SHORT COMMUNICATION

GROWTH INHIBITION OF CLAVIBACTER MICHIGANENSIS subsp. MICHIGANENSIS AND PSEUDOMONAS SYRINGAE pv. TOMATO BY OLIVE MILL WASTEWATERS AND CITRIC ACID

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SUMMARY

Effects of citric acid and olive mill wastewaters on the growth of seed-borne bacterial pathogens of tomato, Clavibacter michiganensis subsp. michiganensis and Pseudomonas syringae pv. tomato were investigated. Bacteria were exposed to citric acid (0.1 mol l$^{-1}$) and 10 fold-diluted filter-sterilized olive mill wastewaters and their growth was determined by the plate count method. Citric acid inhibited the growth of both bacteria. The minimum inhibitory concentration (MICs) of citric acid, determined by the broth dilution method ranged between 1.56 to 3.13 mmol l$^{-1}$ for C. m. michiganensis, and was 1.56 mmol l$^{-1}$ for P. syringae. Olive mill wastewaters inhibited the growth of both bacteria in most experiments. In some, however, a slight growth or reduction in size of the colonies of C. m. michiganensis was observed. Neither treatment showed negative effects on seed germination. Results of this study suggest that citric acid at 0.1 mol l$^{-1}$ concentration can prove useful for the elimination of both pathogens from tomato seeds.

Key words: Plant pathogenic bacteria, organic acids, biophenols, antimicrobial agent, growth inhibitors

INTRODUCTION

Olive mill wastewaters (OMW) cause environmental concerns due to their richness in toxic compounds such as low-degradable biophenols (De Marco et al., 2007). OMW can reduce the population of the soil bacterium Bacillus megaterium (Paredes et al., 1986; González et al., 1990), can be phytotoxic (Capasso et al., 1992), can seep through the soil reaching underground water, and can increase soil salinity (Obied et al., 2007). On the other hand, OMW can be used as fertilizer (Paredes et al., 1999), weed suppressor (Boz et al., 2003) and show antifungal and antibacterial properties due to the high content of phenolics (Capasso et al., 1995; Aziz et al., 1998) and other compounds (González et al., 1990; Capasso et al., 1992). Capasso et al. (1995) studied the effects of raw OMW on Pseudomonas savastanoi pv. savastanoi and Clavibacter michiganensis subsp. michiganensis showing their bactericidal effects. Ciafardini and Zullo (2003) proved that OMW phenols, caffeic acid in particular, had no negative effect on cauliflower seed germination when used to control Xanthomonas campestris pv. campestris.

Citric acid is effective in controlling food-borne yeasts and bacterial pathogens (Ananou et al., 2007; Nielsen and Arneborg, 2007), its antimicrobial properties resting primarily in the chelation of divalent cations (Imai et al., 1970; Graham and Lund, 1986). However, citric acid has not been tested against many plant pathogenic bacteria. A study was therefore conducted for evaluating the effects of OMW and citric acid on the growth of the tomato seed-borne pathogens Clavibacter michiganensis subsp. michiganensis and Pseudomonas syringae pv. tomato.

C. m. michiganensis ICMP 2550 (International Collection of Micro-organisms from Plants, Auckland, New Zealand) and P. s. tomato ICMP 2844 were used in the experiments. Tested inhibitors were 10-fold diluted OMW at pH 4.6, and citric acid (Carlo Erba, Italy) at 0.1 mol l$^{-1}$ concentration and pH 2.0. OMW was obtained from a mill near Çakırbeylı, Aydın, Turkey and was filter-sterilized before use because in preliminary experiments some unidentified bacteria and fungi were observed when it was plated on agar medium.

To test bacterial growth inhibition by the plate count method, C. m. michiganensis and P. s. tomato cultures were grown on nutrient broth yeast extract agar (NBY, Schaad et al., 2001) at 24 °C for 96 h and 48 h, respectively. Four colonies from each culture were transferred to 9 ml of 1% nutrient broth glucose medium (NBG) in glass tubes. Liquid cultures were grown overnight by shaking at 55 rpm with a twist shaker (Isolab, Germany) at 26°C. Aliquots of 0.1 ml from these liquid cultures ($10^8$-$10^9$ cfu ml$^{-1}$) were transferred to tubes containing 0.1 mol l$^{-1}$ citric acid and 10-fold diluted OMW in 10 ml NBG, which were incubated under shaking at 26°C.
for 1.5 and 8 h. Untreated tubes were used as control and 0.1 ml aliquots from treated and untreated tubes were plated onto tryptic soya agar (TSA, Difco, Becton Dickinson, England) plates in triplicate. Plates were incubated for 24 to 48 h at 26°C for allowing the development of _P. s. tomato_ and _C. m. michiganensis_ colonies, respectively. Growth inhibition of _P. s. tomato_ by OMW was also assessed after exposure to treatment for 1 h and 45 min.

To determine the minimum inhibitory concentrations (MICs) of citric acid against both bacteria, the broth dilution method (EUCAST, 2003) was used. Liquid cultures of _C. m. michiganensis_ (1-2 x 10⁸ cfu ml⁻¹) and _P. s. tomato_ (1-2 x 10⁹ cfu ml⁻¹) grown overnight (17 h) in NBG were serially diluted to adjust bacterial concentrations to ca. 1-1.5 x 10⁵ cfu ml⁻¹. To obtain descending half concentrations, citric acid at the initial concentration of 0.1 mol l⁻¹ was prepared and diluted in 4 ml final volume of NBG in glass tubes. Serial dilutions followed so as to reach a final citric acid concentration of 0.024 mmol l⁻¹. Tubes with each citric acid concentration (50-0.024 mmol l⁻¹) were additioned with 0.1 ml of liquid bacterial cultures (1-1.5 x 10⁷ cfu ml⁻¹) and incubated at 26°C for 24 or 48 h for allowing growth of _P. s. tomato_ and _C. m. michiganensis_, respectively. A duplicate set of citric acid concentrations with no bacterial cultures added was prepared in NBG as control. Bacterial growth was assessed visually. MICs were determined in duplicate experiments as the lowest concentration where no visible bacterial growth (turbidity) was observed.

Effects of citric acid and OMW on seed germination were tested on tomato cv. H-2274. Citric acid at 0.1 mol l⁻¹ concentration was dissolved in 10 ml sterile distilled water (SDW) in sterile glass tubes. SDW was used as control. Tomato seeds were added to each tube, mixed briefly and incubated at 26°C for 1 h. Seeds were then removed by filtration and rinsed three times with 10 ml SDW. Rinsed seeds were dried on sterile filter paper, then placed onto sterile double-layered wet filter paper in sterile plastic 90 mm Petri dishes and incubated at 26°C. Each treatment had five replicates. Twenty three seeds were used per replicate. Seed germination was recorded at initial germination (4 days after incubation) and again at 6 to 7 days, when most of the seeds had germinated.

The effect of OMW on seed germination was determined as in citric acid-treated seed assays. Seeds treated with OMW for 1 h at 26°C, were rinsed three times in 25 ml sterile distilled water, dried and incubated as above.

Citric acid and OMW assays were repeated twice. Statistical analysis of the data from the first and second readings on seed germination was done using the SPSS Program (USA). Since no statistical difference was ob-

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*Fig. 1.* Growth inhibition of citric acid (0.1 mol l⁻¹) and OMW (diluted 1:10) on _Clavibacter michiganensis_ subsp. _michiganensis_ ICMP 2590. A. Experiment 1; B. Experiment 2. Results of the third experiment are not shown here since growth was inhibited completely by citric acid and OMW treatments as in Fig. 1B.

*Fig. 2.* Growth inhibition of _Pseudomonas syringae pv. tomato_ ICMP 2844 by citric acid (0.1 mol l⁻¹). This figure is representative of three independent experiments.
served between the two readings, results of first reading only are presented.

 Whereas citric acid completely inhibited the growth of *C. m. michiganensis* and *P. s. tomato* (Fig. 1 and 2). OMW was not equally effective in all experiments, but strongly reduced the growth of both pathogens (Fig 1 and 3). In particular, growth of *P. s. tomato* was completely inhibited in two experiments, while in two others bacterial colonies developed on TSA plates after 8 h incubation. OMW treatment caused approximately 7 log/ml reduction in the growth of colonies as compared with the control (Fig. 3). *C. m. michiganensis* showed very slight growth after 1 and 5 h incubation with OMW in one out of three experiments. In both cases, growth reduction was ca. 5 log/ml in comparison with the control (Fig. 1).

 MICs of citric acid were 1.56 mmol l⁻¹ for *P. s. tomato* and ranged between 3.13 and 1.56 mmol l⁻¹ for *C. m. michiganensis*.

 Citric acid and OMW had no negative effect on the germination of tomato cv. H-2274 seeds (Tables 1 and 2), as shown by the lack of statistical significance between the means of control (SDW), citric acid and OMW treatment data. In the first experiment, citric acid-treated and control seeds showed the same germination level (73%) and in the second experiment, germination of citric acid-treated seeds was 78% compared to 81% of the control (Table 1). OMW-treated seeds had 79% germination, slightly higher than that of the control (74%) in the first experiment and slightly lower in the second (69% versus 80%) (Table 2). The slight reduction in germination detected in the second experiment, may have been caused by residual OMW on the seed surface.

 Plant pathogenic bacteria such as *Pseudomonas savastanoi* pv. *savastanoi* and *C. m. michiganensis* (Capasso et al., 1995) were previously shown to be negatively affected by OMW (up to 100% growth inhibition at 1:1 and 1:2 dilution). A positive although lower effect (55% growth reduction) was also reported for *Pseudomonas tolaasii*.

![Fig. 3. Growth inhibition of *Pseudomonas syringae* pv. *tomato* ICMP 2844 by olive mill waste waters (OMW) (diluted 1:10). A-C. Experiments 1-3 respectively. Results of the fourth experiment are not shown since complete inhibition of bacterial growth was obtained as in Fig. 3B.](image)

### Table 1. Effect of citric acid on tomato seed germination.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Experiment 1⁺ (%) germination</th>
<th>Experiment 2⁺ (%) germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid (0.1 M)</td>
<td>73.40±12.05</td>
<td>77.80±9.39</td>
</tr>
<tr>
<td>Control (SDW)</td>
<td>73.80±8.76</td>
<td>81.20±9.39</td>
</tr>
</tbody>
</table>

⁺Means ± standard deviation.

### Table 2. Effect of OMW on tomato seed germination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1⁺ (%) germination</th>
<th>Experiment 2⁺ (%) germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMW (1:10 diluted)</td>
<td>78.60±11.74</td>
<td>68.60±8.26</td>
</tr>
<tr>
<td>Control (SDW)</td>
<td>74.40±7.64</td>
<td>79.80±8.33</td>
</tr>
</tbody>
</table>

⁺Means ± standard deviation.
Growth inhibitors of bacteria by OMW and citric acid

(Soler-Rivas et al., 2006). This is in line with the results of the present study showing that two seed-borne bacterial pathogens of tomato, i.e. the Gram negative P. s. pv. tomato and the Gram positive C. m. michiganensis are strongly inhibited by 10 fold-diluted OMW.

Some of the OMW-treated C. m. michiganensis colonies showed very slow growth (data not shown). This type of colonies was recognized 18 to 24 h after reading OMW-treated TSA plates. Interestingly, an adverse effect was reported by Della Greca et al. (2001) four days after exposure of algae to OMW, consisting of modification of the shape and colour of the cells.

Examples of the use of citric acid as seed treatment for controlling plant pathogens are few (Haggag and Abd El-Khair, 2007). The present results now show that citric acid can be a potential control agent against some plant pathogenic bacteria. Citric acid is inexpensive, easily applied to and removed from seeds, and does not cause irritation to the operators. Thus, it is a good candidate product to use in organic agriculture (Davies and Maw, 1972).

Although confirmation by further and more extensive experiments would be desirable, it seems plausible to conclude that citric acid and OMW can be safely employed as a seed treatment with no concern for phytotoxicity or negative impact on germination.

REFERENCES


