The effect of olive oil mill wastewater (OMW) on soil microbial communities and suppressiveness against *Rhizoctonia solani*

Maria Kotsou, Ioanna Mari, Katia Lasaridi, Iordanis Chatzipavlidis, Costas Balis, Adamantini Kyriacou

Abstract

The effect of olive oil mill wastewater (OMW), an important pollutant of agricultural origin, on soil suppressiveness against the plant pathogenic fungus *Rhizoctonia solani* was examined. Soil was treated with OMW and the pathogen was introduced into the soil 45 days after the last treatment, while lettuce seeds were planted at four successive time periods after pathogen introduction. The damping-off due to *R. solani* in soil that has been previously treated with OMW was significantly reduced compared to the control (soil treated with water). The effect on soil suppressiveness of the aerobically treated olive mill wastewater (TOMW) was also examined, but was not found to be significantly different from the control. The OMW treated soil exhibited significantly higher respiration compared to the other treatments during the period of waste addition, which immediately decreased when the additions stopped. The bacterial population of r-strategists in the OMW treated soil was significantly higher compared to the soil that received TOMW or water. A transitory phytotoxic effect of the wastes was also observed, but this did not correlate with soil suppressiveness. Results suggest that addition of OMW and initially TOMW to soil creates a nutrient rich environment that is dominated by r-strategists. Such an environment provides unfavourable growth conditions for *R. solani*.

Keywords

Olive mill wastewater; Soil; r/K-selection; Soil suppressiveness

1. Introduction

Olive oil mill wastewater (OMW) constitutes a major environmental problem especially for Mediterranean countries, where most of the world olive oil production takes place. The environmental implications from the uncontrolled disposal of OMW are mainly connected to their high organic load and their antimicrobial and phytotoxic properties (*Paredes et al., 1987, Rodriguez et al., 1988* and *Capasso et al., 1995*). Many researchers have established that these wastes have a high fertilizer value when applied to the soil; OMW is known to increase soil organic matter and the concentration of essential inorganic elements for plant growth resulting in enhanced soil fertility (*Bonari et al., 1993, Cabrera et al., 1996* and *Paredes et al., 1999*).
Several methods (physicochemical and biological) to reduce pollutant problems with OMW have been proposed (Borja et al., 1995, Benitez et al., 1997 and Aktas et al., 2001) and among them aerobic biological treatment has been recognized as the most economical and effective process. A particularly interesting aerobic bioremediation method is the one developed by the late Prof. Balis and his co-workers, using a selected strain of the nitrogen fixing bacterium Azotobacter vinelandii, which can grow well in OMW and transform it into an organic fertilizer and soil conditioner. The method produces treated olive-mill wastewater (TOMW) and has been particularly successful, with several pilot plants in Greece operating for more than 5 years (Balis et al., 1993, Balis et al., 1996 and Chatzipavlidis et al., 1996).

The main constituents of the organic fraction of OMW are proteins and sugars that are easily biodegradable and, to a lesser degree, organic acids, polyalcohols, fats, polyphenols, and others (Fiestas Ros de Ursinos and Borja-Padilla, 1996). The addition of fresh OMW to soil increases the number of soil microorganisms (bacteria, yeasts and fungi), and induces a change in the microbial community (Paredes et al., 1986, Moreno et al., 1987 and Tardioli et al., 1997). High energy organic materials induce a rapid but short term increase in the number of copiotrophic bacteria or r-selected species, which comprise the first colonizers of the newly added organic matter (Stenstrom et al., 2001 and Hu et al., 1999). The r-strategists are also reported to play an important role in the colonization of plant roots. Root tip exudates are absorbed and metabolised initially by copiotrophic bacteria, resulting in a fast increase in their population and a slower increase in oligotrophs (Semenov et al., 1999 and Kozdroj and van Elsas, 2000).

The use of organic amendments as a method for the biological control of soilborne plant pathogenic fungi has been reported by many researchers (Hoitink et al., 1997 and Abawi and Widmer, 2000). The mechanisms responsible involve general suppression, which is related to high microbial activity, and specific suppression, which is related to an increase in the population of specific microorganisms or groups of microorganisms that act as antagonists to the pathogen (Mazzola, 2002).

In the context of this study, the effect of the addition to the soil of (a) untreated olive mill wastewater (OMW) and (b) bioremediated olive mill wastewater using the Azotobacter vinelandii method (TOMW), on soil suppressiveness against the plant pathogen Rhizoctonia solani was investigated. The hypothesis examined was that the high bacterial populations, mainly of r-strategists (fast growing copiotrophic species), which develop in soil treated with OMW, are able to suppress disease caused by R. solani.

2. Materials and methods

2.1. Waste origin

OMW was obtained from a centrifugal olive mill in the area of Messinia, in South Greece and was kept at −20 °C until use (chemical characteristics: pH 4.9; COD: 89 g/l; organic matter content: 6.7%; TKN: 1100 mg/l; K: 8500 mg/l; P: 100 mg/l; Na: 75 mg/l; Ca: 340 mg/l; Mg: 330 mg/l; Cl: 1800 mg/l; Fe: 18.3 mg/l; Zn: 5.9 mg/l; Mn: 4.7 mg/l; Cu: 1.5 mg/l). The treated product (TOMW) resulted from the aerobic biological treatment of OMW using a strain of the diazotroph A. vinelandii. After pH correction to 8.0–8.5 using CaO, the wastewater was inoculated with A. vinelandii to a final concentration of 10⁶ cells/ml and was treated in a 5.5 l capacity rotating biowheel type reactor for approximately 5 days (Chatzipavlidis et al., 1996 and Ehaliotis et al., 1999). Its chemical characteristics were: pH 8.2; COD: 72 g/l; organic matter content: 5.1%; TKN: 1920 mg/l; K: 9800 mg/l; P: 110 mg/l; Na: 96 mg/l; Ca: 2700 mg/l; Mg: 627 mg/l; Cl: 1570 mg/l; Fe: 26.4 mg/l; Zn: 13.6 mg/l; Mn: 5.9 mg/l; Cu: 1.8 mg/l.

2.2. R. solani inoculum preparation

R. solani AG1-IB was kept as a slant culture with sclerotia at 4 °C. To ensure pathogenicity the pathogen was prior tested for the ability to infect lettuce seeds.
was added to the soil as a sand-cornmeal culture using a method adapted from Lumsden et al. (1983). The sand fraction with particle size between 0.5 and 2 mm was moistened with distilled water to 60% of its water holding capacity and autoclaved for 1 h at 125 °C twice, on two successive days. Cornflour (2%) was added and the mixture was autoclaved again for 20 min. The substrate was inoculated with the fungus (plugs from a PDA culture) and incubated for 40 days at 25 °C.

2.3. Soil treatments
A sandy silt soil (silt 49%, sand 39%, clay 12%; pH 7.7; electrical conductivity: 1.58 dS/m; organic matter content: 1.6%; water soluble K: 3.59 meq/l; water soluble Mn: 2.23 meq/l; P>40 mg/l; NO$_3$−: 26 mg/l; exchangeable cations in meq/100 g: Ca 15.23, Mn 1.46, K 0.76, Na 0.6) from the area of Messinia, South Greece, was used. The soil was obtained from a fallow field, was stored outdoors and was air dried and sieved (4 mm diameter) before use. The sequence of the experimental treatments is presented schematically in Fig. 1. The soil was placed in plastic pots (dimensions: 19 cm × 13 cm × 5 cm, 900 g d.w. of soil per pot) and was kept at 60% of its water holding capacity with the addition of OMW, TOMW or water until the total amount of the liquids added reached approximately 400 ml/kg d.w. of soil (phase 1). No other nutrients were added. This was followed by a period of 45 days (phase 2), during which the soil received only water, to eliminate OMW phytotoxicity. Subsequently the pots were inoculated with R. solani (6 g/kg d.w. of soil) and 20 lettuce seeds per pot (Cos Lettuce, Paris White, Pieterpikzen Heerenveen, Holland) were sown at 0, 5, 10 and 15 days after soil infestation (six replicates per treatment and sowing date). Pots were monitored for a period of 4 weeks after sowing. Controls (three replicates) that were not infected with the pathogen were used for every treatment and every sowing period. Experiments were carried out from May to September under ambient conditions.

Pre-emergence damping-off was estimated from the relationship

$$PD = 100 \times \left(1 - \frac{TG}{CG}\right)$$

where PD is the percentage of pre-emergence damping-off, TG is seed germination in the waste treatment and CG is germination in the corresponding control. Survival of lettuce seedlings was estimated from the relationship \(S=100 \times TS/CS\), where \(S\) is the percentage survival of the seedlings, TS is survival in the waste treatments and CS is the survival in the controls after 4 weeks.

Phytotoxicity of the wastes was estimated in the control pots (not inoculated with the pathogen) for each of the three treatments. Seed germination was calculated as the percentage of the sown seeds that germinated.

2.4. Microbial activity
A series of pots that had been subjected to the same protocol of liquid additions but not infested with the pathogen nor seeded, was used for microbial activity determination and bacterial counts (12 replicates per treatment). Microbial activity was assessed using soil
respiration. Soil samples (200 g d.w.) were placed in air-sealed flasks and respiration was measured using the electrolytic respirometer described in Manios and Balis (1983). Measurements were taken on the same day as the waste addition (phase 1) and every 10 days subsequently (phase 2 and 3). The results were expressed as ml O$_2$ per 100 g dry weigh of soil per hour.

2.5. Bacterial counts

The enumeration of bacterial groups of copiotrophs and oligotrophs was carried out in the pots described in Section 2.4, with the spread plate method using a rich carbon source medium (Nutrient Agar-NA) and a dilution 1/100 of this medium. Bacterial colonies were measured after 2, 4 and 6 days of incubation; in this way, three counts (or classes) were generated per plate. The copiotrophs were expressed as the number of colonies grown on NA at 2 days and the oligotrophs as the number of colonies grown on the diluted NA after 6 days incubation.

To characterize the community composition by means of a single value for each treatment from the three measurements at 2, 4 and 6 days, two indices were calculated. The colony development index (CD) adapted from Ahmed and Wardle (1994) and used for bacterial colonies (Kozdrój and van Elsas, 2000) was calculated as follows

$$\text{CD} = \left( \frac{P_2}{2} + \frac{P_4}{4} + \frac{P_6}{6} \right) \times 100$$

where $P_2$, $P_4$ and $P_6$ represent the proportions of bacterial colonies appearing on days 2, 4 and 6. A high CD value would indicate a greater proportion of r-strategists. For three classes measured every other day, as calculated here, CD varies from 50 (if all colonies appear on day 2) to 16.6 (if all colonies appear on day 6).

The eco-physiological (EP) index (Shannon diversity index) as described by De Leij et al. (1993) was calculated as follows:

$$\text{EP} = - \sum (P_i \times \log_{10} P_i)$$

where $P_i$ is the proportion of bacterial colonies in class $i$, i.e. the proportion of colonies appearing on counting day $i$ ($i=2, 4, 6$). The more even the distribution of the classes, the higher the EP index, with $\text{EP}_{\text{max}}=0.477$ and $\text{EP}_{\text{min}}=0$.

2.6. Statistical analysis

Statistical analysis was carried out using SigmaStat 2.03 for Windows. A repeated measures ANOVA was carried out on the data for respiration and microbial populations to assess overall treatment and time effects. Since the data for respiratory activity were not normally distributed the ANOVA results need to be treated with caution in this case. The data for colony development and eco-physiological indices were analysed using one way ANOVA. The effects of treatment and sowing time on pre-emergence damping-off, survival (Table 2) and the seed germination (Table 3) were analysed using two-way ANOVA on data transformed using the arcsin $\sqrt{\frac{x}{N}}$ transformation. All tests were performed at a 0.05 significance level and all pairwise multiple comparisons were performed using the Tukey test. For correlation testing, the Pearson correlation coefficient was used.

3. Results

The addition of OMW and TOMW to the soil led to an increase in the respiratory activity during the whole period of the wastewater addition, which was most pronounced for the OMW treatment (Fig. 2). When the wastewater addition stopped a steep decline in microbial activity was observed for both the OMW and the TOMW treatment, due to the elimination of the readily available organic substrate. The differences in the mean values among the treatment groups were significant ($F=305.4$, $dF=142$, $P<0.001$). Pairwise comparisons indicated that the organic constituents of OMW were able to sustain a
significantly higher microbial activity compared to the control for 60 days after the last liquid addition \((P<0.01)\) while the respiration rate of the TOMW was not significantly different from the control after 10 days \((P>0.05)\) with the exception of day 30.

The effects of the wastewater additions on the soil bacterial populations (copiotrophs and oligotrophs) are shown in Fig. 3. The differences in the mean population size of soil copiotrophs in the three treatments were significant \((F=87.9, \ dF=98, \ P<0.001)\) and so were all pairwise comparisons between the three treatments on the same date \((P<0.05)\) except for OMW and TOMW on day 20 and Water and TOMW on day 60 (Fig. 3a).

The populations of oligotrophs counted in the OMW and TOMW treatments were also greater compared to the control. Differences in the mean population size of the oligotrophs in the three treatments \((F=37.3, \ dF=96, \ P<0.001)\) and all pairwise comparisons between the three treatments on the same date \((P<0.05)\) were significant except for Water and TOMW on day 10 and OMW and TOMW on days 20, 40 and 50 (Fig. 3b).

The copiotroph to oligotroph ratio (Fig. 3c) did not correlate with time (Pearson \(P>0.05\)) for the OMW and the water treatment, varying around 0.5 and 0.1, respectively. In the TOMW treatment the ratio demonstrated a significant \((P<0.05)\) negative correlation with time, declining to the levels of the water treatment in about 2 months after the last TOMW addition.

Both the CD and EP indices indicated a change in the microbial community structure for
the OMW and the TOMW treatments, the difference being more pronounced for the OMW treatment (Table 1). The application of OMW to the soil led to a significant increase of the CD index in both copiotroph and oligotroph populations, indicating a shift to faster growing isolates. In contrast, the CD index for the TOMW treatment was not significantly different from the control. The EP index was significantly decreased in the OMW treatment for both copiotrophs and oligotrophs, indicating a less uniform distribution between the different population classes, while the TOMW treatment affected only the copiotroph population. The CD and EP indices did not correlate with time ($P>0.05$), therefore only the mean values for the 2 month period are reported. The only exception was the CD index for the TOMW treatment, as determined in undiluted NA substrate. In this case, there was a significant negative linear correlation with time ($P<0.05$, $y=0.6-0.0067t$, $r^2=0.97$).

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Copiotrophs (NA)</th>
<th>Oligotrophs (NA 1/100)</th>
<th>Copiotrophs (NA)</th>
<th>Oligotrophs (NA 1/100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WATER</td>
<td>33.9 (±1.0)a</td>
<td>28.0 (±0.7)a</td>
<td>0.438 (±0.007)a</td>
<td>0.443 (±0.006)a</td>
</tr>
<tr>
<td>TOMW</td>
<td>34.6 (±1.7)a</td>
<td>27.9 (±0.5)a</td>
<td>0.393 (±0.013)b</td>
<td>0.433 (±0.009)ab</td>
</tr>
<tr>
<td>OMW</td>
<td>39.4 (±0.6)b</td>
<td>35.8 (±0.9)b</td>
<td>0.362 (±0.009)b</td>
<td>0.411 (±0.009)b</td>
</tr>
</tbody>
</table>

Numbers in parenthesis are the standard error of the means. Different letters indicate significant differences ($P<0.05$) between treatments for the same substrate (ANOVA, multiple comparisons using Tukey’s test).

Soil treatment with OMW or TOMW led to disease suppression for lettuce seeds that were seeded at four different time intervals after soil infection with the pathogen *R. solani* (Table 2). The suppression of disease (pre-emergence damping-off and survival) was significantly affected by both the treatment and the sowing date, while the interaction of the two factors was also significant (two-way ANOVA test). However, the effect of sowing date did not exhibit any coherent trend. All treatments (water, TOMW and OMW) were significantly different from each other ($P<0.001$) in the case of pre-emergence damping-off, but not regarding survival, where only OMW was significantly different from the control. Pairwise comparisons within each sowing time confirm that disease suppression was more pronounced for the OMW treatment, resulting in significant differences from the water treatment in all measurements apart from survival at the second sowing. Although pre-emergence damping-off for the TOMW treatment was overall significantly different from the control, pairwise differences were not significant apart from the first sowing. Pre-emergence damping-off offers a better estimate than survival for disease suppression, because there is no effect from cross contamination of plants during root growth and there is no interference from the delay in the seed germination which was observed in some cases.

Table 2.

<table>
<thead>
<tr>
<th>Time of sowing</th>
<th>Pre emergence damping-off (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>TOMW</td>
</tr>
<tr>
<td>First sowing (day 45)</td>
<td>78.0 (±3.9)a</td>
<td>39.5 (±2.0)b</td>
</tr>
<tr>
<td>Second sowing (day 50)</td>
<td>100 (±0.0)a</td>
<td>96.5 (±1.8)a</td>
</tr>
<tr>
<td>Third sowing (day 55)</td>
<td>83.1 (±8.1)a</td>
<td>65.2 (±14.1)ab</td>
</tr>
<tr>
<td>Fourth sowing (day 60)</td>
<td>78.0 (±4.8)a</td>
<td>59.2 (±6.2)a</td>
</tr>
</tbody>
</table>
The two wastewaters exhibited a phytotoxic effect (Table 3) for about 2 weeks (days 7 and 14) during which the effect of both treatment and sowing time was significant, while neither factor significantly affected phytotoxicity thereafter (days 21 and 28). The phytotoxic effect was more pronounced in plants of the second sowing and was reduced at the third. At the fourth sowing, no phytotoxic effect was observed, indeed both TOWM and TOW treatments preformed significantly better than the control. Phytotoxicity of TOWM and OWM was also not significantly different from the control plants of the first sowing. For all treatments, the observed phytotoxic effect was not significantly correlated with disease suppression, as determined by the pre-emergence damping-off ($P>0.05$). In preliminary experiments (data not shown), it was demonstrated that $R.\ solani$ is able to grow on solid media containing OMW as the sole carbon source.

### Table 3.
Seed germination (%), in the control soil treated with Water, TOMW and OMW for four successive sowings

<table>
<thead>
<tr>
<th>Factor</th>
<th>Day 7 (±1.7)</th>
<th>Day 14 (±2.9)</th>
<th>Day 21 (±3.3)</th>
<th>Day 28 (±4.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First sowing (day 45)</td>
<td>Water: 98.3</td>
<td>98.3</td>
<td>98.3</td>
<td>98.3</td>
</tr>
<tr>
<td></td>
<td>TOMW: 91.7</td>
<td>95.0</td>
<td>95.0</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>OMW: 88.3</td>
<td>88.3</td>
<td>88.3</td>
<td>88.3</td>
</tr>
<tr>
<td>Second sowing (day 50)</td>
<td>Water: 96.7</td>
<td>96.7</td>
<td>96.7</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>TOMW: 53.3</td>
<td>90.0</td>
<td>93.3</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>OMW: 20.0</td>
<td>58.3</td>
<td>71.7</td>
<td>75.0</td>
</tr>
<tr>
<td>Third sowing (day 55)</td>
<td>Water: 96.7</td>
<td>98.3</td>
<td>98.3</td>
<td>98.3</td>
</tr>
<tr>
<td></td>
<td>TOMW: 53.3</td>
<td>93.3</td>
<td>93.3</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>OMW: 20.0</td>
<td>96.7</td>
<td>96.7</td>
<td>96.7</td>
</tr>
<tr>
<td>Fourth sowing (day 60)</td>
<td>Water: 81.7</td>
<td>83.3</td>
<td>83.3</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>TOMW: 100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>OMW: 90.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Numbers in parenthesis are the standard errors of the means. Different letters indicate significant differences ($P<0.05$) between treatments for the same sowing data and at the same day of assessment (two-way ANOVA on arcsin transformed data and all pairwise multiple comparisons using Tukey’s test).
4. Discussion

The observed increase in soil respiration during the whole period of the wastewaters addition, and especially the OMW addition (Fig. 2), was attributed to the higher organic load of the OMW, as part of the organic load of the TOMW has been removed in the form of CO$_2$ during the aerobic treatment. Soil microorganisms responded immediately to wastewater addition, as demonstrated by the direct increase in soil respiration, following the first OMW addition. In both treatments, a fast decline in microbial activity was observed when the wastewater additions were stopped, indicating fast assimilation of the readily available organic fraction of these wastes. The organic constituents of OMW were able to sustain a high microbial activity for a prolonged time period. On the other hand, the treated product led to a short-term increase in microbial activity that was not apparent after the last liquid addition.

The significant disease suppressiveness against *R. solani* that was induced in the OMW treated soil (Table 2) was mainly attributed to the shift in the soil microbial community from K- to r-strategy as was indicated by the EP and CD indices (Table 1), which demonstrated an increase in the population of fast growing bacteria, and from the increase in the copiotroph population (Fig. 3). The EP index is based on a mathematical theory of information, and can be regarded as a measure of the distribution of individuals in the different classes. Higher EP values imply a more even distribution of the proportions of bacteria developing at different times, but do not differentiate between r- or K-strategist dominance (Sarathchandra et al., 1997 and Buyer and Kaufman, 1996). The CD index is of greater relevance and relates better to r–K concepts than the EP index (Sarathchandra et al., 1997). A high CD value would indicate a greater proportion of r-strategists.

The above results suggest that OWM addition induced a microbial community that was dominated by r-strategists over a period of more than 70 days, while addition of TOMW resulted initially in an environment dominated by r-strategists, but within the study period the copiotroph to oligotroph ratio (Fig. 3c) reverted back to the levels observed in the non-amended soil. The addition of OWM creates an r-environment, which results in the enrichment of the soil in r-strategists, as they are more competitive than K-strategists in such environments, and seems to suppress *R. solani* for a prolonged period of time (Table 2). This may be attributed to “general suppression”, which is a function of antagonism for nutrient and energy supply available for the growth of the pathogen through the soil and on the root tip (van Bruggen and Semenov, 2000), a typical r-environment itself.

Addition of TOMW on the other hand, creates initially an r-environment that soon reverts back to a more K-environment (Fig. 3), while it causes significant suppression compared to the control at the first sowing time but not thereafter (Table 2). This further supports the hypothesis that an r-environment is not conductive to *R. solani*.

The use of microbial measurements which allow observation of succession from copiotrophic to oligotrophic organisms or from r- to K-strategists, has been suggested as an indication of soil health and disease suppression potential (van Bruggen and Semenov, 2000). It has been reported that the presence of copiotrophic bacteria in wheat roots was negatively correlated with infection from *R. solani* and this was attributed to the inability of the pathogen to compete with these microorganisms for infection sites and/or nutrients, or to its sensitivity to antibiotics possibly produced by these microorganisms (van Bruggen et al., 2002).

The phytotoxic effect which appeared during the second sowing period, and progressively receded during the third and fourth sowing periods (Table 3), was probably due to the formation of unstable organic matter from the wastewater added. It is likely that the intensive soil mixing for seeding and infestation led to the appearance of a "priming effect" which is a strong but short-term change in the turnover of soil organic matter caused by comparatively moderate treatments of the soil (Kuziakov et al., 2000).
Mechanical treatment or drying-rewetting of the soil can lead to a flush of C and N and to an acceleration of soil organic matter mineralization through improved aeration and destruction of the aggregates. The increased microbial activity can lead to phytotoxic effects through the release of phytotoxins from the microbial breakdown of the organic residues (Bradow, 1993, Stroo et al., 1988 and Tiquia et al., 1996). The assumed priming effect could not be verified by the respiration measurements due to the method followed, which necessarily entailed disturbed samples in all cases.

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