were recorded from Kotsou et al. [32] in the *in vivo* test. However, after the second year of implementation of polyphenols on the soil it appeared transient symptoms of phytotoxicity. Although, it was noted that these transient phytotoxicity symptoms gradually decreased during the third and fourth growing season when polyphenols for OMWW were not applied in the soil.

Also, the results found in the *in vitro* evaluation of the effect of various forms of OMWW polyphenols and especially that of liquid one agree with those of Mavrakis [33] who recorded the positive effect of olive leaves and particular of oleuropein in development of several important plant pathogenic fungal and bacterial pathogens such as *Botrytis cinerea*, *Alternaria alternata*, *Fusarium oxysporum* f.sp., *melonis*, *Rhizopus* species, *Colletotrichum higginsianum*, *Phytophthora parasitica* var. *nicotianae*, *Clavibacter michiganensis* spp. *michiganensis*, *Ralstonia solanacearum*, *Pseudomonas syringae* and *Xanthomonas campestris* pv. *vesicatoria*. However, in the study of Mavrakis [33] oleuropain concentration required for inhibiting the development of the pathogens was much lower than that recorded in our bioassays with liquid polyphenol. Also, indicatively in Mavraki's studies, [33] the minimum inhibitory concentration (MIC), of oleuropain was lower than 0.1% while in our experimental work the same effect was achieved in concentration from 3 % of the liquid polyphenolic compound. At this rate (3%) it was observed that liquid polyphenolic compound especially in three fungi (*B. cinerea*, *S. sclerotiorum* and *A. lentis*) shown inhibitory effect of fungi's spore germination and mycelium growth. In contrast, oleuropain was less effective for fungi *A. alternata*, *F. oxysporum* and *Rhizopus* sp. compared with the growth of *B. cinerea*, *C. higginsianum* and *P. parasitica*.

## 5. Conclusions

The use of low concentration of LFP at 5 and 10 % could control in some cases fungal pathogens. However, higher concentration of LFP (20 and 30%) appear possible phytotoxic effects. Poor root and stem growth are also important factors since fruit development is depending also from water and nutrient absorption from soil. Therefore, all measured parts of the plant are important. It was observed that LFP controlled the growth of fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum.* However, fungi such as *Rhizoctonia solani*, *Penicillium expansum* and *Aspergillus niger* showed affected plant growth mainly due to their rapid mycelium growth, the large number of produced spores and the rapid dissemination into the tissues of the host.

Additionally, in many measurements, the development of plant tissues was favored when the soil contained small amount of LFP at 5 and 10%. This action could show a possible use of LFP as natural component affecting plant growth in a similar way as fertilizer. However, the effectiveness of LFP against plant and fungi was not clear in many cases likely due to external factors such as air temperature and photoperiod affecting plant growth.

Plant pathogenic fungi such as *Fusarium oxysporum* and *Verticillium dahliae* requires longer period of symptoms appearance from first infection due to their development within the plant vascular tissues. For this reason it is proposed at a later stage of examined LFP the *in vivo* experiments to be extended more than 40 days.

From the above it is understood that the LFP has presented encouraging results, but the effectiveness should be studied further. The encapsulation of a polyphenol in encapsulating agent may help to slow the degradation and protection from the frequent watering and runoff due to this action.

Therefore, in order of major protection resulting from the use of polyphenol against major diseases classified the phytopathogenic fungi *Botrytis cinerea, Sclerotinia sclerotiorum* and *Ascochyta lentis.*

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