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Efficacy of olive mill waste water and its derivatives in the suppression of crown gall disease of bitter almond

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Abstract Olive mill waste water (OMW) and some of its indigenous bacterial strains were tested *in vitro* and *in planta* for their efficacy against crown gall disease caused by *Agrobacterium tumefaciens*. OMW and polyphenols displayed a high level of antibacterial activity, however the volatile fraction was less efficient as only a bacteriostatic effect was observed. In pot experiments, the percentage of bitter almond rootstock showing symptoms of crown gall was significantly reduced with the dosage rate of OMW 1% as compared to the control (highly natural infected soil treated with water). Five indigenous bacterial strains isolated from OMW exhibited an antagonistic effect against the bacterium. Based on the gene 16S rRNA sequence analysis, one isolate showed 99.2% similarity to known sequences of *Bacillus subtilis*, one isolate demonstrated high percentage similarities (99.3%) to the genera *Bacillus pumilis*, and two isolates were associated with *Stenotrophomonas maltophilia* and *Pseudomonas putida* 100% and 99.6% similarities respectively. Among these bacteria, the strain B1 proved efficient against the soil borne pathogen *in vitro* and pot experiments. Our study in controlled conditions suggested that the addition of OMW to soil exerts significant disease suppressiveness against *A. tumefaciens*.

Keywords *Agrobacterium tumefaciens* · Crown gall · Indigenous bacteria · Olive mill waste water · Polyphenols

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Introduction

Agrobacterium spp. are ubiquitous telluric bacteria that infect dicotyledonous plants from some 100 plant families (De Cleene and De Ley 1976), including economically important fruit and nut crops, grapes, ornamental and landscape plants. Crown gall is caused when the *Agrobacterium* species infecting the plant contains a large tumour-inducing (Ti) plasmid (Ream 1989). Gall formation results from the integration of a segment of Ti plasmid (T-DNA), into the plant cell genome (Gelvin 1992). Inside the plant cell, genes in the T-DNA are expressed and lead to the synthesis of hormones (auxins

and cytokinins) and to unusual compounds termed opines. Opines play a major role in the epidemiology of crown gall and the ecology of *Agrobacterium* spp. They serve as carbon and nitrogen sources for the tumour-inducing bacterium and some strains induce conjugal transfer of the Ti plasmid to the neighbouring non-tumorigenic agrobacteria (Dessaux et al. 1992).

Crown gall is a chronic and resurgent disease that causes significant economic losses in nurseries and orchards. The disease causes severe annual losses to growers and nursery men worldwide in the form of unsalable nursery stock, low productivity from galled trees, and increased susceptibility of infected plants to other pathogens and to environmental stress generally (Bliss et al. 1999). Root pruning of rootstocks prior to transplanting to nursery fields is a routine practice, but it results in wounds that facilitate infection with tumourigenic agrobacteria that commonly inhabit nursery soils. Crown gall stunts mature plants by reducing root size and/or disrupting the vascular flow in the stems (Moore et al. 2001).

Although the epidemiology of crown gall has been the subject of extensive research in this and the last century, prevention strategies have, for the most part, remained focused on prophylactic methods such as carefully following cultural practices, strict inspection of nursery stock, using sterilised tools when grafting and not planting in infected soils. The use of the antagonistic bacterium *Agrobacterium radiobacter* K84 as pre-planting preventive treatment has been successful for many years, and more recently a genetically engineered derivative of K84 named K1026 has become commercially available (Cooksey and Moore 1982; Lopez et al. 1987; Jones and Kerr 1989; Farrand 1990; Penalver and López 1999; Rhouma et al. 2004). However, these antagonists are only efficient against susceptible strains of *Agrobacterium tumefaciens*. In spite of the success of traditional control strategies, crown gall still causes serious damage, notably in nurseries; in which the percentage of infected plants reaches >50% in some cases.

In recent years, several bacterial and fungal antagonists against soil borne plant pathogens have been described (Howell 2003; Faltin et al. 2004). However, many of these showed inconsistent *in vitro* effects and only very few antagonists were analysed under open field conditions (Grosch et al. 2005). Therefore, other alternatives to control diseases are currently of great importance. The use of organic amendments plays an important role in the outcome of plant–pathogen interactions (Hointik and Beohm 1999; Abawi and Widmer 2000). The decomposition level of organic matter affects the composition of bacterial taxa as well as activities of biocontrol agents (Hointik and Beohm 1999). The decomposition of organic matter increases the population of saprophytic micro-organisms and some of them act as antagonists to plant pathogens (Mazzola 2002; Manici et al. 2004).

Olive mill waste water (OMW) is a major environmental problem because of its high organic load and antimicrobial properties, particularly for Mediterranean countries. Many studies established that these wastes have a high fertilizer value when applied to the soil because of the high organic matter content and some mineral nutrient content (Paredes et al. 1999). However, despite the potential agronomic value, soil amendment with OMW is also known for its antimicrobial activity (Capasso et al. 1995; Kistner et al. 2004). The incorporation of fresh OMW into the soil increases the number of soil micro-organisms and induces a change in the microbial population (Tardioli et al. 1997). In this context, Kotsou et al. (2004) reported that soil treatment with OMW created an r-environment that selectively enhanced and sustained the bacterial population of r-strategists. Consequently, an environmental soil suppressiveness against the plant pathogen *Rhizoctonia solani* was established and possibly also against other telluric pathogens. In the same way, Kistner et al. (2004) found that the addition of OMW to hydroponic nutrient solutions provided an environment suppressing plant pathogens while favouring beneficials. In this work, we investigated the efficacy of OMW against a telluric pathogen *A. tumefaciens*. Thus, we analysed the direct effect of OMW against the bacterium in Petri dishes. This effect was verified in pot experiments containing a highly infected soil by *A. tumefaciens* and amended with one dosage rate of OMW. We isolated some OMW indigenous bacteria in order to verify their possible antagonism against this *A. tumefaciens*.

Materials and methods

Origin of OMW and its characteristics

The OMW water was taken from a three-phase continuous extraction factory located in the region

of Sfax (south east of Tunisia) and kept at −20°C until use. Its physical and chemical characteristics are shown in Table 1.

Plant pathogenic bacterium

Agrobacterium tumefaciens C58 is wild-type strain, harbouring pTi plasmid. Strain C58 was originally isolated from *Prunus avium* (Sciaky et al. 1978) and was kindly supplied by Xavier Nesme (Laboratoire d'Ecologie Microbienne Claude Bernard Lyon 1, France). This strain belongs to an opine group as defined by nopaline strain.

Effect of OMW on multiplication of *A. tumefaciens* cells

The growth of *A. tumefaciens* was tested under different concentrations of OMW 0.5%, 1% and 2% (*v*/*v*) added to mannitol–glutamate (MG) medium (10 g mannitol; 2 g glutamic acid; 0.5 g KH_2PO_4, $3H_2O$; 0.2 g $MgSO_4$,$7H_2O$; 20 g agar; 1 l distilled water). The incorporation of OMW in the MG medium was achieved at 45°C. After solidification, plates were inoculated by streaking a colony from a young pure culture of *A. tumefaciens* (20 h). Petri dishes were further incubated at 28°C and the growth of colonies was detected daily for 3 days (visual observation). Controls were run with inoculated MG without OMW addition.

Table 1 Physical and chemical properties of OMW

Parameters	
pH	4.96
CE (mS cm^{-1})	10.00
MS (g l^{-1})	9.24
MV (g l^{-1})	50.02
DCO (g l^{-1})	100.00
COT (g l^{-1})	38.64
Organic material (g l^{-1})	
Fat matter	10.50
Total polyphenols	5.80
Mineral material (mg l^{-1})	
K	830.00
Fe	3.04
Mg	84.00
P_2O_5	4.20
NO_3^-	17.00
SO_4^-	17.00
Cl	210.00
Ca	12.3
Na	356.00

Antibacterial activity of total polyphenols extracted from OMW

Polyphenol extraction and analysis

OMW was centrifuged at 7,000 rpm for 20 min. The supernatant (SP) was extracted three times with ethyl acetate. The collected organic fraction was dried and evaporated under vacuum. The residue was extracted twice with dichloromethane in order to remove the non-phenolic fraction (lipids, aliphatic fractions and sugars). The liquid phase was discarded, while the washed residue was weighed and re-suspended in ethyl acetate (4.2 mg ml^{-1}). The last compounds were analysed by gas chromatography coupled with mass spectroscopy according to Sampedro et al. (2005) with some modification. Gas chromatography–mass spectrometric (GC–MS) analyses were performed on OMW ethyl acetate extract derivatized with *N*,*O*-bis (trimethylsilyl) trifluoroacetamide in pyridine. Mass spectra were recorded by the use of a Hewlett-Packard 5973 spectrometer equipped with a capillary column HP 5 MS (30×0.25 mm) at 100–280°C with an isothermal programme at 100°C for 2 min, then at 5°C up to 280°C and finally isothermal at 280°C for 5 min. Identification of aromatic compounds was based on comparison with retention times and mass spectra of pure standards.

Effect of polyphenols on bacterial growth

The antimicrobial activity of the polyphenol extract was tested by the disc-diffusion method (NCCLS 1999). The bacterial culture in the exponential phase of growth (100 µl), was spread on sterile MG plates, after which 9 mm diam discs (sterile blank), impregnated with the ethyl acetate extract solution to be tested (20 µl), was placed on the plates. Sterile ethyl acetate served as the negative control. Tetracycline antibiotic biodisc (BioMerieux SA, France) was used as the positive control for the estimation of polyphenol solution antimicrobial activities. The plates were incubated for 24 h at 28°C according to the optimum growth temperature of the microbial strain under aerobic

conditions and the diameter of the inhibition zone around each disc was then measured and recorded.

Effect of volatile fraction

Twenty microlitres of phenolic extract was placed at the centre of half Petri dish containing MG medium and 20 colonies of 1-day pure culture of C_{58} were placed at the surface of MG medium of another Petri dish using a sterile tooth pick. Both half plates were positioned face to face preventing any physical contact between the pathogen and the phenolic extract, and were sealed to isolate the inside atmosphere and to prevent loss of volatiles. Plates were incubated at 28°C for 48 h and the growth of the pathogen was measured and compared to controls growing in the presence of 20 μl of ethyl acetate. Each experiment was repeated at least three times. Results were expressed as means of diameter growth of colonies.

Effect of OMW in suppressing crown gall disease in pot experiments

Pot experiments

A natural highly infected soil by *A. tumefaciens* was taken from a nursery field located in the region of Chbika (Kairouan, central Tunisia). The soil was maintained at 60% of its water-holding capacity, and placed in plastic pots (30×20×15 cm, 2 kg of soil per pot) after adding OMW (1% *w*/*w*). Subsequently, 20 post-emerging bitter almond plants after root pruning were transplanted. Pots were monitored for a period of 3 months after transplantation under ambient conditions. Galls were inspected and weighed.

Microbial estimation

Ten grams of the soil sample were suspended in an Erlenmeyer flask containing 90 ml of a sterile solution (0.2% sodium polyphosphate $(Na\ PO_3)n$ in distilled water, pH 7). The flask was shaken at 250 rpm for 2 h. Serial tenfold dilutions of the samples in 0.9% NaCl solution were plated in triplicate on MG medium at 45°C and PCA (peptone of casein: 5 g, yeast extract: 2.5 g, dextrose: 1 g, distilled water: 1 l) for *Agrobacterium* and total bacterial counts respectively. Each soil sample was analysed twice and the dilution series were plated in triplicate for each experiment. All these counts were expressed as colony-forming units (cfu) g^{-1} of dried soil. Individual colonies on PCA medium were re-streaked on PCA, verified for purity and identified using the Gram coloration, API test (BioMerieux SA, France).

Isolation of bacteria from OMW

For isolation of bacterial cells, 10 g of stored OMW was suspended in 90 ml of sterile distilled water and shaken for 10 min at 250 rpm. One millilitre of this suspension was used to prepare serial tenfold dilutions in 0.9% of NaCl. Aliquots (100 μl) of a dilution of each suspension were spread on Luria–Bertani Agar (tryptone: 10 g, yeast extract: 5 g, NaCl: 5 g agar: 18 g and distilled water: 1 l). Some representative colonies, which differed morphologically, were selected from the countable plates and re-streaked on a new plate of the same media to obtain pure colonies. Purified bacterial isolates were stored in 30% glycerol at −20°C.

In vitro antagonistic activity of bacteria isolated from OMW

All isolated bacteria from OMW were transferred individually to 50 ml of Luria–Bertani broth medium (LB broth) in 250 ml Erlenmeyer flasks and incubated by shaking each culture at 200 rpm for 4 days under ambient conditions. The bacterial cultures were centrifuged at 15,000 rpm for 30 min to remove cell debris. After centrifugation, 20 ml of each sample was filtered through 0.45 μm filters under sterile conditions and kept at 4°C until impregnation of blank discs. The ability of the OMW indigenous bacterial strains to produce diffusible metabolites was tested according to the disc diffusion assay as reported by NCCLS (1999) with some modification. MG medium (20 ml) was poured into each sterile Petri dish (90 mm diam). A suspension of C_{58} was spread on the plates containing solidified MG medium (1 ml of fresh liquid culture at 10^9 cfu ml^{-1} + 3 ml of MG medium at 0.6% agar), and 9 mm diam discs (sterile blank), impregnated with the supernatant of each bacterium to be tested (20 μl), were placed on the plates. The plates were incubated for 24 h at 28°C and then examined for haloes of inhibition around the discs. Sterile LB broth served as the negative control. Four replicates of each bacterial isolate were used, and the experiment was repeated three times.

Identification of the potential antagonistic bacteria

The identification of bacterial strains was achieved by sequencing the 16S rRNA gene (*rrs*). Amplification was carried out by polymerase chain reaction (PCR) with primers F667-pA-rrs AGAGTTTGATCCTGGC TCAG and F668-pH-rrs AAGGAGGTGATCCAGCC GCA designed by Bruce et al. (1992). Standard PCR conditions were 1 min DNA denaturation at 94°C, 1 min annealing at 57°C, and 1 min extension at 72°C for 35 cycles. The 16S rDNA sequences were compared to sequences in the GenBank database with the Basic Alignment Search Tool (Altschul et al. 1990).

Effect of the potential antagonistic bacteria against *A. tumefaciens* in pot experiments

The pathogenicity of C_{58} strain was verified by tested induced galls on tomato 3 weeks after stem inoculation following the method of Zoina and Raio (1999). To investigate the effect of suppression for crown gall caused by *A. tumefaciens*, 10 µl of SP of each antagonistic bacterium (prepared as described above) was placed on 1 cm long longitudinal wounds made with a sterile scalpel at the internodes. After water evaporation, 10 µl of C_{58} suspension (10^8 cfu ml^{-1}, DO=0.4) was poured in treated wounds. Wounds were covered with sterile cotton impregnated with water to prevent drying. Positive control inoculations were made with C_{58} suspension and the negative ones were made with distilled water.

Data analysis

Data were subjected to analysis of variance using SPSS software (version 11). Mean values among treatments were compared by Duncan's multiple range test at the 5% (P=0.05) level of significance.

Results

In vitro effect of OMW and polyphenols against *A. tumefaciens*

The incorporation of OMW into the culture medium resulted in a potent antibacterial activity against *A. tumefaciens* and complete inhibition of cell multiplication was observed for the tested does of 1% (Fig. 1) and 2%. However, the dose of 0.5% was less efficient in preventing cell multiplication. These results indicated that *A. tumefaciens* was probably more sensitive at the dosage rate of 1% of OMW than at 0.5%.

The results with OMW were confirmed by the effect of polyphenols. This effect was expressed as a large inhibition zone around the disc (34 mm±0.1) compared to the negative control (0 mm with ethyl acetate) and to the positive control where the inhibition zone was 15.4±0.1 (tetracycline).

The reduction of the diameter of streaked colonies treated with volatiles (>50% for 2 days of incubation) suggests a bacteriostatic effect of volatile compounds (Fig. 2). GC–MS analysis of polyphenols revealed the presence of vanillin, M-hydroxyphenylethanol, 4-hydroxyphenylethanol, 1,2-dihydroxyl-4-(1-propyl) benzene, 4-hydroxyphenyl-propionic acid, vanillethanediol, 2-hydroxyphenyl acetic acid and 3,4-dihydroxyphenylglycol (Fig. 3).

Suppression of crown gall disease by OMW in pot experiments

Plants of bitter almond potted in a highly infected soil treated with OMW (1%) did not show symptoms of crown gall, while plants transplanted in untreated soil showed galls both on stems and roots. The weight of collected galls was approximately 15 mg/plant.

The enumeration of the *A. tumefaciens* population in the treated soil by the addition of OMW was significantly reduced (0 cfu g^{-1} of soil). However, the population of beneficial bacteria identified as *Pseudomonas* spp. (90%) was significantly increased. Moreover, the bacterial count in the untreated soil

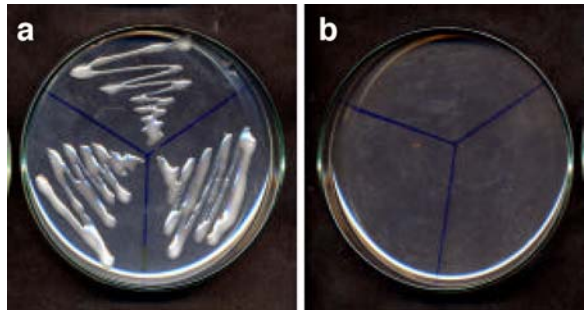

Fig. 1 Efficacy of OMW incorporating C_{58}. **a** multiplication of C_{58} in controls (0% of OMW). **b** Inhibition of C_{58} growth at the dosage rate 1%

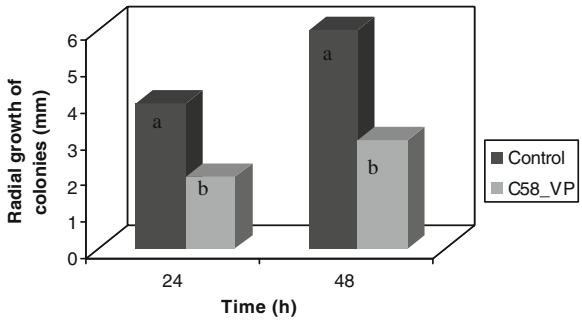

Fig. 2 Radial growth of colonies of *A. tumefaciens* treated with volatile fraction of ethyl acetate extract (C58_VP: C58_Volatile Polyphenol). *Histograms* followed with different *letters* are significantly different according to the test of Duncan (P=0.05)

demonstrated that the *Agrobacterium* population increased to 3×10^8 cfu g^{-1} of soil (3×10^6 cfu g^{-1} of soil before the start of the experiment) while *Pseudomonas* spp. became stable (Fig. 4).

In vitro and in vivo effect of indigenous bacterial strains of OMW

Five bacterial strains isolated from OMW exhibited antibacterial activity towards *A. tumefaciens* in Disc diffusion assay (Table 2). Based on 16 S rRNA sequences analyses, the strain B_1 associated to the genus *Bacillus* BM_{11} was identified as *Bacillus pumilis*, BM_2 as *Bacillus subtilis*, BM_{17} as *Pseudomonas putida* and BM_6 was associated with strain of *Stenotrophomonas maltophilia*. The presence of a wide inhibition zone with the disc diffusion test indicated that the antagonism could be related to extra-cellular metabolites released in the culture medium.

Under field conditions, the isolate BM_{17} was not efficient against *A. tumefaciens*. The percentage of gall formation was not significant compared to controls inoculated with C_{58}. The strain BM_{11} was more efficacious and the galls formed were significantly decreased. The most efficient isolate was B_1 with the lowest percentage of galls formed (Fig. 5). The approximate weight of galls formed in plants treated with B_1 and BM_{11} was significantly lower than that obtained with controls and plants treated with BM_{17} (Fig. 6).

Discussion

The results obtained demonstrate that suppression of crown gall of the most used rootstock in Tunisia (bitter almond) using OMW is due to chemical compounds such as polyphenols and probably to some of its indigenous bacterial strains that act as antagonists.

The complete growth inhibition of *A. tumefaciens* demonstrated that this bacterium can not grow on

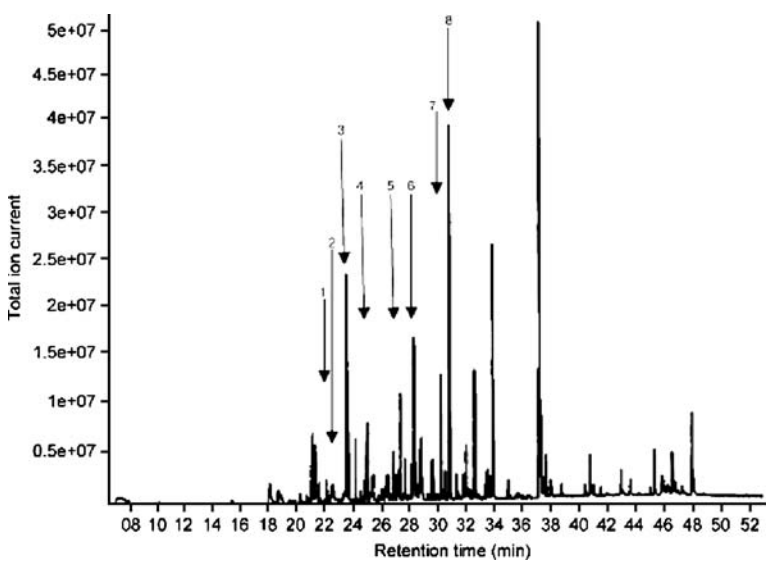

Fig. 3 GC–MS chromatogram (total ion current) of phenolic compounds extracted from OMW. The following identified compounds have been numbered according to their retention times: vanillin [*1*], M-hydroxyphenylethanol [*2*], 4-hydroxyphenylethanol [*3*], 1,2-dihydroxyl-4-(1-propyl) benzene [*4*], 4-hydroxyphenyl-propionic acid [*5*], vanillethanediol [*6*], 2-hydroxyphenyl acetic acid [*7*], 3,4 dihydroxyphenylglycol [*8*]

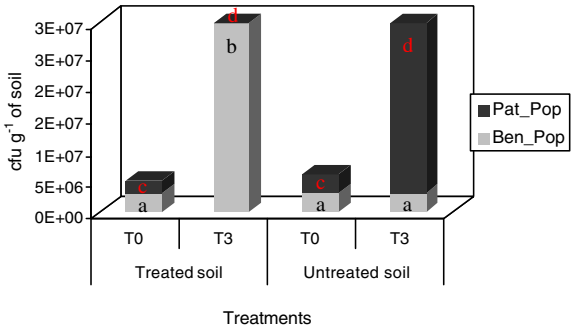

Fig. 4 Bacterial estimation of treated (amendment of 1% OMW) and untreated soil. *T0* before start of experiment, *T3* 3 months after plant transplantation, *Pat_Pop* pathogen population, *Ben_Pop* beneficial population. *Histograms* followed with different *letters* are significantly different according to the test of Duncan (P=0.05)

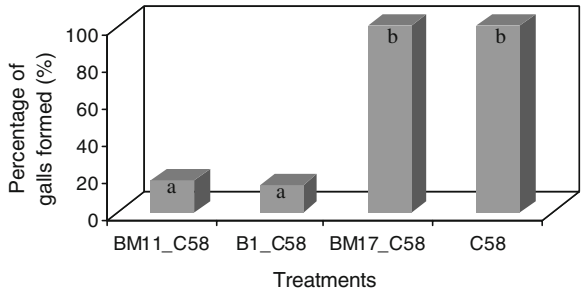

Fig. 5 Reduction of galls formed in bitter almond rootstock treated with indigenous bacteria of OMW under field conditions. *Histograms* followed with different *letters* are significantly different according to the test of Duncan (P=0.05)

media containing >0.5% OMW. The total inhibition could be due to the indirect effect of acidification of the medium (acid pH of OMW) and to chelating transition metals by polyphenols. Wong and Kitts (2006) reported that phenolic compounds are able to chelate transition metals and also lower the reactivity of metal iron by forming an inert metal-ligand complex. Chelation of transition metals, such as iron and copper, reduces bioavailability for phytopathogen growth. The growth inhibition of bacteria cells could be due to phenolic compounds that can potentially impair cellular function and membrane integrity (Ciafardini and Zullo 2003). It has been demonstrated that phenolic compounds bond tightly on the cell walls, being generally classified as surface-active compounds, thus causing disruption of cell peptidoglycan or leakage of cytoplasmic constituents such as protein, glutamate, potassium, phosphate from bacteria or damage of the cell membrane (Sousa et al. 2006; Rodriguez Vaquero et al. 2007). Phenolic compounds are also characterized by very strong protein cross-linking and protein-denaturizing activity (Bais et al. 2002; Ciafardini and Zullo 2003; Sousa et al. 2006). Puupponen-Pimiä et al. (2001) reported that different bacterial species (Gram-positive and Gram-negative) exhibit different sensitivities towards phenolics and phenolic acids in which our extract is rich. Due to their acidic side chain, phenolic acids are much less polar. This property might facilitate the transport of these molecules across the cell membrane which might be related in turn to the stronger inhibitory effect of phenolic acids. On the other hand, they are known to interact with membrane lipids by a neutralization of the membrane's electric potential,

Table 2 Diameter of inhibition zone of *in vitro*-treated *A. tumefaciens* caused by different bacteria (± indicates standard error of the mean)

Treatment	Ø of inhibition zone (mm)
B_1: *Bacillus sp.*	34.0±0.10
BM_2: *Bacillus subtilis*	19.2±0.11
BM_6: *Stenotrophomonas maltophilia*	13.8±0.2
BM_{11}: *Bacillus pumilis*	25.0±0.13
BM_{17}: *Pseudomonas putida*	26.0±0.23
Ethyl acetate	0.0±0.0
Tetracycline	15.4±0.10

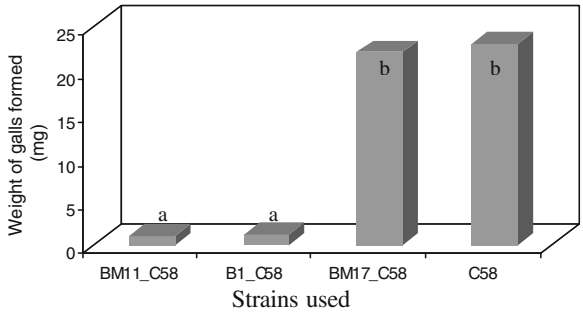

Fig. 6 Reduction of gall weight of treated bitter almond rootstock with isolates of OMW by-product under field conditions. *B1 Bacillus* sp., *BM11 B. pumilis*, *BM17 P. putida*. *Histograms* followed with different *letters* are significantly different according to the test of Duncan (P=0.05)

following penetration of the molecule. A similar effect could occur in the bacterial cell membrane affecting their energy metabolism.

Additionally, the inhibition of radial growth of colonies of *A. tumefaciens* indicated the possible presence of bactericidal compounds in the volatile fraction of the extract. Volatile polyphenols could be stronger and react with nucleotides and proteinaceous materials. Among the polyphenols, flavonoids such M-hydroxyphenyethanol and 4-hydroxyphenylethanol (compounds found in our extract) inhibited phytopathogenic growth by reacting with DNA and disrupting DNA replication (Wong and Kitts 2006). Thus, this could explain the observed growth inhibition of the soil borne pathogen in this study.

The significant reduction of crown gall incidence on bitter almond rootstocks using the OMW amendment was attributed to the effect of polyphenols and probably other chemical compounds. Several researchers have demonstrated that only a few micro-organisms are able to survive in this by-product, because it contains various simple and complex phenolic compounds characterized by high antimicrobial activity (Capasso et al. 1995; Kistner et al. 2004). Some phytopathogenic bacteria like *Pseudomonas syringae* pv. *savastanoi*, *Corynebacterium michiganense* and *Xanthomonas campestris* are inhibited by polyphenols present in OMW in their original concentration (Ciafardini and Zullo 2003).

In addition, despite the potential agronomic value (Paredes et al. 1999; Mekki et al. 2006), soil amendment with OMW is also known for its antimicrobial properties (Kotsou et al. 2004). In fact, several studies reported that soil organic materials (OM) play a pivotal role in the outcome of the plant–pathogen interaction (Hointik and Boehm 1999; Whipps 2001; Bailey and Lazarovits 2003). OM amendments affect the pathogen viability and distribution, nutrient availability and the release of biologically active substances from both crop residues and soil micro-organisms (Bailey and Lazarovits 2003). Indeed, considerable evidence has accumulated to support the view that ammonia liberation following application of high-N amendments is responsible for killing plant pathogens (Huber and Watson 1970; Shiau et al. 1999). Bailey and Lazarovits (2003) reported that organic amendments rich in nitrogen may reduce soil borne diseases by releasing allelochemicals generated during product storage or by subsequent microbial decomposition. This may explain why amendments with OMW (NO_3^- 17 mg l^{-1}) are most often found to suppress crown gall disease.

Cook (1999) has also argued that utilising novel sources of waste products as organic amendments directly and indirectly influence the balance of beneficial and detrimental microbial residents in soil. In fact, the multiplication of the fluorescent *Pseudomonas* spp. population in the soil treated by OMW suggests a suppressive environment for *A. tumefaciens* which could be related to the competition for nutrients, antibiosis and enhanced resistance of plants. This result was consistent with that reported by Kistner et al. (2004), who demonstrated that there was a positive correlation between the concentration of fluorescent *Pseudomonas* spp. and resistance to microbial impact with the untreated OMW having the highest protection ability. On the other hand, Garbeva et al. (2004) reported that this genus includes several functional groups of environmental interest, such as plant growth promoters, plant pathogens and xenobiotic degraders. Moreover, *Pseudomonas* species can also play important roles as biological control agents against soil borne plant pathogens. Different mechanisms may be involved, such as the production of secondary metabolites (Dowling and O'Gara 1994; Keel et al. 1996; Molina et al. 2003; Garbeva et al. 2004; Kistner et al. 2004), cellulolytic and chitinolytic activity, and the induction of systemic resistance against phytopathogens in the host plant (Whipps 2001; Garbeva et al. 2004; Kistner et al. 2004). Molina et al. (2003) demonstrated the ability of some *Pseudomonas* species to degrade the quorum-sensing molecules of *A. tumefaciens*.

With regard to the effect induced by the microbiological component of OMW, it is possible that indigenous bacterial strains were also involved. The present work showed that some OMW indigenous bacteria played a major role against *A. tumefaciens*. The study conducted by Kotsou et al. (2004) demonstrated that the significant disease suppressiveness against a telluric fungus, *R. solani*, induced in the OMW-treated soil, was mainly attributed to the shift in the soil microbial community from K to r-strategy. Our results showed that species of *Bacillus*, *Pseudomonas* and *Stenotrophomonas* were isolated from OMW and exhibited antimicrobial activity against the soil borne plant pathogenic bacterium *A. tumefaciens*. The antagonists may have different mechanisms of action including competition for

nutrients or for space (Yoshida et al. 2001; Whipps 2001). Biocontrol by antibiosis could occur (Whipps 2001; Lopez et al. 2000). The inhibition of cell multiplication could be due to the antibiotics secreted by these bacteria which may have bactericidal activity. This confirms the hypothesis of Penyalver et al. (2001) who considered that the biocontrol of *A. tumefaciens* using the non-pathogenic strain K84 was by the production of the antiagrobacterial antibiotic agrocin 84.

The suppressive effect of the antagonists B_1 and BM_{11} against the soil borne plant pathogen *A. tumefaciens* is likely to be due to antibiosis by strains B_1 and BM_{11} that would play a major role in the suppression of crown gall disease caused by *A. tumefaciens*, because the number of diseased plants was significantly less when treated with BM_{17} or with the C_{58} pathogen strain. In addition, inhibition of crown gall disease on bitter almond rootstock by the supernatant was observed when it was applied before bacterial inoculation suggesting that the filtrate had a therapeutic effect. Further studies are required to extend our knowledge about the management of OMW for soil sanitation.

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