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Potential of hydroxytyrosol-rich composition from olive mill wastewater as a natural disinfectant and its effect on seeds vigour response

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ABSTRACT

Hydroxytyrosol-rich olive mill wastewater (HROMW) and hydroxytyrosol-rich composition (HRC) were prepared from olive mill wastewater using hydrolysis and post-hydrolysis purification processes. The HROMW and HRC showed powerful bactericidal and fungicidal activities against phytopathogens, and their minimal inhibition concentrations for fungi and bacteria were 7.18–57.4 mg l^{-1} and 7.18–14.4 mg l^{-1}, respectively. After 5 min of contact time, the disinfectant properties of the HROMW and HRC added at concentrations of 1.25% (dw/v) allowed for a reduction in bacterial viability by greater than 5 log units. However, a higher concentration of 1.5–3% (dw/v) or a longer contact time of 30 min were needed to achieve values for fungal viability reduction that were higher than the 4 log units recommended by EN 1275 [EN 1275 (1997a). Chemical disinfectants and antiseptics. Basic fungicidial activity. Test method and requirements (phase 1)]. HROMW and HRC were less effective with the most ubiquitous fungi, *Fusarium* spp., which needed 10% dw/v. The addition of HRC at 10% (dw/v) showed that the composition was a potent exogenous enhancer of growth that stimulated the seedling vigour of tomato and muskmelon, according to the conventional agronomic parameters for seed vigour. Compared to the control, the germination percentage, shoot weight, shoot height, and root length were all significantly enhanced in the HRC-treated seed plants. HRC was found to have effective disinfectant properties against seed-borne diseases. In treated seeds, the composition had significant effects on the control of damping-off disease groups at the pre-germination stage. HRC was also equally effective in the control of root rot diseases caused by *Fusarium sambucinum* and *Alternaria solani* as well as of wilts and even bacterial seed-borne pathogens. HROMW was also found to be as effective as HRC in terms of its efficacy against the three seed-borne diseases mentioned above.

1. Introduction

Root rots are reputed to be among the most devastating plant diseases. They are caused by a complex of fungal pathogens resident in the soil and have often been considered a major constraint to the establishment and development of plants in nurseries, glass houses and crops worldwide (Agrios, 2005). They are often associated with significant yield losses or decline in productivity (Tu, 1992). Studies have often reported that those fungi, either individually or associated, have severe effects on seed germination and young seedlings; observations of antagonism and synergism phenomena would then follow (Davet, Ravise, & Baroduy, 1980). Reports have also emphasised that those pathogens constitute the major factors behind the low productivity rates in many plantation fields. Based on the plant organ or growth stage that they affect, fungal disease pathogens can be divided into three groups: damping-off, root rot and wilts. Damping-off cause seed rot in pre-germination or germination stages or plant death after germination. Root rots cause rot and/or destruction of the root, restricting the absorption of water and nutrients. Wilts appear after flowering, and rarely at the juvenile stage of growth. Accordingly, many seed phytopathogenic bacteria are able to survive on dried seeds for extended periods (Ciafardini & Zullo, 2003). They, therefore, do not influence the establishment of plants but are the major means for the long-term spread of the disease. Rapid emergence can reduce root rot (Phillips, 1989); hence the presence of root rot can be directly related to the use of different sowing techniques, particularly those that support a favourable microclimate for their development (Valenciano, Casquero, & Boto, 2004). The disinfection of seeds enhances seed germination rates, increases plant growth, improves plant emergence and reduces damping-off (Gupta, Mathew, Shyam, & Sharma, 1999).

The application of pesticide seed treatment on various crops results in the improvement of plant emergence because it reduces plant mortality and losses caused by damping-off and root rot

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(Tu & Zheng, 1993), thus improving the overall yield. Nevertheless, disinfectants, such as sodium hypochlorite, or fumigants, such as methyl bromide, can cause serious toxicity problems in young plants, as well as present risks to handlers and the environment (Soriano, Porras-Piedra, & Porras-Soriano, 2006). They can also cause irreparable damage to the metallic structure of green houses (Ciafardini & Zullo, 2003). Physical methods, such as heat treatment, are not adequately appropriate for application and often produce large amounts of unviable seeds (Soriano et al., 2006).

During the last few decades, the severe limitations of the use of methyl bromide prior to its complete phase-out seem to have triggered immense interest among researchers in the search of alternative solutions. In fact, the use of natural substances as alternative substitutes to synthetic chemical pesticides has often been recommended, particularly because they are less persistent and are known to have fewer non-targeted toxic impacts than traditional agrochemical aggregates. More pertinent to the aims and objectives of the current study, olive mill wastes, particularly olive mill wastewaters, have recently been reported to offer promising opportunities to overcome the problem at hand.

Olive mill wastewaters (OMW) are known to contain a number of biologically-active substances with promising potentials. The phytotoxic and antimicrobial properties of these residues have been extensively investigated and are associated with the presence of phenolic compounds (Mekki, Dhouib, Aloui, & Sayadi, 2006; Obied et al. 2005). Several investigators have reported on the inhibition of plant and microbial growth by low-molecular-weight phenols present in OMW (Fiorentino et al., 2003). Hydroxytyrosol has been identified as one of the major natural phenolic monomers present in OMW that has powerful antimicrobial activity (Fiorentino et al., 2003). Many compounds, however, still remain unidentified and controversy still persists over the exact type and amounts of phytotoxic components in OMW. Raw OMW exhibits a broad spectrum of toxicity against bacteria, fungi, plants, animals and human cells (Capasso et al., 1995; Obied, Bedgood, Prenzler, & Robards, 2007). However, OMW fractional extracts and isolated biophenols demonstrate selective or minimal toxicity (Capasso et al., 1995). For instance, Gonzalez, Moreno, Quevedo-Sarmiento, and Ramos-Cormenzana (1990) found that the antimicrobial activity of OMW phenolic acids (tested separately) did not coincide with the inhibitory effect of OMW. Some investigators have evaluated the recovery of the biological activity in soil after its treatment with OMW. Piotrowska, Iamarino, Rao, and Gianfreda (2006) observed a complete recovery of seed germination 42 days after OMW had been applied at 40 m^3 ha^{-1}. Yangui, Rhouma, Gargouri, Triki, and Bouzid (2008a, 2008b) reported on the phytopathogen suppression capacity of OMW. This helped demonstrate that OMW could be safely used as a pre-plant or seed disinfectant in plantation fields with no phytotoxic effect on plant development and crop yield.

This being so, the present study was undertaken to evaluate the potential that natural phenolic aggregates taken from olive mill wastewater might have as a biobased pesticide against a variety of seed infections. It aimed to evaluate the hypothesis that a series of biochemical compounds that are naturally-occurring in OMW could act against various seed infections. Treatment with purified and unpurified hydroxytyrosol-rich composition, and vigour-related growth parameters were also evaluated.

2. Materials and methods

2.1. Plant material

Seeds of tomato (*Lycopersicon esculentum*) and muskmelon (*Cucumis melo*) were obtained from Agricultural Garden Seeds (Sfax, Tunisia) in 11/2007 and were also obtained from the "National Institute of Agronomic Research of Tunisia (INRAT), under organic conditions and stored under normal conditions. Seeds were washed five times with sterile water, surface sterilised with 10% (v/v) commercial bleach for 15 min, and subjected to 3–4 further washes in sterile distilled water.

2.2. Preparation of hydroxytyrosol-rich composition

2.2.1. Preparation of hydroxytyrosol-rich OMW (HROMW)

A sample of about 20 l of fresh olive mill wastewater (FOMW) was taken in February 2006 from a three-phase continuous extraction factory located in Sfax, Tunisia. A fraction of 500 ml of this FOMW sample was used for physico-chemical characterisation. The pH and electrical conductivity (EC) were determined according to the standard of Sierra, Marti, Montserrat, Cruanas, and Garau (2001). Total organic carbon was determined by dry combustion (TOC Analyser multi N/C 1000). Total nitrogen was determined by Kjeldahl (1883) method. Chemical oxygen demand (COD) was determined according to Knechtel (1978) standard method. Phosphorus, iron, magnesium, potassium, sodium, calcium and copper were determined by atomic absorption (AAnalyst 200, Perkin–Elmer, Waltham, MA). The main characteristics of FOMW are: Total C measured 18.2 g l^{-1}, $N_{Kjeldahl}$: 0.5 g l^{-1} with a C:N of 36.4, P: 36.1 mg l^{-1}, K: 1.45 mg l^{-1}, Fe: 2.58 mg l^{-1}, Mg: 1.08 mg l^{-1}, Ca: 11.51 mg l^{-1}, Cu: 0.15 mg l^{-1}, Na: 1.59 mg l^{-1}, pH: 5.2, EC: 7.8 dS m^{-1}, and chemical oxygen demand was 63.5 g l^{-1}. The remainder of the FOMW sample was used to produce HROMW, according to the methods of Bilter et al. (2005) and Crea and Mateo (2008), by acidification with acetic acid to pH 3 and incubation for 6 months (from February to July) in the dark and at room temperature (varying between a minimum of 8–10 °C by night in February and a maximum of 38–40 °C by day in July). The supernatant was ultrafiltered using a 100 kDa pore size.

2.2.2. Preparation of hydroxytyrosol-rich composition (HRC)

In order to achieve the deodorisation, decolourisation and, above all, the removal/recovery of the hydroxytyrosol-rich composition, the filtered HROMW was passed through a series of XAD4, XAD7HP, and XAD16 adsorbent resins according to the procedure of Agalias et al. (2007). The hydroxytyrosol-rich composition obtained was concentrated 10 times to honeyed liquid under vacuum at 45 °C using a Büchi Rotavapor (Büchi Laboratories, Flawil, Switzerland).

2.2.3. Characterisation of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC)

Total phenols, total flavonoids and total flavonols were determined using Folin–Ciocalteu reagent according to the methods of Miliauskas, Venskutonis, and Van Beek (2004), Singleton and Rossi (1965) and Zqhishen, Mengcheng and Jimming (1999), respectively. Results were expressed on a dry weight basis as gallic acid equivalents (GAE), catechin equivalents(CE) and rutin equivalents (RE), respectively.

The identification and quantification of phenolic monomers were carried out by HPLC and LC–MS analysis as described by Bouaziz, Fki, Jemai, Ayadi, and Sayadi (2007). The main characteristics of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC) are given in Table 1 and Fig. 1.

2.3. Microorganisms

Fusarium sambucinum, *Verticillium dahliae*, *Alternaria solani* and *Pseudomonas syringae* pv tomato were originally isolated in 2003 from tomato plants that exhibited the main characteristic symp-

Table 1
Physicochemical characteristics of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC).

Characteristics	FOMW	HROMW	HRC
pH (25 °C)	5.2 ± 0.2	4.5 ± 0.2	6.5 ± 0.2
Total solids (%) (w/v)	6.28 ± 0.9	2.87 ± 1.9	13.1 ± 0.2
Mineral matter (%) (w/v)	0.68 ± 0.3	1.14 ± 0.1	5.7 ± 0.2
Volatile solids (%)(w/v)	5.7 ± 0.5	1.81 ± 0.2	7.4 ± 0.3
Total phenols (PyE) (%, w/dw)	5.45 ± 0.3	7.39 ± 0.38	27.5 ± 1.8
Flavono (CaE) (%, w/dw)	1.13 ± 0.06	2.30 ± 0.11	20 ± 0.8
Flavonols (RuE) (%, w/dw) 0.05	± 0.01	0.06 ± 0.01	0.8 ± 0.3
Hydroxytyrosol (%, w/dw)	0.3 ± 0.1	29.3 ± 3.4	52.7 ± 4.3
K (%, w/dw)	0.44 ± 0.02	0.28 ± 0.02	ND
Fe (%, w/dw)	0.26 ± 0.03	0.07 ± 0.01	0.41 ± 0.05
Ca (%, w/dw)	1.15 ± 0.1	0.21 ± 0.02	ND
Cu (%, w/dw)	0.02 ± 0.002	0.01 ± 0.002	0.01 ± 0.002
Mg (%, w/dw)	0.11 ± 0.01	0.11 ± 0.01	ND

Values are mean ± standard deviation of three repetitions.
CaE: catechin equivalent.
ND: not detected.
PyE: pyrogallol equivalent.
RuE: rutin equivalent.

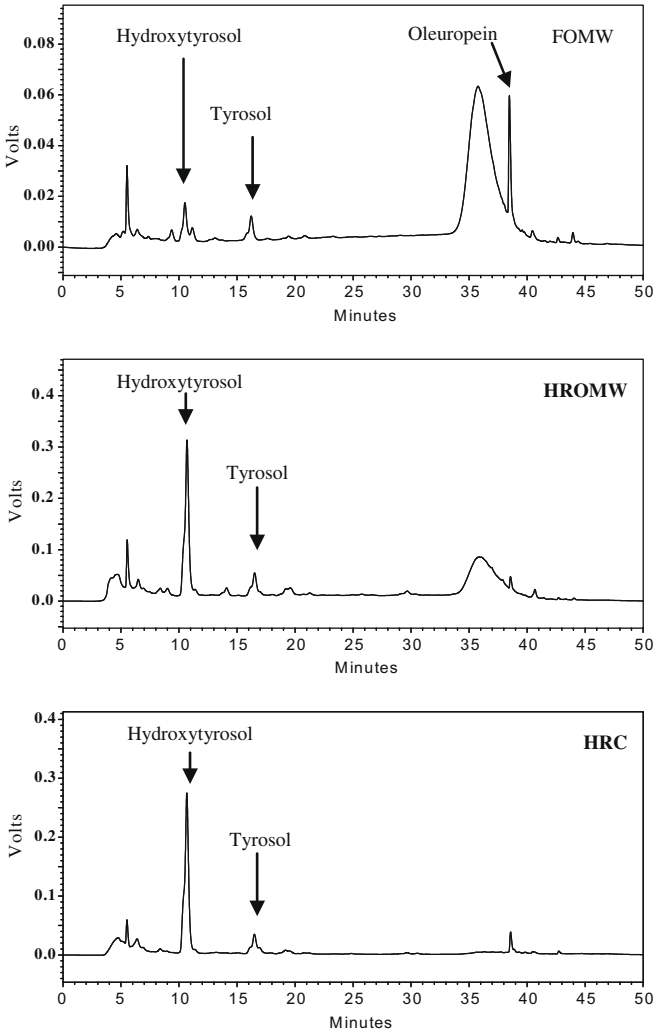

Fig. 1. HPLC chromatogram (UV 280 nm) of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC).

toms and were identified and provided by Institut de l'Olivier de Sfax, Tunisia. *Xanthomonas campestris* seed-borne phytopathogenic bacterium producing leaf spot symptoms in cruciferous plants was kindly provided by Pr. Maria Lopez from IVIA, Spain. For long-term conservation, the isolates were deposited at the Centre of Biotechnology of Sfax culture collection. For short-term conservation, bacteria were subcultured on nutrient agar (Difco; BD, Franklin Lakes, NJ) and fungi on malt extract agar (Difco) slant tubes at 25 °C for 72 h, then stored at 4 °C. Prior to use, the bacterial strains were cultured overnight on nutrient broth (Difco) and inocula were prepared by adjusting the turbidity of each bacterial culture to reach an optical comparison to that of a 0.5 McFarland standard, corresponding to approximately $1–5 \times 10^6$ CFU ml^{-1}. Fungi were cultured on malt extract agar plates for 7 days until sporulation, then the spore suspension of each fungal culture was prepared by pouring sterile buffered dilution water (SBDW) containing 0.1% of Tween 80 over the individual fungal cultures. The concentration of spore suspensions was determined by using a haematocytometer (Thoma cell) and adjusted to $1–5 \times 10^6$ spores per ml.

2.4. Bioassays

2.4.1. Antimicrobial activity

Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC).

MICs, MBCs and MFCs were determined by NCCLS (2000) broth dilution method. The HROMW and HRC were 2-fold serially diluted for bacteria in nutrient broth or for fungi in malt extract broth. MIC was taken as the interval of concentration between the highest dilution of HROMW and HRC that showed no detectable growth and the subsequent dilution. MFC was determined by sub-culturing all tubes that showed no visible growth.

2.4.2. Bactericidal and fungicidal activities

The bactericidal and fungicidal activities of the HROMW and HRC as natural disinfectants were evaluated according to the European Standard methods EN 1276 (1997b) and EN 1275 (1997a) under dirty conditions (3 g l^{-1} bovine albumin, 300 mg kg^{-1} $CaCO_3$) and the method of choice was the dilution-neutralisation method with 3% Tween 80, 3% saponin, 0.1% histidine, 0.1% cysteine as neutraliser and sterile hard water (300 mg kg^{-1} $CaCO_3$) as diluents. The HROMW and HRC-test concentrations were 0.5, 0.75, l.0, and 1.25% (w/v) for bactericidal activity and 1.25, 1.5, 3.0, and 6.0% (w/v) for fungicidal activity. The contact time and test temperature were: t = 5 min ± 10 s and θ = 20 °C ± 1 °C, respectively.

2.4.3. Phytotoxicity

Phytotoxicity was assessed by the determination of the germination index at two times, 36–72 h for tomato and 25–50 h for muskmelon, according to the standard method of Zucconi, Forte, Monac, and Beritodi (1981). Post-germinated seeds were transplanted in a sterile potting mix, as described in Section 2.4.4 below. Traditional seed vigour biomarkers were determined based on the total numbers of seedlings that fully emerged, shoot height, shoot weight and root length.

2.4.4. Seeds disinfection bioassays

Seed disinfection was carried out in two steps. The first step consisted of assessing all microorganisms in Petri dishes; the second involved the transplantation of the germinated seeds of fungi that exhibited symptoms of damping-off, root rot and wilt at the juvenile stage of tomato growth in plug trays.

For each microorganism, 50 g of bleached seeds were infected with 1% (v/w) of the microbial suspension which contained approximately 10^6 spores ml^{-1} for phytopathogenic fungi or 10^8 cfu ml^{-1} for phytopathogenic bacteria, and the infected seeds were then dried at room temperature for about 8 h. The contaminated seeds were divided into four parts. The first part was disinfected by placing the seeds in contact with a 10% (dw/v) sodium

hypochlorite solution for 30 min and then rinsed three times with sterile distilled water. The second part was mixed with HROMW at the ratios of 10% and 1% (dw/v) for fungi-infected and bacteria-infected seeds, respectively. The third part was mixed with HRC, using the same dosage rate used for HROMW. The fourth fraction was used, untreated, as control. After 24 h of incubation at room temperature, the seeds were transplanted into Petri dishes (10 seeds/Petri dish of 9 cm in diameter) containing sterile potting mix (twice autoclaved). The Petri dishes were then incubated at 27 °C for 52 and 72 h for muskmelon and tomato seeds, respectively. After that, the contaminated seeds were counted and the germination index was calculated, according to the method of Zucconi et al. (1981). This step was assessed to determine whether HROMW and HRC had disinfestation or disinfection properties. Accordingly, post-germinated tomato seeds were planted in a sterile potting mix (twice autoclaved) and grown in seedling plug trays (plug size 3 cm × 3 cm × 5 cm, 120 plugs per tray). The plug trays were placed under greenhouse conditions and monitored for a growing period of 4 weeks. The plants showing characteristic symptoms of the disease were noted.

2.5. Statistical analysis

The trial was established according to the randomised plots experimental design with three triplicates, including 30 plants in each replicate. Data were subjected to analysis of variance using SPSS software (Version 11; SPSS Inc., Chicago, IL). Mean values among treatments were compared by the Duncan's multiple range test at the 5% ($p = 0.05$) level of significance.

3. Results

3.1. Concentration of hydroxytyrosol in FOMW, HROMW and HRC

The analysis (Table 1 and Fig. 1) showed the effect of hydrolysis of complex phenols, such as oleuropein, into simple phenols, such as hydroxytyrosol, which was observed as a reduction in the peak height at 34–40 min and the amplification of the hydroxytyrosol peak at 10.5 min of retention. Moreover, an almost complete reduction in complex phenols detectable at 280 nm was observed for HRC with the phenols removed by separation on resin. Hydrolysis occurred during the storage of acidified FOMW for 6 months, and purification steps was also observed in the analyses. As the stages of processes progressed a significant increase in the content of hydroxytyrosol, total phenols, flavonoids and flavonols was observed (Table 1). After hydrolysis, the hydroxytyrosol concentration shifted from 0.2 g l^{-1} corresponding to 0.3% (w/dw) in FOMW to 8.4 g l^{-1} corresponding to 29.3% (w/dw) in HROMW, and the hydroxytyrosol yield of this step was 0.82% (w/v) (Table 1). After decantation, filtration and separation on resins and 10-fold concentration by water evaporation, the hydroxytyrosol concentration increased to 69 g l^{-1}, corresponding to 52.7% (w/dw) in the HRC.

3.2. Fungicidal and bactericidal potential of HROMW and HRC

Table 2 shows MICs, MBCs or MFCs of HROMW and HRC determined on the five microorganisms tested. Both HROMW and HRC inhibited the two bacteria tested, with MIC and MBC values ranging from 7.18 to 14.4 mg l^{-1}. For fungal strains, the HROMW MIC values ranged from 7.18 to 28.7 mg l^{-1} and the HRC MIC values ranged from 14.4 to 57.4 mg l^{-1}. As for those of HROMW and HRC, the MIC values were equal to the MFC values for *A. solani* and *V. dahlia* but only half those of the MFC values for *F. sambucinum*.

HROMW and HRC were bactericidal against both types of phytopathogens, *Pseudomonas* and *Xanthomonas*. Indeed, more than 5 log units of reduction in viability of *P. syringae* were achieved at a dosage rate of at least 1%, even after 5 min (Table 3). But, higher HROMW and HRC concentrations (1.25%) were actually needed to obtain 5 log units of reduction in viability for *Xanthomonas* spp. (Table 3). In the case of phytopathogen fungi, the antifungal activity shown by HROMW and HRC was moderately significant when compared to bactericidal activity. The fungicidal activity of HROMW and HRC against *A. solani* was obtained at the concentration of 3% after 5 min of contact. When the lowest dose recommended by the standard test method EN 1275 1.5% (w/v) was employed, the fungicidal activity against *V. dahlia* was achieved with the two products tested after 30 min of contact time (Table 4). *F. sambucinum* was not sensitive to the two extracts tested. In fact, a dosage rate (10%) that was higher than the one recommended by the standard test method EN 1275, 6% (w/v) was necessary to achieve the 4 log units of reduction in the viability of this fungus (Data not shown).

3.3. Phytotoxicity effect of HROMW and HRC on plant species

Seed germination was conducted on both HROMW (one dose was applied, 10%, which was used for the disinfection treatment) and HRC, and were used in comparison to those treated with water (negative control) and with sodium hypochlorite (positive control). The results showed that seed germination was strongly inhibited for the two species studied when treated with both sodium hypochlorite and HROMW (Fig. 2). HRC did not show any inhibitory effect on seed germination and the two crops represented close ratios of germination when compared to the control subjected to water treatment (Fig. 2). The application of HRC improved the germinative energy of the seeds. The index of germination at the half-period of germination was 36 and 25 h for tomato and muskmelon, respectively, and was significantly higher than that of the negative controls (Fig. 2). Treatments with HROMW and sodium hypochlorite, however, have yielded a germinative energy that was lower than the optimum (ratio <50%).

Initial vigour response was measured using traditional agronomic parameters, such as germination percentage, root length and shoot weight and height. Results indicated that vigour response was, indeed, higher in response to HRC than the other treatments (Fig. 3). HROMW, on the other hand, negatively affected the

Table 2
Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of HROMW and HRC using NCCLS (2000) method.

Microorganisms	HROMW		HRC	
	MIC (mg l^{-1})	MBC/MFC (mg l^{-1})	MIC (mg l^{-1})	MBC/MFC (mg l^{-1})
Alternaria solani	7.18–14.36	7.18–14.36	14.36–28.72	14.36–28.72
Fusarium sambicinum	14.36–28.72	28.72–57.44	28.72–57.44	57.44–114.88
Verticillum dahliae	7.18–14.36	7.18–14.36	14.36–28.72	14.36–28.72
Pseudomonas syringae	7.18–14.36	7.18–14.36	7.18–14.36	7.18–14.36
Xanthomonas campestris	7.18–14.36	7.18–14.36	7.18–14.36	7.18–14.36

Table 3
Evaluation of bactericidal activity of HROMW and HRC using European Standard EN 1276 (1997b) method in dirty conditions and 5 min of contact time.

Bacteria test	N: Bacterial test suspension (cfu ml^{-1})	HROMW		HRC	
		Test procedure at concentration % (dw/v) .			
		1.0	1.25	1.0	1.25
		Reduction in viability (cfu ml^{-1})			
Pseudomonas syringae	N: 2.1×10^8	1.5×10^5	2.6×10^5	1.3×10^5	1.9×10^5
Xanthomonas campestris	N: 1.3×10^8	$<10^5$	1.3×10^5	$<10^5$	1.1×10^5

Table 4
Evaluation of fungicidal activity of HROMW and HRC using European Standard EN 1275 (1997a) method in dirty conditions and 30 min of contact time.

Fungi test	N: Spore test Suspension (cfu ml^{-1})	HROMW			HRC		
		Test procedure at concentration % (dw/v)					
		1.5	3.0	6.0	1.5	3.0	6.0
		Reduction in viability (cfu ml^{-1})					
Fusarium sambicinum	N: 5.5×10^6	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$
Alternaria solani	N: 1.1×10^6	$<10^4$	1.4×10^4	6.9×10^4	$<10^4$	1.1×10^4	2.7×10^4
Verticillum dahliae	N: 2.0×10^6	2.0×10^4	3.5×10^4	1.2×10^4	1.5×10^4	2.3×10^4	2.3×10^5

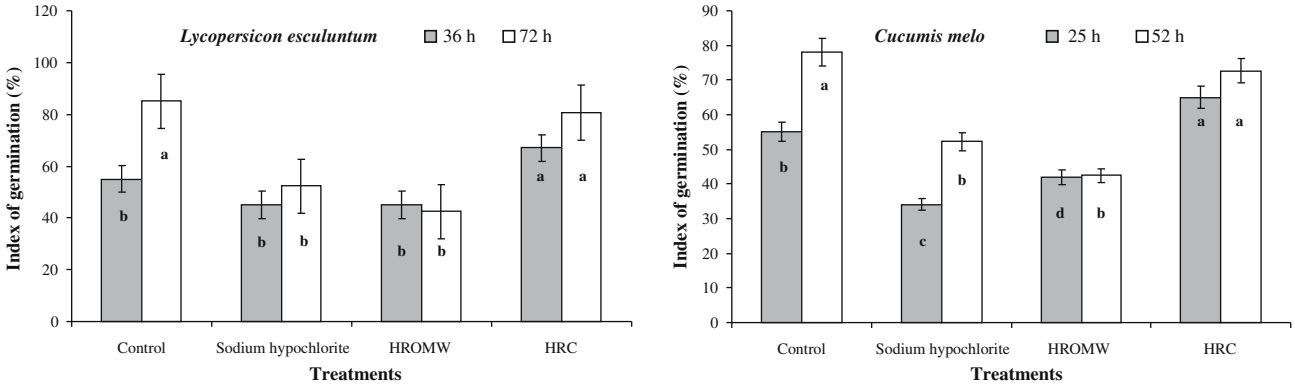

Fig. 2. Germination index of *Lycopersicon esculuntum* and *Cucumis melo* determined on distilled water (control), sodium hypochloride, HROMW, and HRC. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

vigour response of seeds with a significant reduction in root heights as well as in shoot heights and weights. These results were comparable to those of sodium hypochlorite treatment, where the seed vigour biomarkers were drastically affected.

3.4. Disinfectant property

The trials carried out in Petri dishes demonstrated that HRC was able to inhibit the growth of the seed-borne diseases used. In fact, HRC allowed for a highly significant control of the damping-off disease groups on the treated seeds in the pre-germination stage (Figs. 4 and 5). With regard to the application of HROMW, highly significant differences were observed with the treated seeds, as opposed to the inoculated seeds (Figs. 4 and 5). A higher damping-off control was observed in the seeds subjected to HRC + *X. campestris* when compared to those subjected to HRC + *P. syringae* pv tomato (Fig. 4).

Furthermore, the trials carried out in plug trays demonstrated that HRC was able to control root rot caused by *F. sambucinum* and *A. solani*. Under greenhouse conditions, HRC also allowed for a highly significant control of this group of seed diseases (Fig. 5). Wilts were also significantly decreased in seeds subjected to HRC + *V. dahliae* treatment (Fig. 5). Likewise, HROMW was noted to be as effective as HRC against the three seed-borne diseases under investigation.

4. Discussion

Given the fact that OMW is generated for only 3–4 months a year, and to assure a continuous industrial activity of hydroxytyrosol production, a sufficient quantity of OMW must be provided during the year. In a previous survey Feki, Allouche, Bouaziz, Gargoubi, and Sayadia (2006) demonstrated that the storage of OMW is doubly beneficial; first, hydroxytyrosol concentration increased from 0.98 to 3.5 g l^{-1} during 5 months of OMW storage. As well, OMW storage resulted in the abolition of the centrifugation step necessary for the elimination of suspended matter. Bilter et al. (2005) and Crea and Mateo, (2008) also demonstrated the addition of acid to OMW in an amount effective to produce a pH between 2 and 4, and incubating OMW for a period of 6–12 months until at least 75–90% of the oleuropein originally present in OMW has been converted to hydroxytyrosol. The HROMW remained black-coloured; consequently, further purification steps through resin separation were deemed necessary to recover the HRC from the HROMW.

The antimicrobial effect of HRC was revealed by MIC and proved through basic method against most phytopathogenic fungi and

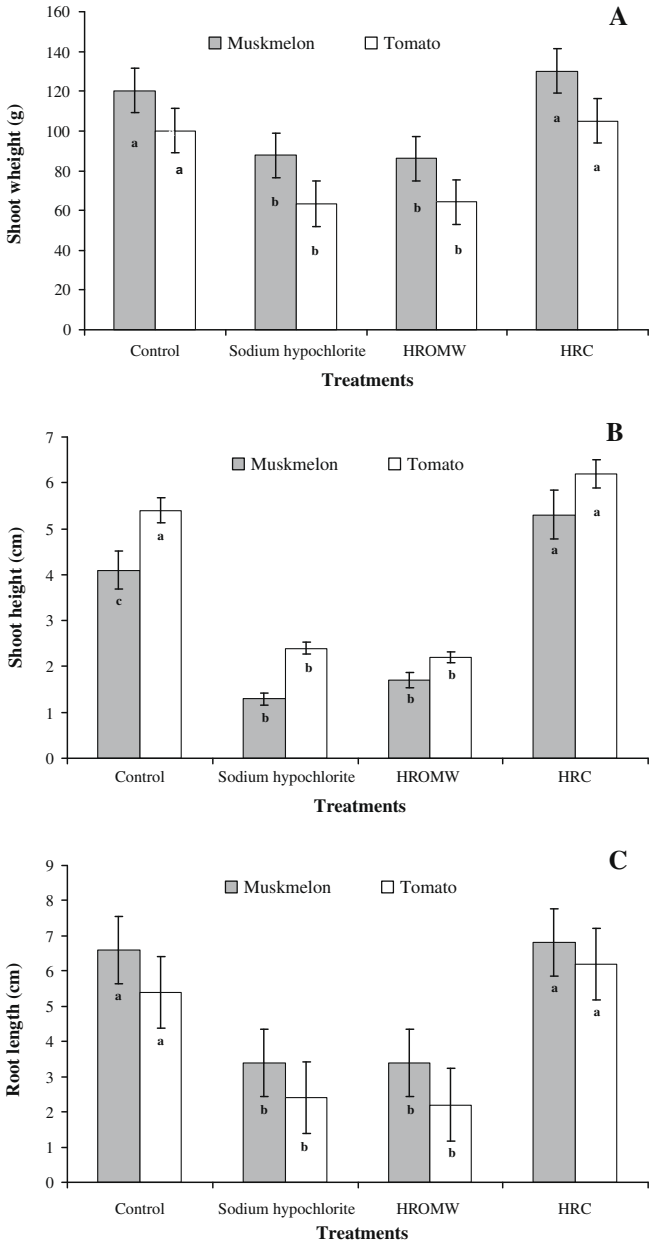

Fig. 3. The effect of germination index of distilled water (control), sodium hypochloride, HROMW, and HRC on (A) shoot weight; (B) shoot height; and (C) root length at 2 months after transplantation of tomato (*Lycopersicon esculuntum*) and muskmelon (*Cucumis melo*) plants. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

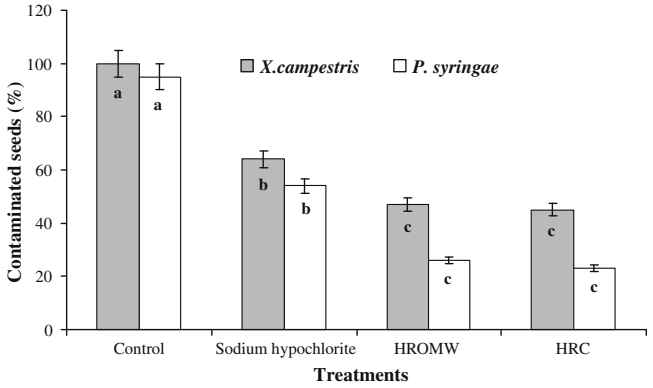

Fig. 4. Disinfection effect of water (control), sodium hypochloride, HROMW, and HRC on percentage of contaminated seeds by *Xanthomonas campestris* and *Pseudomonas syringae* pv. Tomato. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

bacteria. Hence, most plant extracts show activity against Gram-positive bacteria. Activity against Gram-negative bacteria and fungi is also considered a critical measure of success (Ezzoubeiri et al., 2005). It was pointed out in previous studies, however, that Gram-positive bacteria are more susceptible to antimicrobial extracts of plants than the Gram-negative ones (Ezzoubeiri et al., 2005). The different activities against Gram-negative and Gram-positive bacteria may be rationalised by considering differences in cell wall composition. Gram-negative bacteria have a lipopolysaccharide component in their outer membrane that makes them more resistant to antibacterial compounds. As far as the current study is concerned, the hydroxytyrosol-rich composition inhibited the growth of Gram-negative bacteria within the recommended doses of application. The effectiveness of HRC could be attributed to the chelating of transition metals by polyphenols. Wong and Kitts (2006) reported that phenolic compounds are capable of chelating transition metals and lowering the reactivity of metal iron by forming an inert metal–ligand complex. Chelation of transition metals, such as iron and copper, reduces bioavaibility for microbial growth.

The control of tested phytopathogenic fungi is a very difficult process, even with the application of pre-harvest fungicides (Polashock, Ehlenfeldt, Stretch, and Kramer, 2005). Sampedro, D'Annibale, Ocampo, Satazi, and Gargia-Romera (2005)) demonstrated the capability of a saprophytic *Fusarium* to grow on a medium rich with phenolic compounds. This can explain the inefficacy of hydroxytyrosol-rich solutions against the soil-borne *Fusarium* strain. The effectiveness of HRC against other phytopathogenic fungi, namely *Verticillium* and *Alternaria*, can be related to the altering of microbial permeability, which can permit the loss of cytoplasmic macromolecules.

It is widely acknowledged, and as is found in numerous references, products that are rich in oleuropein demonstrate antimicrobial activity against a variety of viruses, bacteria, yeasts and fungi (Aziz, Farag, & Mousa, 1998; Bisignano et al., 1999; Fleming, Walter, & Etchells, 1973; Tassou & Nychas, 1995; Tassou, Nychas, & Broad, 1991). Since higher antibacterial effects were observed using HRC, the latter should be examined in terms of its powers as a new antimicrobial intensifier. The present *in vitro* study demonstrated that the experimental antiseptic and/or disinfectant were undeniably effective in inhibiting plant pathogens. This could be of particular interest to the nurserymen whose plants have always suffered from fungal disease pathogens. It is common for the nurserymen in Tunisia to reuse their propagation trays, which may contain the remains of contaminated substrates. Several approaches have been tried to remove the pathogens from those trays (Soriano et al. 2006), none of which is fully satisfactory. Disinfectants, such as sodium hypochlorite or formalin, can cause serious toxicity problems in young plants and create severe risks to handlers and the environment (Ciafardini & Zullo, 2003; Soriano et al., 2006). HRC can, therefore, be considered a strong alternative candidate for the current inadequate methods involving the removal of the harmful disinfectants in both seed and propagation trays.

HRC did not exhibit any inhibitory effects on seed germination and two crops presented a high germination ratio (>50%) even at half-period of germination. It can be inferred that the germination inhibition is basically due to the phenolic compounds that are present at high levels in HROMW. This is in line with previous findings by Mekki, Dhouib, Feki, and Sayadi (2008), Komilis, Karatzas, and Halvadakis (2005) and Piotrowska et al. (2006), which demonstrated that the germination inhibitory effect is partly due to the

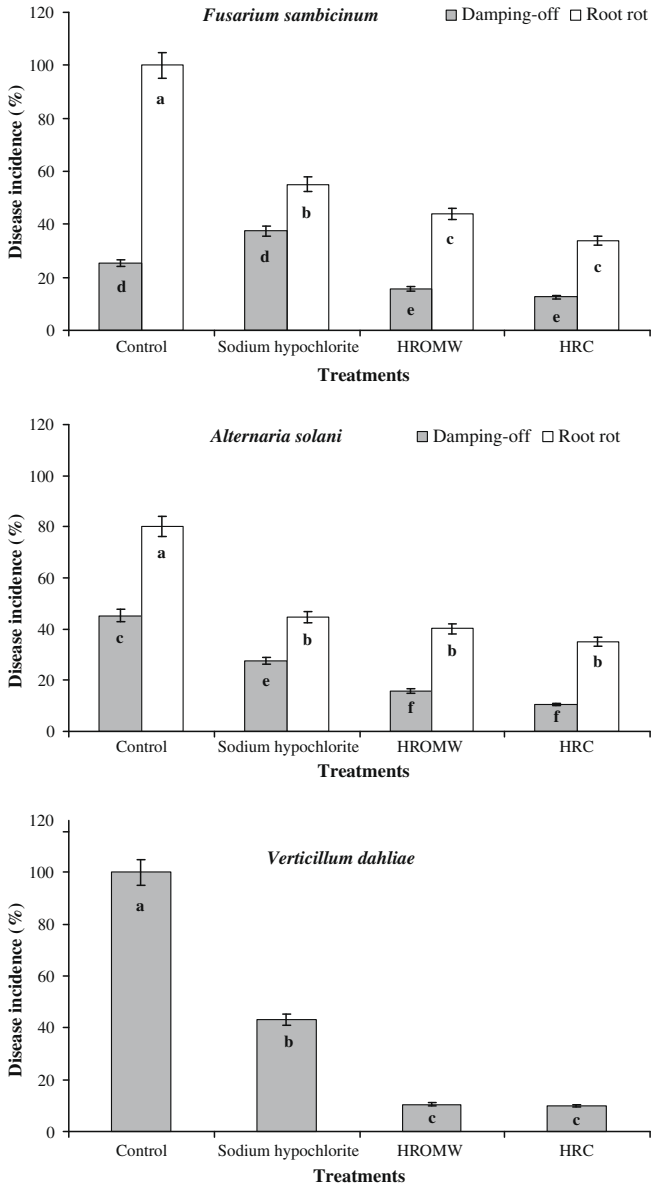

Fig. 5. Effect of treatments by distilled water (control), sodium hypochloride, HROMW, and HRC on the percentage of damping-off and root rot incidence of tomato (*Lycopersicon esculuntum*) plants caused by *Fusarium sambicinum* and *Alternaria solani* and on the percentage of *Verticillum* wilt incidence of tomato (*Lycopersicon esculuntum*) plants caused by *Verticillum dahliae*. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

high amount of phenolic compounds and partly to the high molecular weight of phenolics present in untreated OMW. Gonzalez et al. (1990) reported that the antimicrobial activity of phenolic acids (tested separately) did not coincide with the inhibitory effect of OMW. Obied et al. (2007) also reported that crude OMW has a broad spectrum of toxicity against bacteria, fungi, algae, plants, animals and human cells and added that fractionated OMW extract and isolated biophenols, on the other hand, demonstrate selective or minimal toxicity. This could explain the reason why HRC had an antimicrobial effect with no phytotoxic effects (Table 1). In addition, the relatively high salinity of the HROMW solution may affect the germinability of the crops. This seems similar to the findings of Ramana, Biswas, Kundu, Saha, and Yadava (2002) who reported that in some crop species, such as tomato, cucumber, chilli and onion, the increase in the concentration of the highly saline effluent was paralleled by a similar decrease in the percentage of germination. The germination rate, in particular, is immensely important, for the increase in germination is usually followed by an improvement in overall seedling performance (Parera & Cantliffe, 1991). The findings of the current study indicated that the tomato and muskmelon seedlings treated with HRC similarly showed higher and average germination rates, when compared to the HROMW solution and control seedlings, respectively. Root lengths and shoot weights and heights are all vital indices of seeds' vigour. Results indicated that vigour response, in terms of those factors, is a useful approach to meeting productivity of crops (Perry, 1978). With regard to improving seed vigour through the stimulation of phenolic synthesis, it has often been suggested that exogenously applied phenolic antioxidants may be able to stimulate the endogenous phenolic content in plants (Randhir & Shetty, 2003). This stimulation is hypothesised to involve the concurrent activation of antioxidant enzyme metabolites which are dependent upon reductants (NADPH) obtained as a by-product of carbon flux through the pentose phosphate pathway, which is, itself, the source of sugar phosphate precursors required for phenolic synthesis (Randhir & Shetty, 2003).

Quick seedling emergence and even stands are essential in maximising the yield of all crops. The use of high quality, disease-free seed is the first step to producing stands. The application of HRC treatment improved the germinative energy measured at half-period of germination. Germinative energy can play an important role in achieving quick and uniform seedling emergence and in reducing damping-off incidence, thus improving the yield. Gupta et al. (1999) reported that the use of fungicides is effective in enhancing germination, emergence and growth as well as in reducing damping-off. In addition, accelerated germination is reported to help improve stress resistance and enhance overall plant growth and productivity (Pattan, Gothkar, Joshi, Chivasa, & Nyamudeza, 2001). This could account for the usefulness of the HRC solution for seed disinfection.

A further transplantation of post-germinated seeds demonstrated the compelling disinfection effect of both HRC and HROMW, and not a mere surface disinfestation of seeds. Yields were higher when seeds were treated by HRC, which led to earlier and better emergence and to improved control of both damping-off/root rot and wilt caused by *Fusarium*/*Alternaria* and *Verticillium*, respectively. Valenciano et al. (2004) demonstrated that the use of pesticides for the protection and/or disinfection of seed improved yields because it increased the number of established plants. A negative correlation exists between yield and the presence of disease in the soil and/or in the seeds because the loss of plants from root rot has a huge impact on yield (Pedroza, Teliz, de la Torre, & Campbell, 1994). Thus treatment is a fundamental operation because the initial stage in plant development is the most susceptible to adverse environmental conditions (Valenciano et al., 2004). Sodium hypochlorite is the most used solution for the disinfection of seeds as well as of the equipment used in the production of plants (Soriano et al., 2006). Nevertheless, and due to its highly corrosive nature, sodium hypochlorite must be used with extreme caution. As a matter of fact, it can seriously hurt the seeds and reduce germination. This justifies the urgent need for research on efficient alternative substitutes for the current disinfestations, disinfections or protections used for seed and propagation equipment. The results obtained demonstrate that HRC represents a class of natural products that offers an efficient substitute for the currently used commercial corrosive disinfectants such as sodium hypochlorite. In fact, it can be used to control the most resistant fungal structure (chlamydospores) produced by the species of *Fusarium* (Mavrogianopoulos, Frangoudakis, & Pandelakis, 2000), *Alternaria*, *Verticillium*, and even *Xanthomonas* and *Pseudomonas*, without causing any damage to the germination ability of crops.

In short, HRC has proven to be a solution that: (1) has high element content; (2) promotes early vigour and improves plant health; (3) provides protection against pathogens which attack germinating seeds and emerging seedlings; (4) aids in the production of healthy vigorous crops and higher yields; (5) promotes quick emergence and crop uniformity; (6) is easy to use and simple to clean up with water; and (7) is environmentally safe and not harmful for plants, animals and humans.

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