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Effect of olive mill wastewater on growth and bulb production of tulip plants infected by bulb diseases

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Abstract

The effect of olive mill wastewater (OMW) on growth of tulip plants infected by common diseases as well as on their new bulbs production is analyzed in this work. Filtered and sterilized OMW was tested as growth inhibitor of *Botrytis tulipae*, *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium* spp. mycelium. The effect of filtered OMW on uninfected tulip bulbs was also tested as well as on the growth of bulbs infected with the fungus *B. tulipae and A. niger in vivo*. The mycelium length, severity of scab-like lesions, plant height (PH), fresh mass (FM) and dry mass (DM) of plants and production of new bulbs were recorded. Only the filtered OMW inhibited the *in vitro* mycelium growth of all tested fungi. However filtered OMW caused infections when it sprayed on uninfected bulbs, malformations on 30% of the plants grown from these bulbs and decrease PH, FM and DM as well as new bulbs production at 75%, 72.4%, 79.1% and 50% respectively. The treatment of *B. tulipae* infected bulbs with filtered OMW reduced further the PH, FM, DM and the production of new bulbs in 92.1%, 81.4%, 78.7% and 97% respectively. In contrast the treatment of infected bulbs by *B. tulipae* + *A. niger* with filtered OMW did not affect PH, FM and the number of new bulbs produced and significantly improved plants DM and the mass of new bulbs.

Additional key words: OMW; *Tulipa* spp.; scab-like lesions; malformation; dry mass; biofungicides.

Introduction

Damage during mechanical harvesting and planting of tulip bulbs as well as handling during storage may result in significantly infected bulbs by fungi, bulb rot, reduction of root growth and increasing floral abortion (Saaltink, 1971; Piwoni, 2007). A common practice to reduce the rotting of bulbs is to disinfect them with chemical fungicides. However, several benzimidazol-resistant fungi strains of *Penicillium* spp., *Botrytis elliptica* and *Fusarium oxysporum* have been already isolated on many bulb ornamental plants like tulips (*Tulipa gesneriana* L.), lilies (*Lilium* sp.) and gladiolus (*Gladiolus communis* L.) (Migheli *et al.*, 1990; Bus *et al.,* 1991; Chung *et al.*, 2009). To overcome this problem and reduce the pesticide use, alternative methods based on the use of organic amendments for the biological control of soilborne plant pathogenic fungi has been reported (Hoitink *et al.*, 1997; Abawi & Widmer, 2000). One alternative and economical solution is the use of olive mill wastewater (OMW), to reduce soil-borne plant pathogens in many crops and postharvest rot diseases of flower bulbs (Smid *et al.*, 1995; Kotsou *et al.*, 2004). In addition, aerobic biological of OMW and their use in agriculture to protect plant material from storage fungi (Bonanomi *et al.*, 2006) will contribute to the reduction of the environmental pollution, since OMW constitutes a major environmental problem due to its high content in phenolic compounds as tannins and flavonoids (Hamdi, 1992; Gonzales *et al.*, 1999).

However, apart from its antimicrobial properties, OMW may become phytotoxic (Bonari *et al.*, 1993; Capasso *et al.*, 1995), since uncontrolled OMW ap-

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Abbreviations used: AFP (antifungal protein); CMI (Commonwealth Mycological Institute); DBU (dibutylurea); DM (dry mass); FM (fresh mass); GAE (gallic acid equivalents); OMW (olive mill wastewater); PDA (potato dextrose agar); PH (plant height); SDW (sterilized distilled water); TDS (total dissolved solids); TP (total polyphenols); WP (wettable powder); Un (untreated bulbs).

plication could inhibit seed germination (Casa *et al.*, 2003; D'Annibale *et al.*, 2004), shoot and root elongation and biomass production (Asfi *et al.*, 2012), plant growth (Ouzounidou *et al.*, 2010), uptake and translocation of nutrients (Ouzounidou *et al.*, 2008) and cause various physiological changes (Mekki *et al.*, 2006; Ouzounidou *et al.*, 2008; El Hassani *et al.*, 2010) including decreased water use efficiency mainly due to decreased CO_2 assimilation rate (Asfi *et al.*, 2012). The presence of phenols as well as short and long-chain fatty acids is believed to contribute to the phytotoxic nature of OMW (Casa *et al.*, 2003; Kistner *et al.*, 2004; Isidori *et al.*, 2005). Accordingly, the current scientific knowledge seems to be insufficient to support the safe agricultural use of untreated OMW (Azbar *et al.*, 2004), since the existing results are sometimes contradictory and not always useful to draw practical conclusions.

The main objective of this study was to investigate the effect of OMW on growth and new bulb production of tulip plants, when these wastes were used for biological control of common tulip bulb diseases (*Botrytis tulipae* and *Fusarium oxysporum*) and tulip bulb moulds, caused by *Aspergillus niger* and *Penicillium* spp. as an alternative to disinfection of the bulbs with chemical fungicides.

Material and methods

The study site

The experiments were carried out at the Plant Pathology Laboratory of the Technological Education Institute of Larissa, Greece and in an experimental polyethylene covered greenhouse, N-S oriented, located at the University of Thessaly near Volos, (Velestino: Latitude 39° 22', longitude 22° 44', altitude 85 m) on the continental area of Eastern Greece.

Olive mill wastewater characterization

Olive mill wastewater was obtained from a 3-phase olive mill plant, located near the city of Volos, central eastern Greece. The OMW was stored in PVC vessels tightly closed at 18°C until use.

Total polyphenols (TP) were determined with Folin-Ciocalteau reagent (Singleton & Rossi, 1965) and were expressed as gallic acid equivalents (GAE). Organic carbon was analysed by chemical oxidation with 1 mol L^{-1} $K_2Cr_2O_7$ and titration of the remaining reagent with 0.5 mol L^{-1} $FeSO_4$. The organic carbon content was transformed in organic matter content by multiplying by 1.724, which is an experimental factor, reported by Hesse (1972). Total N, K and P content were determined by using Kjeldhal device, flame emission spectroscopy and molecular absorption spectroscopy, respectively, after sulphuric digestion. The concentration of Cu and Zn were measured by using atomic absorption spectroscopy, after the digestion of OMW with HNO_3 and $HClO_4$ (in a 2:1 portion). The characteristics of OMW used in this experiment are summarized in Table 1.

Table 1. Main physical and chemical characteristics of olive mill wastewater (OMW)

Physicochemical characteristics	3-Phase OMW
pH	5.1
EC (dS m^{-1})	1.83
TDS[a] (mg L^{-1})	63,800
Total organic matter (mg kg^{-1})	60,000
Total phenols (mg GAE[b] L^{-1})	4,340
Total N (mg kg^{-1})	540
Total P (mg kg^{-1})	302
Total K (mg kg^{-1})	3,820
Total Zn (mg kg^{-1})	5.1
Total Cu (mg kg^{-1})	12.7

[a] TDS: total dissolved solids concentration of olive oil mill wastewater. [b] GAE: gallic acid equivalents.

Pathogen preparation used for infections

Botrytis tulipae, Fusarium oxysporum, *Aspergillus niger* and *Penicillium* spp. isolated from infected tulip bulbs were used for this experiment. Potato dextrose agar (PDA) and malt extract medium was used for fungi isolation. The identification of fungi was made based on Hopkins (1921) description for *B. tulipae* and CMI descriptions of Pathogenic Fungi and Bacteria according to Brayford (1987) for *F. oxysporum*, Onions (1966) for *A. niger* and Kozakiewicz (1992) for *Penicillium* spp. (Sheets No. 921, 94, 111 respectively).

In vitro procedures

The antifungal effect of OMW against *B. tulipae, F. oxysporum*, *A. niger* and *Penicillium* spp. mycelia was tested *in vitro* by using two procedures. In the first one, 25 mL of OMW were added into 1 L of PDA and further sterilized by autoclaving (121°C for 20 min). These substrates were placed in 9 cm Petri dishes. In the second procedure, Petri dishes containing PDA were sterilized by autoclaving (121°C for 20 min) and afterwards 50 μL of filtered OMW (using syringe filler 0.2 μm) was added onto the centre of each Petri dish. Fifteen Petri dishes per treatment were inoculated with a mycelium plug (5 mm in diameter) colonized by the fungus taken from the periphery of 7 days old fungal colonies. Mycelia plugs were placed onto the centre of each Petri dish or next to the OMW drop. Equal Petri dishes numbers were used as control (without OMW). All cultures were incubated in darkness at 21°C for 6 days. At the end of this period, fungus mycelium length (mm) was measured.

Bulbs inoculation

Spore suspension was prepared from 7 days old cultures of *Botrytis* and *Aspergillus* species. Six Petri dishes with *Botrytis* and *Aspergillus* species cultures on PDA media (three dishes per fungus species) were used as spores' sources. Spores were collected by washing the agar surface with 3 mL distilled water. The produced solution was filtered through sterilized muslin in 250 mL conical flask that contained distilled water. In each flask spores, suspension was adjusted for a final concentration 10^6 spores mL^{-1}. A total of eighty healthy precooled tulip bulbs cv. Kees Nelis (with circumference of 11-12 cm) were divided in two groups (40 bulbs each) and sprayed with 50 mL of the above mentioned solution, containing spores of *B. tulipae* or spores of *B. tulipae* + *A. niger*.

Treatments of plant material

One hour after inoculation, ten bulbs from each group (infected with *B. tulipae* or *B. tulipae* + *A. niger*) as well as 10 healthy uninfected bulbs, were sprayed with: a) 20 mL filtered OMW, b) 20 mL sterilized distilled water (SDW) or c) 20 mL Carbendazim (methyl 1H-benzimidazol-2-ylcarbamate) 50 WP (wettable powder) solution 120% w/v, while 10 bulbs from the infected groups and 10 healthy uninfected bulbs remained without any farther treatment. All bulbs were placed in plastic box chambers and kept for 12 days at 21°C. The plastic box chambers were spayed every two days with SDW to achieve high humidity (>85% RH). After the incubation period, the scab-like lesions on each tulip bulb were recorded and classified in six damage rating scales (0-5), where 0 is equal to healthy tulip bulb, 1 = brown halos without scab lesion, 2 = slightly visible scab lesion, 3 = scab lesions up to 1 mm, 4 = scab lesions up to 2 mm, and 5 = heavily infected tulip bulb with large scab-like lesions (>4 mm).

All the above mentioned tulip bulbs, were planted individually in 0.7 L plastic pots. A mixture of sterilized black pit and perlite (3:1 v/v) was used as substrate. The top 1-2 cm of substrate was removed to facilitate the exposures of bulb tip and stem measurements.

Pots were placed on benches inside the greenhouse with natural daylight and without any other OMW and Carbendazim application. Minimum day and night temperature inside the greenhouse was 18°C, with automatic ventilation at 22°C. The plants were irrigated every two days with distilled water, while, once a week, 2 g L^{-1} of $Ca(NO_3)_2$ was added with the irrigation water (Hanks, 1984). The pH of the irrigation solution was adjusted to 6.5. Pots were set out in dense rows. A plot was a pot and all experiments were complete randomised blocks with 10 replications.

At the end of the experiment (50 days after planting): a) the number of malformed plants, b) the plant height (PH) from the bulb tip, c) the fresh mass (FM) and dry mass (DM) of shoot, leaves and flowers and e) the number, FM and diameter of new bulbs produced from each plant were measured.

Statistical analysis

Data were analyzed using the Minitab statistical package (Ryan *et al.,* 2005). One-way analysis of variance (ANOVA) was applied to test the effect of OMW on: a) mycelium length of *B. tulipae, F. oxysporum*, *A. niger* and *Penicillium* spp., grown *in vitro*, b) diameter of scab-like lesions on tulip bulbs caused from *B. tulipae* or with *B. tulipae* + *A. niger* infections and c) growth characteristics and new bulb production of plants grown in pots. Significance was evaluated at $p < 0.05$ for FM, DM and new bulbs production and b) $p < 0.001$ for all other parameters measured.

Results

In vitro assessment of antimicrobial activity of OMW

The mycelia growth of *B. tulipae*, *F. oxysporum*, *A. niger* and *Penicillium* spp., decreased ($p < 0.001$) when filtered OMW was added on the agar surface where fungi were growing (Fig. 1). Mycelia growing on untreated PDA and on substrate with autoclave sterilized OMW had similar growth or a slight growth reduction. In contrast, filtered OMW strongly affected the fungi growth (Fig. 1).

Effect of olive OMW on scab-like lesions of tulip bulbs

Untreated bulbs as well as uninfected bulbs sprayed with SDW remained without scab-like lesions 12 days after inoculation (Fig. 2). However, uninfected bulbs sprayed with OMW, showed scab-like lesions probably caused from microbial flora present in OMW (Amaral *et al.*, 2008). Infected with *B. tulipae* or with *B. tulipae* + *A. niger* bulbs showed a great number of scab-like lesions with large diameter, which decreased by about 50% after the treatment with filter sterilized OMW. Bulbs treated with Carbendazim shown only few scab-like lesions with shorter diameter (Fig. 2).

Effect of olive OMW on plant growth

Treatment with OMW did not affect shoot emergence from the bulbs unless they were infected with *B. tulipae*, when shoot emergence decreased by 10%. In addition, 33.3% of uninfected bulbs sprayed with Carbendazim as well as 30.1% and 70% of bulbs infected with *B. tulipae* or *B. tulipae* + *A. niger*, respectively, and treated with Carbendazim, failed to emerge. The bulbs from the other treatments showed 100% emergence.

A great percentage of malformations occurred in plants grown from control bulbs sprayed with OMW (30%), control bulbs infected only with *B. tulipae* (20%) and untreated bulbs (16%) (Fig. 3). In contrast, plants from control bulbs infected with *B. tulipae* + *A. niger*, sprayed with SDW or Carbendazim did not show any abnormality. Bulbs infected with *B. tulipae* and treated with OMW resulted in 90% of plants with

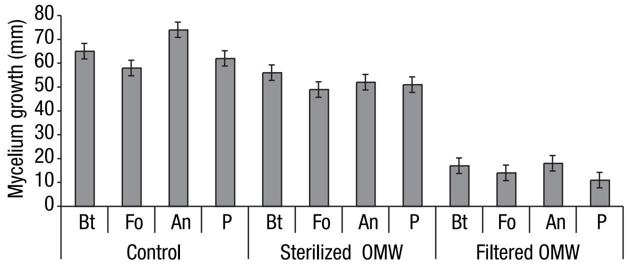

Figure 1. Effect of sterilized and filtered olive mill wastewater (OMW) on mycelia growth of *B. tulipae* (Bt), *F. oxysporum* (Fo), *A. niger* (An) and *Penicillium* spp. (P).

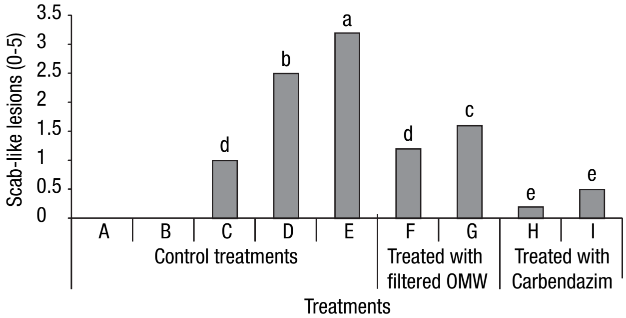

Figure 2. Scab-like lesions on tulip bulbs: A) untreated, B) sprayed with sterilized distilled water (SDW), C) uninfected sprayed with olive mill wastewater (OMW), D) infected with *Botrytis tulipae* (Bt), E) infected with Bt + *Aspergillus niger* (An), F) infected with Bt and treated with filtered OMW, (G) infected with Bt + An and treated with filtered OMW, H) infected with Bt and treated with Carbendazim and I) infected with Bt + An and treated with Carbendazim. Treatments with the same letter were not significantly different at the 0.001 threshold. All treatments were significantly different ($p < 0.001$) from control (A and B treatments).

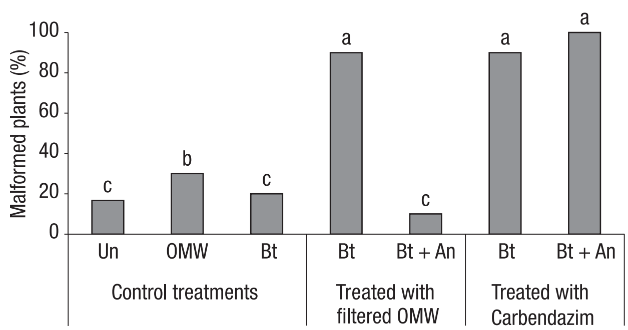

Figure 3. Malformed plants grown from untreated tulip bulbs (Un), control bulbs sprayed only with olive mill wastewater (OMW), control bulbs infected with *B. tulipae* (Bt) and bulbs treated with filtered OMW or Carbendazim after infection with Bt or Bt + *Aspergillus niger* (Bt + An). Treatments with the same letter were not significantly different at the 0.001 threshold.

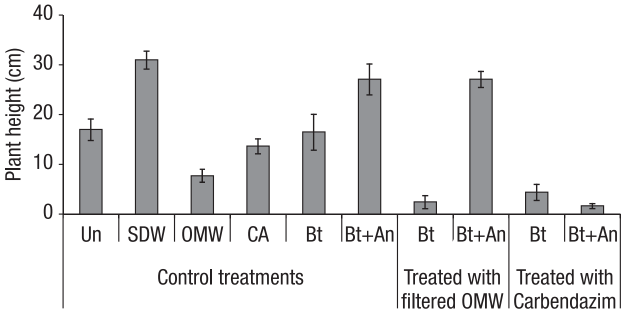

Figure 4. Height of control plants grown from untreated tulip bulbs (Un), sprayed only with sterilized distilled water (SDW), sprayed only with mill wastewater (OMW), sprayed only with Carbendazim (CA), infected with *B. tulipae* (Bt) or Bt + *Aspergillus niger* (Bt + An) and treated with filtered OMW or Carbendazim after infection. Vertical bars indicate standard deviation.

malformed shoot, leaves and flowers, while bulbs infected with *B. tulipae* + *A. niger* and treated with OMW showed very few malformations (10%), mainly on leaves (Fig. 3). The results also indicate that treatment of infected bulbs with Carbendazim failed to reduce plants organ malformations, which were 90% and 100% for bulbs infected with *B. tulipae* or with *B. tulipae* + *A. niger*, respectively.

The effect of OMW application on PH, FM and DM production was also significant. Plants from control bulbs sprayed with OMW were shorter by 75% (Fig. 4), and had reduced FM and DM production by 72.4% and 79.1% respectively, compared to plants grown from bulbs sprayed with SDW with maximum height, FM and DM (Table 2). Bulbs infected with *B. tulipae* + *A. niger* gave plants of similar height ($p < 0.001$) to plants grown from SDW treated bulbs, and reached almost the maximum height plants of this variety can reach (26 to 35 cm). In contrast, plants grown from untreated bulbs (Un) and control bulbs sprayed with Carbendazim or infected with *B. tulipae* were shorter, having around half the height of SDW plants (Fig. 4). However (except of plants grown from control bulbs sprayed with OMW), only plants grown from *B. tulipae* infected bulbs suffered significant FM and DM reduction (Table 2).

Nevertheless, OMW treatment of bulbs did not limit final plant height, when bulbs were previously infected with *B. tulipae* + *A. niger*. OMW application resulted in plants with homogeneous height (Fig. 4) and improved their DM by about 18.2% compared to those grown from infected with *B. tulipae* + *A. niger* bulbs without any further treatment (Table 2). In contrast,

Table 2. Fresh mass (FM) and dry mass (DM) of tulip plants grown from treated bulbs

Treatment	FM (g)	DM (g)
Control		
Untreated	24.72[b]	1.62[b]
Sprayed only with SDW	33.47[a]	2.35[a]
Sprayed with filtered OMW	9.23[d]	0.49[d]
Sprayed only with Carbendazim	21.60[b]	1.57[b]
Infected with *B. tulipae*	14.59[c]	0.95[c]
Infected with *B. tulipae* + *A. niger*	24.56[b]	1.87[b]
Sprayed with filtered OMW		
Infected with *B. tulipae*	6.21[e]	0.5[e]
Infected with *B. tulipae* + *A. niger*	31.51[a]	2.21[a]
Sprayed with Carbendazim		
Infected with *B. tulipae*	10.05[d]	1.64[d]
Infected with *B. tulipae* + *A. niger*	6.45[e]	0.52[e]

Means in each column followed by the same letter are not statistically significant (Duncan, $p \leq 0.05$).

bulbs infected with *B. tulipae* produced plants with 46.7%, 56.4% and 59.6% height, FM and DM reduction, respectively, compared to plants grown from SDW bulbs. These reductions became greater (92.1%, 81.4% and 78.7%, respectively) after their treatment with OMW (Fig. 4 and Table 2). Treatment with Carbendazim decreased PH and FM by about 73.3% and 31.1%, respectively, compared to plants grown from SDW bulbs, but increased DM by 42% compared to plants grown from *B. tulipae* infected bulbs and decreased plants height, FM and DM by 93.9%, 73.7% and 72.2%, respectively, compared to plants grown from *B. tulipae* + *A. niger.*

Effect of olive OMW on new bulb production

New bulb production was strongly influenced by OMW or Carbendazim spraying, since plants grown from control bulbs sprayed with these solutions produced almost half of the number of bulbs in the next generation, compared with untreated and SDW treated bulbs (Table 3). In addition, almost 40% of plants from the above mentioned treatments did not produce any new bulb. OMW or Carbendazim spraying had slightly different influence on the FM of bulbs of the next generation. The production of new bulbs remained almost unaffected when control bulbs were sprayed with Carmendazim, but reduced by 37.5% when

Table 3. Number of new bulbs, fresh mass (FM), maximum diameter and percentage of plants without new bulb production

Treatment	Bulbs per plant (No.)	FM (g)	Maximum diameter (cm)	Plants without new bulbs (%)
Control				
Untreated	4.2[a]	10.7[a]	2.2[a]	0.0
Sprayed only with SDW	3.8[a]	11.2[a]	2.3[a]	0.0
Sprayed with filtered OMW	2.1[b]	7.0[b]	2.1[a]	40.2
Sprayed only with Carbendazim	2.1[b]	8.2[ab]	1.9[a]	40.1
Infected with *B. tulipae*	3.4[a]	13.7[a]	2.6[a]	28.6
Infected with *B. tulipae* + *A. niger*	3.6[a]	6.1[b]	2.2[a]	40.0
Sprayed with filtered OMW				
Infected with *B. tulipae*	0.1[c]	2.4[c]	1.8[a]	89.5
Infected with *B. tulipae* + *A. niger*	3.6[a]	11.5[a]	2.2[a]	30.2
Sprayed with Carbendazim				
Infected with *B. tulipae*	2.8[a]	8.3[ab]	1.8[a]	30.4
Infected with *B. tulipae* + *A. niger*	0.0[d]	—	—	100.0

SDW: sterilized distilled water. OMW: olive mill wastewater. Means in each column followed by the same letters are not statistically significant (Duncan, $p \leq 0.05$).

control bulbs sprayed with OMW, compared to SDW treated bulbs.

Infection of control bulbs with *B. tulipae* did not affect new bulb production as well as their FM and diameter, whereas 28.6% of the plants grown from these bulbs did not produce new bulbs. However, new bulbs FM decreased by 45.5%, when control bulbs were infected with *B. tulipae* + *A. niger*. During this treatment 40% of the growing plants did not produce new bulbs.

The application of OMW on infected with *B. tulipae* bulbs significantly reduced (97%) the new bulb production, as well as 82.5% the FM of new bulbs and increased 62.7% the number of plants without any new bulb compared to SDW treated bulbs (Table 3). In contrast, *B. tulipae* infected bulbs treated with Carbendazim left unaffected the mentioned parameters. In the other hand, plants from bulbs infected with *B. tulipae* + *A. niger* had similar new bulb production, new bulbs FM and their maximum diameter to plants from control treatments, when bulbs sprayed with OMW; but there was no new bulb production, when these bulbs were treated with Carbendazim (Table 3).

Discussion

The antimicrobial activity of OMW against *B. tulipae*, *F. oxysporum*, *A. niger* and *Penicillium* spp. indentified *in vitro* experiments can be attributed to the phenol content (0.43%) and antioxidants remaining in the filtered OMW (Fodale *et al.*, 1999; Paredes *et al.*, 1999; Kotsou *et al.*, 2004). A solution with similar phenol concentration can kill 75% of *Botrytis* sp. spores in almost 120 min (Henderson, 1982). In contrast, many *Aspergillus*, *Penicillium* and *Fusarium* species are resistant to phenol since characterized from their ability to degrade aromatic compounds (García *et al.*, 2000; Millan *et al.*, 2000; Mendonça *et al.*, 2004). The decreased mycelia growth observed *in vitro*, when filtered OMW was added on the agar surface where the fungi were grown, could be also attributed to Cu^{2+} concentration (12.7 mg kg^{-1}) measured in OMW, since minimum Cu^{2+} inhibitory concentrations for fungi colony growth is 10 mg L^{-1} for *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp. (Iqbal *et al.*, 2010; Rathod *et al.*, 2010; Askarne *et al.*, 2011). Olive mill wastewater seems to lose its properties when is sterilized at 121°C for 20 min, since normal mycelia growth was observed on substrate containing autoclave sterilized OMW. This is due to the decreased antioxidant activity of phenolic compounds with increasing temperature (Réblová, 2012), but this is not similar for all phenolic compounds, which can be more than 30 in olive mill wastewater, as reported by several authors (Greco *et al.*, 2006; De Marco *et al.*, 2007). However, use of OMW on uninfected bulbs may cause primary infections, probably due to the presence of moulds like *Penicillium* sp. and *Aspergillus* sp. detected and isolated from the olive mill waste water (Mouncif *et al.*, 1993). Hamdi *et al.* (1991a,b), Hamdi & Ellouz (1992a,b) and Martínez-Nieto *et al.* (1992), studied the ability of *A. niger* to grow in the presence of OMW. Scab-like lesions observed on tulip bulbs caused from these infections were limited, without any further evolution compared to those caused from *B. tulipae* or *B. tulipae* + *A. niger* infections, probably because of the phenols and antioxidants content of OMW that hinder their further evolution. It is noteworthy that these scab-like lesions had similar evolution to those developed on tulip bulbs infected with the above mentioned fungus and treated with filter-sterilized OMW.

Shoot emergence was the only parameter that remained unaffected after OMW application, probably because shoot emergence depends mainly from internal physiological factors, such as dormancy and maturity. However, shoot emergence was slightly negatively affected (10% decrease) only when bulbs were previously infected with *B. tulipae*. This must probably be attributed to the action of *B. tulipae*, which attacks mainly to the emerged shoot, rather to the OMW toxicity. In contrast, a great percentage (33.3%) of uninfected bulbs sprayed with Carbendazim failed to emerge shoots. Benomyl (methyl (1-(butylamino-carbonyl)-1H-benzimidazol-2-ylcarbamate) and its main metabolite Carbendazim have been shown to negatively affect root growth and shoot development of several ornamental plants (Klingensmith, 1961; Hocking & Thomas, 1979; Lee *et al.*, 1983). In addition, increased fungicide concentration may cause abnormalities in mitotic cell divisions and mitosis decreases (Dane & Dalgiç, 2005) as well as significant changes in morphology of bulb plants (Reyes, 1975). An additional reduction (about 36.7%) of shoot emergence observed in Carbendazim-treated bulbs previously infected with *B. tulipae* + *A. niger*, whereas there was no significant further reduction when bulbs were previously infected only with *B. tulipae*. It seems that the net effect of Carbendazim on shoot emergence and growth was the result of interplay between its disease controlling and phytotoxic effects.

Spraying uninfected (control) bulbs with OMW significantly increased shoot, leaf and flower malformations and reduced plants height (by about 75%) and FM and DM compared to SDW control bulbs. A common adverse effect of OMW treatment is the reduction in fresh and dry biomass production in different crops and other plants such as tomato, spearmint, peppermint, and wheat (Mekki *et al.*, 2006; Ouzounidou *et al.*, 2008; El Hassani *et al.*, 2009a,b). This can be attributed to a number of factors such as: a) decrease of leaf chlorophyll and carotenoids concentrations (Asfi *et al.*, 2012), which can affect plant photosynthesis, b) root growth reduction, since phytotoxic substances existing in OMW such as phenols, organic acids, and fats affect root membrane structure and modify its functions including metabolic efficiency and stability (El Hadrami *et al.*, 2004), c) nutrients depletion which severely affects metabolism and resulting in growth inhibition (Wulff-Zotelle *et al.*, 2010) and d) low water use efficiency mainly due to decreased CO_2 assimilation (Asfi *et al.*, 2012). However, the intensity of this phytotoxicity is related to the concentration of OMW and the sensitivity of plant species to OMW. Increased phytotoxicity after OMW treatment can be attributed also to the increased microbial activity that can lead to phytotoxic effects, through the release of phytotoxins from the microbial breakdown of the organic residues (Stroo *et al.*, 1988; Tiquia *et al.*, 1996). Spraying Carbendazim on control bulbs did not result to the production of malformed plants however may cause plants height, FM and DM reduction compared to OMW application. The negative effect of Carbendazim on plant growth can be probably attributed to their metabolites like dibutylurea (DBU), which can decrease the net photosynthesis of the plant. Although there have been no published reports of the effects of DBU on the gas exchange of higher plants, Van Lersel & Bugbee (1996) reported that DBU decreased the dry matter production and shoot height of cucumber.

According to the present results the extent of shoot and leaf malformation as well as plants height, FM and DM reduction in plants grown from infected bulbs, depend on infection by one or two fungi as well as on the treatment (OMW or Carbendazim). Infection of bulbs with *B. tulipae* + *A. niger*, did not cause plant malformations or PH and FM reduction, probably because of the production of antifungal substances by *A. niger* (Moreno *et al.*, 2003; Brzezinska & Jankiewicz, 2012) and/or antagonism between the two fungi. The mold *Aspergillus giganteus* is known to produce and secrete a basic low molecular weight protein, the antifungal protein (AFP). This protein strongly inhibits mycelial growth as well as conidial germination of *B. cinerea*. The antagonism between *B.*

tulipae and *A. niger* may strongly be influenced by pH, diameter of colonies and soil environment conditions (Sahile *et al.*, 2011). In our experiments, infection of bulbs with *B. tulipae* + *A. niger* without any other treatment caused a slight plant FM and DM reduction, compared to plants grown from SDW sprayed bulbs. However, this FM and DM reduction was lower than the reduction caused when bulbs were infected only with *B. tulipae*. The above mentioned results confirm the inhibitory action of *A. niger* against *B. tulipae*. The presence of *A. niger* in bulbs microflora probably restrict *B. tulipae* action and consequently can reduce *B. tulipae* effect on plants FM and DM reduction. Plant FM and DM reduction caused from *B. tulipae* + *A. niger* infection was deterred after the treatment of the infected bulbs with OMW. The beneficial role of *A. niger* in this treatment seems to be twofold since it acts against *B. tulipae* and also it can biodegrade OMW (Millán *et al.*, 2000), reducing the total phenol content in OMW and decreasing their phytotoxicity. Nevertheless, we observed malformation in 10% of plants, probably as an adverse effect of OMW treatment. In contrast, when bulbs were previously infected with *B. tulipae*, OMW treatment increased shoot, leaf and flower malformations, FM and DM reduction. These results indicate that OMW treatment of bulbs infected with *B. tulipae* + *A. niger* was more effective than OMW treatment of bulbs infected with *B. tulipae* alone. Inoculation of bulbs with *B. tulipae* resulted in the formation of primary infections but this happened only where the shoot tip came into contact with the fungi (Coley & Javed, 1972), preventing shoot and leaf emergence and causing leaf malformations and blemishes flowers. The treatment with OMW did not reduce malformations caused from *B. tulipae* infections, but it seemed to act synergistically increasing the percentage of abnormalities in plants. The same negative effect was observed with Carbendazim treatments, which not only failed to improve the height, FM and DM of plants produced from *B. tulipae* or *B. tulipae* + *A. niger* infected bulbs, but it seemed to further decrease them.

The reduced new bulb production and new bulb weight produced from plants grown from control bulbs sprayed with OMW can be attributed probably to the decreased plant DM and increased leaf malformation due to OMW (Timmer & Van Der Valk, 1973; Benschop & Van Der Valk, 1984). In contrast, limited plant FM and DM reduction, as well as the absence of malformations in plants grown from control bulbs

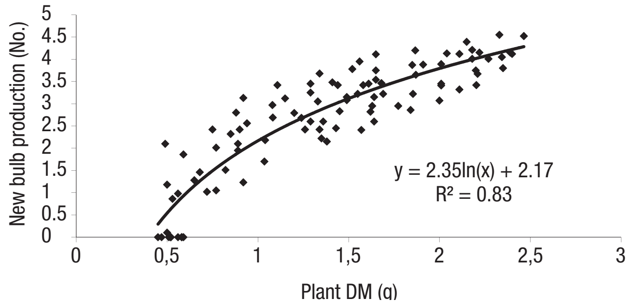

Figure 5. Correlation between the number of new bulbs and green plant dry matter (DM).

sprayed with Carbendazim, resulted in a higher number of new bulbs with higher FM. According to the same pattern, the inability of OMW and Carbendazim treatment to limit DM reduction of plants grown from infected by *B. tulipae* or *B. tulipae* + *A. niger* bulbs, respectively, resulted in a proportional reduction of the number and the FM of the new bulb produced from the plants of these treatments. In general, new bulbs production followed a logarithmic correlation with plant DM reduction ($R^2 = 0.8$), regardless of the cause of this reduction (fungal infection, type of treatment solution used or both), whereas plants with DM lower than 0.6 g did not produce any new bulb (Fig. 5).

Olive mill wastewater can reduce the growth of *Botrytis tulipae*, *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium* spp., when it is used with *in vitro* cultured fungi, probably because of its phenolic and antioxidant components, but this ability will be lost if OMW is sterilized at 121°C for 20 min. Consequently, treatment of infected bulbs with OMW may decrease significantly the scab-like lesions resulting from these infections on bulb surface. The treatment of *B. tulipae* infected bulbs with OMW seems to be unable to reduce malformations caused from infections. The OMW seems to act synergistically to fungi infection, increasing the malformations of plants and decreasing further plant height and dry mass as well as new bulb production (their number and weight). In contrast, treatment of *B. tulipae* + *A. niger* infected bulbs with OMW, does not affect plants height, their fresh mass and the number of new bulbs produced from these plants and improve significantly plant dry mass and daughter bulb weight. However, spraying of uninfected bulbs with OMW may cause primary infections because of the microbial flora (like *Penicillium* sp. and *Aspergillus* sp.) that had been yet detected from other researchers in OMW. The scab-like lesions from these infections will probably remain limited, without any further evolution. OMW application does not affect shoot emergence but will cause shoot, leaves and flowers malformations and will decrease significantly plant height as well as fresh and dry mass production. Due to leaf malformations and plant dry mass reduction caused from bulbs spraying with OMW, the number of new bulbs will be reduced.

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