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## Acceptance Letter

Dear Stefanos Leontopoulos,

Congratulations! As a result of the reviews and revisions, we are pleased to inform you that your following paper has been accepted for publication.

Paper Title: Effectiveness of Olive Fruit Polyphenol Extract Combined with Aqueous Extracts of Solid Wastes of Pomegranate or/and Orange Juice Against Important Plant Pathogens - Part 2 (in vivo studies)

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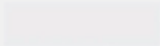
Contributor (s): Stefanos Leontopoulos, Konstantinos Petrotos, Christina Papaioannou, Ioannis Vasilakoglou

It is scheduled for publication on Universal Journal of Agricultural Research, Vol 9, No 1.

The publication fee \$ 380 should be paid within 2 weeks.

Should you have any questions, please feel free to let us know by quoting your **Paper ID** in any future inquiries.

Best wishes,

  
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# Effectiveness of Olive Fruit Polyphenol Extract Combined with Aqueous Extracts of Solid Wastes of Pomegranate or/and Orange Juice Against Important Plant Pathogens - Part 2 (*in vivo* studies)

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**Abstract** Olive fruit extracts, combined with pomegranate and orange waste extracts, isolated using microwave assisted extraction technique, were used as a potential alternative crop protection method against *Verticillium dahliae*, *Rhizoctonia solani*, *Botrytis cinerea* and *Pyrenochaeta lycopersici* on tomato plants. For the evaluation of the antimicrobial activity, various mixtures of the extracts were applied on 15 days old tomato plants, variety "Bella Dona", infected by the above-mentioned pathogens. After 40 days of treatments, plant growth parameters such as plant fresh and dry weight, root weight, plant height, number of blossoms and number of formed fruits were calculated in order to assess the extract effectiveness. The results indicated that the four pathogens dramatically reduced tomato growth. In contrast, most of the evaluated extracts provided satisfactory control of the pathogens resulting in tomato growth similar to that of the uninfected control plants. Conclusively, the use of these polyphenolic extracts could protect tomato plants in case of fungal infection.

**Keywords** Polyphenols, Pomegranate, Orange, Olive Fruit Extracts, Biocontrol, Plant Diseases, Tomato Plants

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## 1. Introduction

A significant increase in food supply is needed due to the emerging global growing population which is estimated to reach 9.7 billion by 2050 [1]. Crop losses from pests and diseases, it is estimated to range from 17 to 30% worldwide [2]. Furthermore, the use of several plant species for energy production may limit fatherly crop areas cultivated for food production purposes [3-7].

Despite all actions aimed to sustainable agriculture, environmental problems are increasing due to the increasing use of agrochemicals and the high amount of the produced wastes and heavy metals resulting in adverse effects on human health affecting as well environment quality and food-chain [8-14]. Therefore, it is necessary to seek for alternatives of agrochemical products and take further action to explore the potential of agricultural wastes [15]. These actions reflect the ever-increasing interest in maximizing the use of agricultural by-products for various purposes [16]. In recent years, there has been

significant interest in linking waste management and use of more environmental friendly crop protection products. Thus, several research studies are focused on using these wastes and natural compounds in innovative applications [17-27]. Plant tissues as well as fruit residues after proper treatment have been found to be effective against several human diseases due to the anti-inflammatory, antioxidant and antimicrobial properties [28-42]. In order to extract efficiently these bioactive compounds, many methods have been proposed [43-49]. This study is in continues to previous *in vitro* study where it was proved that the use of 100%-pure olive fruit polyphenol extract affected the mycelium growth of *B. cinerea*, *V. dahliae*, *R. solani* and *P. lycopersici*, while, pomegranate polyphenol extract at 100% concentration inhibited mycelium growth of *B. cinerea* suggested that various olive-pomegranate-orange polyphenol aqueous extract combinations may be used in order to control the mycelium growth of the majority of the tested microorganisms. The aim of the research study was the evaluation of mixed liquid extracts of olive, pomegranate and orange wastes used as natural phytoprotective agents in organic farming. The assessment of different concentrations and mixtures of polyphenols from olive oil wastes-orange and pomegranate as plant protection materials against economically important plant pathogenic fungi in *in vivo* tests was the main research area of this research work. Tomato plant growth used in order to assess the effectiveness of these polyphenolic mixtures against 4 important fungal pathogens.

## 2. Materials and Methods

### 2.1. General

Agricultural wastes by three plant species were examined for their antimicrobial potential activity in an *in vivo* study. These wastes were aqueous microwave assisted isolates extracted by olive fruit, pomegranate and orange peels.

The *in vivo* antimicrobial activity of polyphenols was evaluated against *V. dahliae*, *B. cinerea*, *R. solani* and *P. lycopersici*. All fungal microorganisms used in this study were obtained from the Benaki Phytopathological Institute (B.F.I.) in Athens Greece. The plant pathogens, their code and the source of collection are listed in the following Table 1.

**Table 1.** Tested fungal species for the *in vivo* experiments

Fungal pathogen	Code	Source of Collection
<i>Verticillium dahliae</i>	2509	B.F.I.
<i>Botrytis cinerea</i>	1948	B.F.I.
<i>Pyrenochaeta lycopersici</i>	2587	B.F.I.
<i>Rhizoctonia solani</i>	2531	B.F.I.

B.F.I.: Benaki Phytopathological Institute, Greece

The potential antimicrobial activity of liquid olive fruit polyphenol extract with aqueous extracts of solid wastes of pomegranate or/and orange juice industry against fungal phytopathogenic species were examined in previous *in vitro* research study where results showed that the use of olive fruit and pomegranate polyphenol extract affected the mycelium growth of *B. cinerea*, *V. dahliae*, *R. solani* and *P. lycopersici* [50]. The concentrations and combinations of liquid olive fruit pomegranate and orange extracts used were as follows in Table 2.

**Table 2.** Ratios of olive, pomegranate and orange polyphenol extracts

Number of sample	Olive fruit (%)	Pomegranate (%)	Orange (%)
11	80	-	20
28	90	7	3
34	50	25	25
37	80	10	10
38	90	5	5
47	80	6	14
57	80	12	8
58	90	6	4
67	80	8	12
68	90	4	6
70	100	-	-

Subsequently, the liquid solutions were placed in sterile commercial plastic containers, numbered accordingly and kept in frozen at -23.3 °C. Whenever needed a small amount of the samples were used in order to examine their antifungal activity against important plant pathogenic microorganisms *in vivo*. The phytopathogenic fungi were grown in Petri dishes filled with PDA and kept at 4°C,

### 2.2. Preparation of Plants-Inoculums Application

As mentioned above 15 days old tomato plant, variety “Bella Dona” were used to ascertain the effectiveness of the antimicrobial activity of different concentrations and combinations of the examined polyphenolic compounds against 4 important fungal pathogens on tomato plants. The possible phytotoxic effects of these extracts were also evaluated. The method followed was described in previous work completed by Leontopoulos et al., [50-52]. More specific the tomato plants were transplanted in disposable plastic pots (19 x 18 cm) 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 with pH: 5,5-6,5; EC: 45mS/m and organic matter: 90-95%. Temperature in glasshouse varied from 22-27°C according to the electronic thermometer located in the greenhouse. Light could not be measured. However, light duration was the standard one observed in Greece during April and at the end of August. Humidity was varied from 65-77%. The irrigation type was drip irrigation with drips made to each

pot. The transplanted plants were watered in order to avoid dryness. The application of different concentrations-combinations of the examined extracts was applied after two days of transplanting. After 24 hrs of the extract application, a hole near the root system was made in order to apply 2ml of fungal spore suspension for the plant infection. Similar amount (2ml) of the examined polyphenolic extract was also applied in order to evaluate their effectiveness in plant growth and fungus action. Plant controls (plants without infestation or extract) were not infected and did not receive extract. Finally, chemical controls were used to which only extract was applied, in order to evaluate a possible phytotoxic effect. In case of application of *B. cinerea* a spore suspension was sprayed on the plants. The same treatment was followed for extract's application in plant leaves using a sprayer. Then, the plants were placed in greenhouse and were irrigated when needed. Four plants were used in each treatment and the experiment was repeated twice. After 40 days of application, plants were harvested and fresh and dry plant weight, fresh and dry root weight, plant height, number of blossoms and number of tomato fruits was measured. For the determination of dry root and plant weight the tomato plants were placed on paper bags and drying at 80 °C for 48 hours.

### 2.3. Statistical Analysis

A randomized complete block experiment design was selected for the study. Data are expressed as the means of 4 measurements. Statistical differences among the mean values were detected by ANOVA. Duncan's test was used in order to group the examined samples at 0.05 level. SPSS software was used as a tool to perform the statistical analyses. The experiment was repeated twice.

## 3. Results

In this experimental work the activity of various concentrations and mixtures of polyphenolic compounds of olive mill orange and pomegranate wastes were tested against important phytopathogenic fungi. Stem, leaves fresh weight, plant height, number of blossoms and tomato fruits were measured in order to consider the most suitable polyphenolic mixture and the possible phytotoxic effect on tomato plants "Bella Dona" variety. However, *in vivo* results could differ from cultivation reality in both in terms of effectiveness as well as application form, since it is still difficult to implement the polyphenolic compound as it is, in large scale in field or in greenhouse cultivations.

### 3.1. *In vivo* Evaluation of Polyphenolic Compounds on Tomato Growth Inoculated with *P. lycopersici*

From Figure 1A, average height of tomato plants infected with the fungus *P. lycopersici* and different

concentrations of olive-pomegranate-orange polyphenolic extracts (OPO-PE), differed statistically from the average height of tomato plants where no infection was applied (control-pathogen). In more details treatments in which the average height of the tomato plants did not differ statistically between them and the treatment of "control-plant" were containing polyphenolic extracts of olive-pomegranate-orange in concentrations: 50-25-25, 80-10-10, 90-5-5, 80-6-14, 80-12-8, 90-6-4, 90-4-6 and 100-0-0. Average height of the "control-plant" treatment differed statistically important from the average height of tomato plants treated with 80-0-20, 90-7-3, 80-8-12 olive-pomegranate-orange concentrations, as well as from the average height of the tomato plants infested with *P. lycopersici* only (control-pathogen).

Regarding, the evaluation of the results of Figure 1B, it was observed that the average fresh weight of the tomato plants used as "control-plant" did not differ statistically from the average fresh weight of the tomato plants treated with OPO-PE at 80-0-20, 80-10-10, 90-5-5, 80-6-14, 80-12-8, 90-6-4, 90-4-6 and 100-0-0%. However, average fresh weight of the "control-plant" treatment differed statistically important from the average fresh weight of the OPO-PE at: 90-7-3, 50-25-25, 80-8-12%, as well as from the average fresh weight of the "control-pathogen" treatment. Although, average fresh weight of the tomato plants in treatments containing OPO-PE at concentrations of 90-5-5 and 100-0-0%, were slightly higher even from those observed in the "control-plant" treatment.

The presence of the pathogen caused a reduction of the dry weight of the aboveground part of the tomato plants by more than 50% (Figure 1C). On the contrary, in the tomato plants where simultaneously applied the extracts 80/10/10, 90/5/5, 80/8/12 and 100/0/0, there was no significant reduction of the dry aboveground weight of the crop. However, limited reduction in dry plant weight was observed on the rest of the treatments.

Regarding dry root weight it was observed that *P. lycopersici* affected root growth of tomato plants inoculated only with the phytopathogenic fungus (Figure 1d). In contrast, in the infected plants where the extracts were applied, the root weight was not negatively affected or it was slightly (treatments 80/10/10, 80/6/14 and 90/4/6).

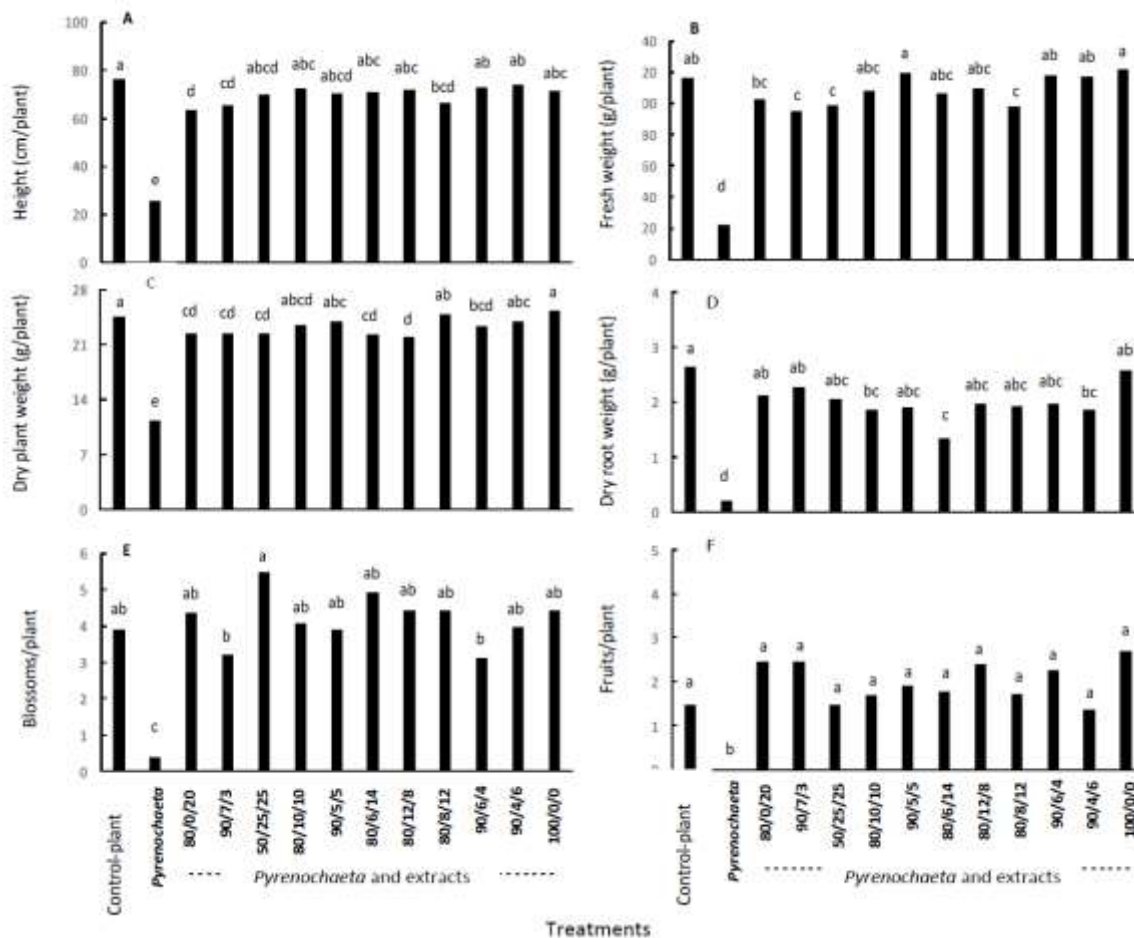
Due to the large variability among observed data, average number of tomato flowers did not differ statistically between the treatments applied except one treatment (Figure 1E). It is worth mentioning that the number of flowers in treatment containing OPO-PE at concentration of 50-25-25% was the largest and differed statistically significant from the treatments of OPO-PE at concentration of 90-7-3, 90-6-4% and from the treatment in which the phytopathogenic fungus was applied.

Regarding the average number of tomato fruits formed, it was observed that there was no statistically significant difference between treatments of the polyphenolic

substances and the control-plant (Figure 1F). A statistically significant difference was observed only on the tomato plants to which the phytopathogenic fungus was applied (control-pathogen).

Therefore, it is believed that polyphenolic extracts can limit the growth of *P. lycopersici* on tomato plants and significantly reduce the losses caused by the presence of

the pathogen in this crop. Thus, the use of polyphenolic extracts it is recommended as alternative bio-chemical compound in order to reduce *P. lycopersici* infection on tomato plants as it affect the phytopathogenic microorganism and improve and maintain fresh weight and height of the infected tomato plants compared to the untreated plants.



**Figure 1.** Fresh and dry plant and root weight, plant height, number of flowers and fruits of tomato plants infected with the fungus *P. lycopersici* to which different concentrations of olive-pomegranate-orange polyphenolic extracts were applied

## 2.2. *In vivo* Evaluation of Polyphenolic Compounds on Tomato Growth Inoculated with *Rhizoctonia solani*

Figure 2A represents the average height of tomato plants infected with the fungus *R. solani*, to which different concentrations of olive-pomegranate-orange polyphenolic extracts have been applied. Tomato plants of the treatment “control-plant” (in which no infection or extracts were applied) grown in height, differing statistically important from the tomato plants to which the infection had been applied (control-pathogen). Tomato plants in treatment where 80% olive-6% pomegranate-14% orange was applied showed the highest growth in terms of height, but did not differ statistically from the tomato plants of the “control-plant” treatment. Moreover, there was no statistically important difference between the “control-plant” treatment and the treatments containing polyphenolic extracts in combination of oil-pomegranate-orange at concentrations (%) of 50-25-25, 80-12-8, 90-7-3, 80-10-10, 90-5-5, 80-8-12, 90-6-4 and 90-4-6. However, average height of tomato plants in treatments containing polyphenolic extracts at concentrations (%) 80-0-20 and 100-0-0 differed statistically from the average height observed in “control-plant” treatment as well as on tomato plant treatment that infection of the phytopathogenic fungus was applied.

Regarding fresh weight, Figure 2B represents that average fresh weight of tomato plants, to which neither concentration of polyphenolic extracts of olive-pomegranate-orange nor the fungus *R. solani* was applied, differed significantly from the average fresh weight of the infected plants, to which polyphenolic solution at a rate of 80-0-20, 50-25-25, 80-10-10, 90-5-5, 80-10-10, 90- 5-5, 80-6-14, 80-12-8, 80-8-12, 90-6-4, 90-4-6 and 100-0-0 were applied. Although they did not differ statistically important from the “control-plant” treatment, tomato plants treated with OPO-PE at a concentration of 80% olive-6% pomegranate-14% orange showed the highest biomass production. In contrast, treatment containing OPO-PE at a concentration of

90-7-3% differed statistically important from the treatment “control-plant” as well as from the tomato plants infected by the phytopathogenic fungus.

The presence of the pathogen caused a reduction of the dry weight of the aboveground part of the tomato plants by more than 50% (Figure 2C). On the contrary, on the tomato plants that were also applied the extracts 80/0/20, 80/10/10, 80/6/14, 80/12/8, 80/8/12, 90/6/4, 90/4 / 6 and 100/0/0 there was no significant reduction in dry ground weight of the crop. However, limited reduction in dry plant weight was observed on the rest of the treatments.

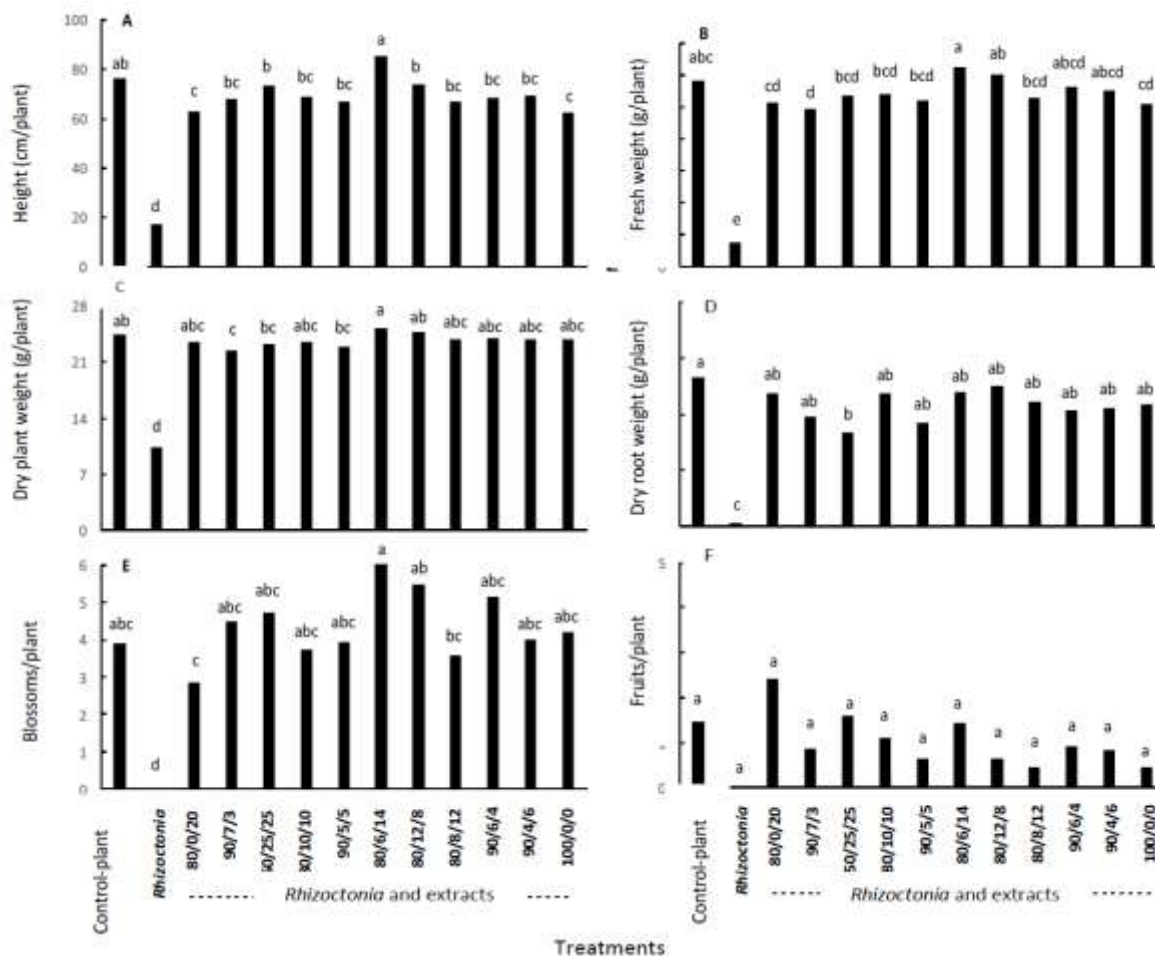
Regarding dry root weight it was observed that *R. solani* affected root growth of tomato plants inoculated only with the phytopathogenic fungus (Figure 2D). In contrast, in infected plants to which the extracts were applied the root weight was not adversely affected or it was slightly affected (50/25/25 treatment).

Regarding the evaluation of the tested polyphenolic extracts in average number of blossoms formed/tomato plant, due to the large variability of the blossoms, no statistically significant differences were observed between examined treatments (Figure 2E). However, average number of blossoms in treatment with OPO-PE, at a concentration of 80-6-14% was slightly higher than all others; it was also differed statistically important from treatments of OPO-PE, at a concentration of 80-0-20 and 80-8-12, as well as from the “control-pathogen” treatment.

Likewise, average number of tomato fruits formed did not differ statistically important among examined treatments due to the large variability (Figure 2F). Application of OPO-PE at concentration of 80-6-14% represents quite promising results in terms of the growth of tomato plants (average height and average fresh weight). In addition, treatment of OPO-PE at concentration of 80-12-8%, showed also quite promising results.

Therefore, these extracts could be applied in order to control infection of *R. solani* on tomato plants, since it was proved that they can reduce the adverse effect of the phytopathogenic microorganism affecting tomato plant growth.





**Figure 2.** Fresh and dry plant and root weight, plant height, number of flowers and fruits of tomato plants infected with the fungus *R. solani* to which different concentrations of olive-pomegranate-orange polyphenolic extracts were applied.

### 3.3. *In vivo* Evaluation of Polyphenolic Compounds on Tomato Growth Inoculated with *Verticillium dahliae*

Figure 3A represents average height of tomato plants infected with the fungus *V. dahliae*, in which different concentrations of OPO-PE have been applied. The results showed that average height of tomato plants that had not been treated (control-plant) differed statistically important from the average height of infected with the pathogen (*V. dahliae*) plants. This treatment was also differed statistically important from the treatment where OPO-PE, at a concentration of 80-0-20% was applied. In more details, average plant height of the treatments where OPO-PE, applied at a concentration of 90-7-3, 50-25-25, 80-10-10, 90-5-5, 80-6-14, 80-12-8, 90-6-4, 90-4-6 and 100-0-0 did not differ statistically important from the treatment where no application was applied. Average height of OPO-PE treatment of 90-4-6% was slightly higher even than the average height observed in the treatment of the “control-plant”.

Figure 3B represents average fresh weights of tomato plants infected with the fungus *V. dahliae*. The results

have shown that the fresh weight of the treatment “control-plant” did not differ statistically important from the average fresh weights of the tomato plants treated with OPO-PE, at a concentration of 90-7-3, 50-25-25, 80-10-10, 90-5-5, 80-6-14, 80-12-8, 90-6-4, 90-4-6 and 100-0-0%. However, average weigh of fresh tomato plants in treatment “control plants” differed statistically from the average fresh weight of the OPO-PE, at a concentration of 80-0-20, as well as from the average fresh weight of the treatment infected with the plant pathogenenic fungus. Average fresh weight of tomato plants treated with OPO-PE, at a concentration of 90-4-6 was slightly higher even than the average fresh weight observed on the “control-plant” treatment.

The presence of the pathogen caused a reduction of the dry weight of the aboveground part of the tomato plants by more than 50% (Figure 3C). In contrast, in tomato plants where applied almost all of the extracts at the same time, no significant reduction in the dry ground weight of the crop was observed. However, a slight decrease in dry weight was observed in the extractions of the extracts 80/0/20 and 80/6/14.

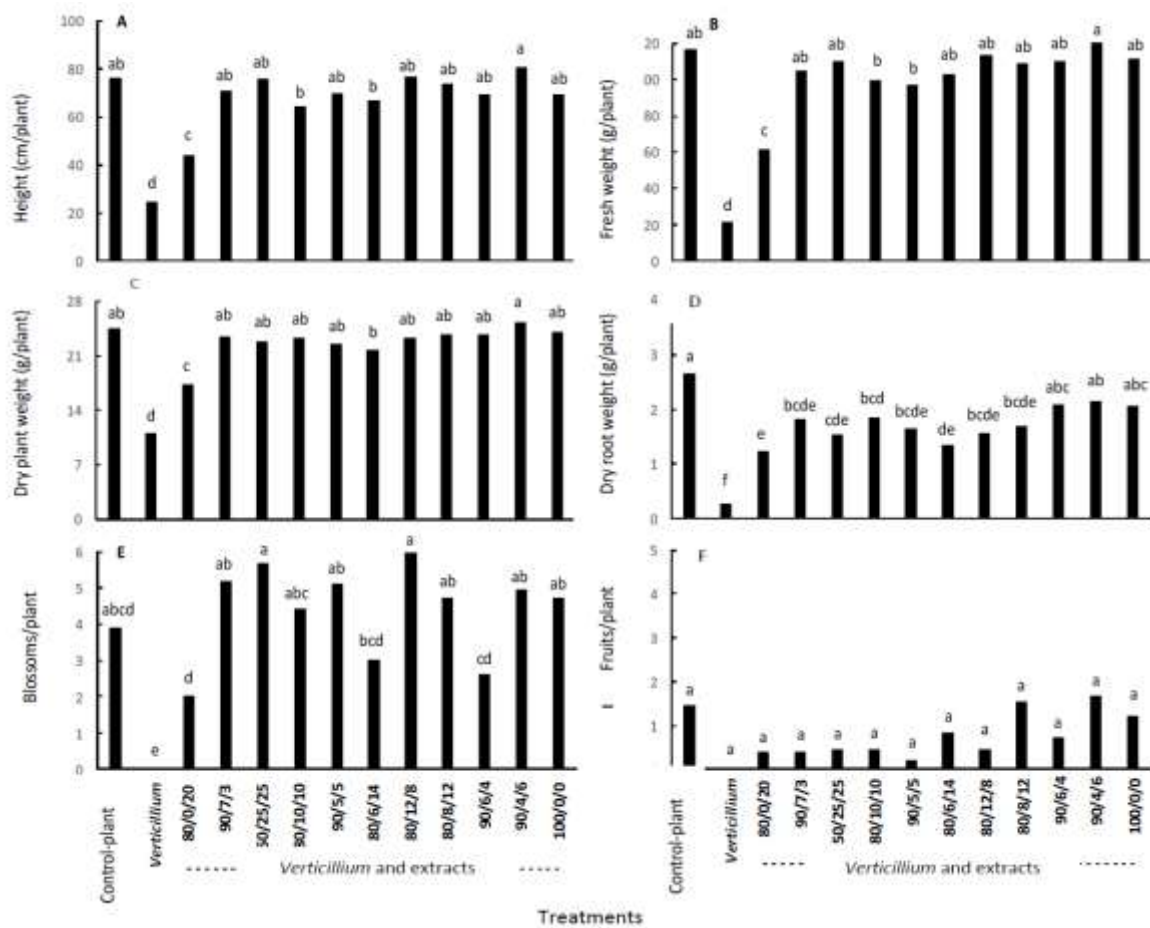


Regarding dry root weight, it was observed that *V. dahliae* affected root growth of tomato plants inoculated only with the phytopathogenic fungus (Figure 3D). In contrast, in the infected tomato plants where OPO-PE treatment containing the 90/6/4, 90/4/6 and 100/0/0% were applied, the weight of dry roots was not negatively affected. However, in the infected plants to which the remaining extracts were applied, the weight of the tomato roots showed an intermediate decrease, relative to the root weight of the tomato plants used as control.

Regarding the number of blossoms formed on tomato plants, due to the large variability of formed blossoms, there was no statistically important difference among the examined treatments beside the treatment where the plant

pathogen was applied (Figure 3E). Number of flowers in OPO-PE treatment containing 50-25-25 and 80-12-8% was higher than the rest and differed statistically important from the treatments containing OPO-PE at a concentration of 80-6-14, 90-6-4, 80-0-20, as well as the treatment where “control-pathogen” was applied. Likewise, due to the large variability in number of formed tomato fruits/plant, it was observed no statistically significant difference among the examined treatments (Figure 3F).

Therefore, the use of polyphenolic extracts can be used in order to treat tomato plants infected by *V. dahliae*, as these extracts limit the growth of the fungus, resulting a satisfactory plant growth.



**Figure 3.** Fresh and dry plant and root weight, plant height, number of flowers and fruits of tomato plants infected with the fungus *V. dahliae* to which different concentrations of olive-pomegranate-orange polyphenolic extracts were applied.

### 3.4. In vivo Evaluation of Polyphenolic Compounds on Tomato Growth Inoculated with *Botrytis cinerea*

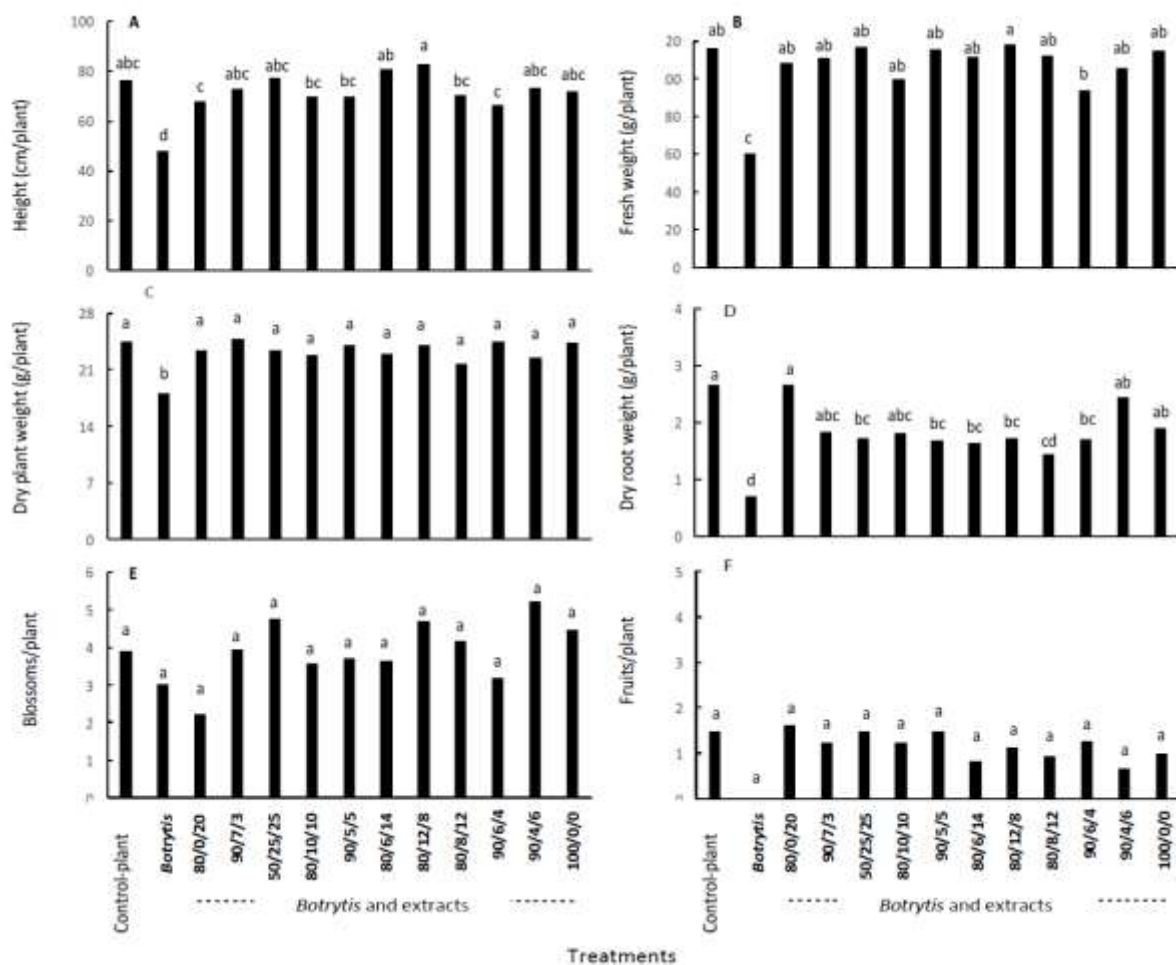
Figure 4A represents average height of tomato plants infected with the fungus *B. cinerea*, in which different concentrations of OPO-PE have been applied. The results showed that average height of tomato plants that had not been treated (control-plant) differed statistically important from the average height of infected with the pathogen (*B. cinerea*) plants. In more details, average plant height of the treatments where OPO-PE, applied at a concentration of 80-0-20, 90-7-3, 50-25-25, 80-10-10, 90-5-5, 80-6-14, 80-12-8, 90-6-4, 90-4-6 and 100-0-0 did not differ statistically important from the treatment where no application was applied. Average height of OPO-PE treatment of 80-12-8 and 80-6-14 were slightly higher even than the average height observed in the treatment of the “control-plant”.

Figure 4B represents the average fresh weights of tomato plants infected with the fungus *B. cinerea*. The results have shown that the fresh weight of the treatment “control-plant” did not differ statistically important from the average fresh weights of the tomato plants treated with

OPO-PE, at a concentration of 80-0-20, 90-7-3, 50-25-25, 80-10-10, 90-5-5, 80-6-14, 80-12-8, 90-6-4, 90-4-6 and 100-0-0%. However, average weigh of fresh tomato plants in treatment “control plants” differed statistically from the average fresh weight of the treatment infected with the plant pathogenic fungus. Average fresh weight of tomato plants treated with OPO-PE, at a concentration of 80-12-8 was slightly higher even than the average fresh weight observed on the “control-plant” treatment.

The presence of the pathogen caused a reduction in the dry weight of the aboveground part of the tomato plants by 27% (Figure 4C). In contrast, in the tomato plants where the extracts were applied at the same time, no significant reduction in the dry plant weight of the crop was observed.

The pathogen infestation caused an important reduction in the weight of the root system in treatment where *B. cinerea* was applied as control (Figure 4D). In contrast, in the infected tomato plants where the tested extracts were applied, dry root weight was not affected or it was affected slightly (treatments with extracts 50/25/25, 90/5/5, 80/6/14, 80/12/8 and 90/6/4).



**Figure 4.** Fresh and dry plant and root weight, plant height, number of flowers and fruits of tomato plants infected with the fungus *B. cinerea* to which different concentrations of olive-pomegranate-orange polyphenolic extracts were applied.



Regarding the number of blossoms formed on tomato plants, due to the large variability of formed blossoms, there was no statistically important difference among the examined treatments (Figure 4E). Number of flowers in OPO-PE treatment containing 90-4-6, 50-25-25, 80-12-8, 100-0-0 and 90-7-3 were slightly higher even than the treatment of the “control-plant” although they did not differ statistically. Likewise, due to the large variability in number of formed tomato fruits/plant, it was observed no statistically significant difference among the examined treatments (Figure 4F). Therefore, polyphenolic extracts can be used to treat *B. cinerea* infestation on tomato plants.

### 3.5. Evaluation of the Phytotoxic Effect of Different Concentrations of Olive-Pomegranate-Orange Polyphenolic Extracts on the Growth of Tomato Plants

The results of the evaluation of the possible phytotoxic activity of the 11 polyphenolic extracts on tomato plants are presented in Figure 5. Results mentioned plant height showed that the average height of the plants treated with

OPO-PE, at a concentration of 80-0-20, 80-12-8, 90-6-4, 90-4-6 and 100-0-0% were lower than the average height of the tomato plants treated as “control plant” (Figure 5A). In contrast, no statistically significant differences were observed between average height of the tomato plant treated as “control plants” and the average height of the OPO-PE, at a concentration of 90-7-3, 50-25-25, 80-10-10, 90-5-5 and 80-6-14%.

Regarding, fresh weight of tomato plants treated as “control plant” did not differ statistically from the fresh weight observed on tomato plants treated with OPO-PE, at a concentration of 90-7-3, 50-25-25, 80-10-10, 90-5-5, 80-6-14 and 90-6-4% (Figure 5B). In contrast, fresh weight of tomato plant treated as “control plants” differed statistically from the average fresh weight observed on OPO-PE, at a concentration of 80-0-20, 80-12-8, 80-8-12, 90-4-6 and 100-0-0%, resulting a possible phytotoxic effect.

The extracts did not cause a significant reduction (phytotoxicity) in the dry weight of the aboveground part of the tomato plants (Figure 5C). In addition, the extracts did not cause a significant reduction in the dry weight of tomato root (Figure 5D).

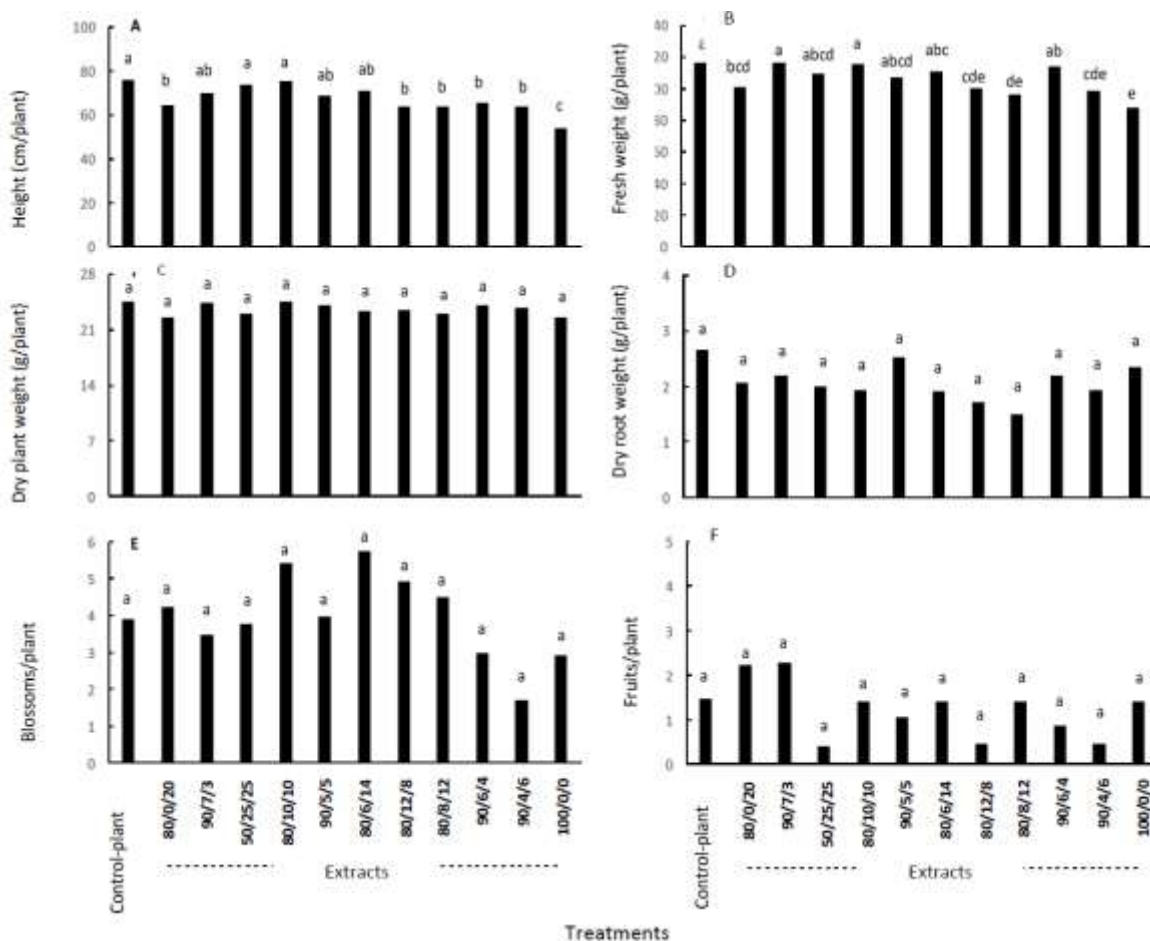


Figure 5. Phytotoxic effect of different concentrations of olive-pomegranate-orange polyphenolic extracts on the growth of tomato plants



Number of blossoms/plant observed in the examined treatments appeared high variability and thus no statistically important difference was observed (Figure 5E). However, it is worthily to be mentioned that the number of blossoms in the OPO-PE, at a concentration of 80-6-14, 80-10-10, 80-12-8 and 80-8-12% were slightly higher than the number of blossoms observed in “control-plant” treatment. The lowest number of blossoms/plant was observed in the treatments containing OPO-PE, at a concentration of 90-4-6 and 100-0-0%. Likewise, due to the large variability of number of tomato fruits/plant formed and in this case, it was observed no statistically important difference between examined treatments (Figure 5F).

Therefore, polyphenolic extracts can be applied on tomato plants used in order to treat various phytopathogenic fungi, as long as their phytotoxicity ranges from zero to very low and is offset by the benefit of effective treatment of pathogenic fungi.

Regarding the selection of the most suitable polyphenolic solution that can be used in order to control the 4 phytopathogenic fungi studied in this research

project, the following table 3 emerges.

## 4. Discussion

Alternative crop protection methods are based on the use of beneficial microorganisms capable of producing inhibitory bioactive substances against important pests and plant pathogens [53-57]. Natural compounds can be extracted from tissues or from agro-industrial wastes of various plant species. As it was mentioned above, the ongoing challenge is to protect plant production and consequently food production with natural antimicrobials to prevent degradation, especially during plant production and fruit storage and / or processing. This situation creates the need to find solutions that lead to maintaining the quality of plant products. In a recent study, van Long et al., [58] studied the effect of essential oils, preservatives, natural products, chemical fungicides, and coated nanoparticles *in vitro* and *in vivo* on different membranes to extend shelf life of fresh fruits and vegetables while many other studies have mentioned the potential use of medicinal plants as biological crop protection agents [27].

**Table 3.** Evaluation of polyphenolic extract of olive-pomegranate and orange wastes used as bio-chemical agent against important phytopathogenic fungi on tomato cultivation

Polyphenolic extract Olive-Pomegranate-Orange	<i>Pyrenochaeta lycopersici</i>	<i>Rhizoctonia solani</i>	<i>Vreticillium dahliae</i>	<i>Botrytis cinerea</i>
80-0-20	-	-	-	-
90-7-3	-	-	-	-
50-25-25	-	-	-	x
80-10-10	-	-	-	
90-5-5	x	-	-	x
80-6-14	-	x	-	
80-12-8	x	x	x	x
80-8-12	x	-	-	
90-6-4	-	-	-	x
90-4-6	x	-	x	
100-0-0	x	-	-	x



During the process of juicing, large amounts of bark and seeds (which make up about 50% of the total weight) are discarded as waste [59]. Pomegranate residues of juicing industry it has been calculated that although pomegranate peel is amounted about 24% of the fruit, it can be responsible for the 40-50% of its total weight [60]. Disposal of these solid wastes cause serious environmental problems. Thus, further treatment is needed. So far many efforts have been completed in order to use these wastes. Quagli et al., [61] studied the influence of pomegranate fruit tissues and liquid waste mills against phytopathogenic fungi such as *Alternaria*, *Aspergillus*, *Botrytis*, *Colletotrichum*, *Coniella*, *Nematospora*, *Penicillium*, *Pestalotia* Syphance and *Sphaidi*, Pilidi concluding that compounds included in these wastes may appear antifungal activity. Furthermore, according to Rosas-Burgos et al., [62] the untreated methanolic extracts of the sweet and sour varieties of pomegranate peel showed significant inhibition of fungi *A. flavus*, *F. verticillioides*, *A. alternata* and *B. cinerea*. Another study, completed by Elsherbiny et al., [63] investigated the efficacy of methanol extract from pomegranate peels to control the growth of *F. sambucinum*. In *in vivo* experiments, methanol extract from pomegranate peels results a significant reduction in the development of the disease in potato tubers suggesting that this extract could be used as an alternative plant protection agent to control dry rot in potato tubers. Furthermore, Tayel et al., [64] documented in his study that extracts of solid pomegranate juice residues can also be used for plant protection applications. In particular, the above author used pomegranate extracts as an alternative, low-risk, antifungal preparation for the treatment of green mold (*P. digitatum*) in orange fruits suggesting that they can be used as natural antifungals to disinfect orange storage chambers and to disinfect the fruit itself by immersing it in an aqueous solution. Similar study has been completed by Tehranifar et al., [60] who studied the antioxidant and antifungal properties of pomegranate peels and extracts against *Penicillium italicum*, *Rhizopus stolonifer* and *B. cinerea* that cause post-harvest fruit rot. The results of their study concluded that the peels and extracts of the seeds had a greater inhibitory effect than the extract of the leaves. Similarly, Glazer et al., [65] studied the antifungal action of solid pomegranate juice residue extracts on a number of known phytopathogenic fungi responsible for spoiling fruits and vegetables during their storage and specifically against *A. alternata*, *Stemphylium botryosum*, and *Fusarium* spp. The use of pomegranate peel extract (PGE) has been also shown to be very effective in inhibiting the growth of fungi after artificial vaccinations in lemon, grapefruit and apples by *P. digitatum* and *P. italicum*, [66] playing an important role in plant protection actions [67].

Regarding the use of OMWW, it is well known that OMWW are the main pollutant parameter responsible for

the severe environmental impact of wastewater mills especially in the Mediterranean countries. However, several research studies have demonstrated the potential use of these compounds as environmental friendly plant protective method applied on several plant species producing fruits, vegetables and flowers during the growing season and postharvest period under storage conditions [68-71]. Furthermore, Kotsou et al., [72] reported that OMWW shown antifungal action against *R. solani* in field experiments while Mavrakis, [73] also reported that various forms of OMWW polyphenols and especially the liquid one affect the development of several important plant pathogenic fungi such as *B. cinerea*, *A. alternata*, *F. oxysporum* f.sp., *melonis*, *Rhizopus* species, *C. higginsianum*, and *P. parasitica* var. *nicotianae*. Despite their antimicrobial activity OMWW wastes can also be used as herbicides since in high concentrations appear phytotoxic effects affecting seed germination [74]. However, this attribute may limit their application in cultivated plant species.

Citrus fruits are the most abundant fruit crop in the world, with annual production estimated from 110-125 million tons [75]. Oranges are the fruit produced in the largest proportion of all citrus fruits (70.6 tsp), about 33% of total production is subjected to industrial processing, resulting in the creation of about 15 e.t. waste, which corresponds to 50% of the initial mass of the processed fruits. Their use is necessary, both to avoid environmental pollution and because the value of the by-products that can be produced from them is important. Despite their use as bioactive extracts and food ingredients, it is believed that these wastes can be also applied as organic fertilizer and plant protective compounds suppressing damage caused by pest and diseases [76-78]. According to De Corato, [79] in a study completed by Bernal-Vicente et al., [80] it was commented that composted citrus fruit residues have shown slight increasing of total biomass in a melon cropping system cultivated under greenhouse nurseries, due to bioactive action of auxin-like and cytokine-like molecules.

Small size of the olive mills have resulted in a difficulty in utilization of liquid wastes. However, the potential of their use as well as the limited industries that are involved in the production-extraction of polyphenolic components from various sources makes it particularly encouraging. The utilization of all quantities of liquid wastes produced, except for technological difficulties, requires the promotion of policies to encourage and develop a National Action Plan for olive mill wastes. Further research is recommended on the acceptance of these products by consumers, as well as the benefits arising from their use as new organic plant protection products and fertilizers in order to increase their dissemination. Technologies that utilize liquid mill wastes and extract their polyphenolic components also need to be improved. In particular, *in vitro* evaluation of different phytopathogenic species is



recommended while test panels can be used in order to evaluate the taste of the products to which these polyphenolic extracts have been applied in food industry. Concluding, as it is suggested by Dávila et al., [81], by improving isolation, marketing and use of compounds presented in plant residues that have high bioactivity may be the way to better use and utilize these residues. This can lead to independence on agrochemicals used in crop production.

## 5. Conclusions

The findings of this study demonstrate the potential activity of aqueous extracts of olive-pomegranate-orange fruits isolated by microwave assisted extraction. Especially, these polyphenolic compounds could protect tomato plants from fungi infections and provide adequate tomato growth and yield. Form previous work it demonstrated that the fungi *P. lycopersici*, *R. solani*, *V. dahliae* and *B. cinerea* showed growth sensitivity at the tested extracts. Based on these results it is understood that the use of the tested extracts might be an alternative, bio-control method of plant diseases caused by these pathogens. From the above it is clear that the olive fruit polyphenol extract, combined with pomegranate and orange extracts, has presented encouraging results, but the effectiveness should be studied further.

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## Author Contributions

S.L., conducted the experiments and wrote the manuscript. C.P., conducted the experiments. I.V., reviewed the manuscript and conducted the data analysis, K.P., supervised the experiments. All authors have read and agreed to the published version of the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

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