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Changes in Leaf Phenols and other Physiological Parameters of Peppermint in Response to Olive Mill Wastewater Application

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ABSTRACT

We studied changes in physiology of peppermint (*Mentha piperita*) exposed to olive mill wastewater (OMW) toward the moment of OMW application, the dose applied and the stage of plant growth. The biomass, hydrous state, chlorophylls in leaves and total proteins in roots were generally reduced by OMW spreading except for spreading 218 d before implantation. Peppermint shoots were more sensitive to phytotoxicity than roots. Determination of leaf phenols showed that mint roots facilitate the absorption and transfer of OMW phenols to leaves that are more sensitive to OMW toxicity. Phenols accumulation in leaves was as high as the dose spread was higher. Significant variations in leaf phenols were obtained depending on time of OMW spreading.

Key Words: Olive mill wastewater; Leaves; Phenols; *Mentha piperita*; Phytotoxicity

INTRODUCTION

Mediterranean countries produce annually about 10 million tons of olives (Barranco *et al.*, 1997). The olive oil production in these countries reach 98% of the total world's production. Consequently, about 30 million m^3 of olive mill wastewater (OMW) are annually produced in the region during a short duration (Casa *et al.,* 2003) causing the most serious threats to the environment (Paraskeva & Diamadopoulos, 2006). Progress has been made in the processes of olive oil extraction allowing a production of less quantities of more concentrated OMW without production of pomace (Jones *et al.,* 2000). However, widely used processes are still the three phase systems producing OMW, pomace and oil.

Many physicochimical and biological treatments could remove toxic substances existing in OMW (Paraskeva & Diamadopoulos, 2006). However, their practical application is generally limited due to high cost of treatments (Casa *et al.*, 2003). Nowadays, the principal destinies of OMW are direct spreading to agricultural soils and storage in evaporation ponds. The OMW value as a fertilizer is depending on removal of the toxic compounds that OMW contains (Paraskeva & Diamadopoulos, 2006; Saadi *et al.,* 2007), which are restrained or chemically modified by soil (Piotrawska *et al.,* 2006), degraded by soil microflora and soil free enzymes (Jones *et al.,* 2000; Mekki *et al.,* 2006a; Altieri & Esposito, 2008) and absorbed and metabolized by cultivated plants (Martin *et al.*, 2002).

The OMW spreading to soil essentially changes in physico-chemical and microbiological properties of soil (Zenjari & Nejmeddine, 2001; Sierra *et al.,* 2001; Mekki *et al.,* 2006a; Altieri & Esposito, 2008; Jarboui *et al.*, 2008). Earlier studies on plant response to OMW were generally limited to seeds germination and plant yield (Di Giovacchino *et al*., 2001; Tsioulpas *et al.*, 2002; Rinaldi *et al.*, 2003; Casa *et al.,* 2003; Ben Sassi *et al.,* 2006; Mekki *et al.,* 2006b). Recent studies are more and more interested to understanding mechanisms of phytotoxicity and plant response to OMW application (Ouzounidou *et al*., 2008). Little information available about OMW spreading to soils is still piecemeal, insufficient and sometimes contradictory and more studies should be done in order to master this way of valorization (Saadi *et al.*, 2007).

Phenols are considered as the principal cause of OMW phytotoxicity (Casa *et al.,* 2003; Kotsou *et al.*, 2004; Mekki *et al.,* 2006b; McNamara *et al.,* 2008). OMW phenolics are coming from the olive fruit. During triturating, just 2% of phenols pass in the oil, 53% in OMW and 45% in pomace (Rodis *et al.,* 2002). OMW composition of phenols is complex and highly variable depending on culture conditions in orchards, degree of ripeness of olive fruit, climatic conditions, storage conditions and oil extraction system (De Marco *et al.,* 2007).

Martin *et al.* (2002) showed that during OMW spread, plant roots could absorb OMW phenols. However, we don't know what is their destiny and if any accumulation in the plant exists. Aim of this work was to deepen our knowledge about the relationship between the contributions and capabilities of the entire peppermint to accumulate, transport

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and tolerate toxicities of OMW when grown in contaminated soil.

MATERIALS AND METHODS

Plants culture and OMW application. Black plastic pots filled with soil as 2 kg each one were prepared. Soil was previously dried in open air then sieved through 4 mm. soil pH was 7.82 ± 0.44, electrical conductivity 0.21 ± 0.01 ms cm^{-1} and humification degree of 10.68 ± 2.96%.The soil was lime comprising 51.16± 5.88% sand, 32.56± 3.92% silt and 16.25± 1.96% clay. Pots filled with soil received raw OMW as 9, 22.5 and 54 mL 100^{-1} g soil (corresponding to 180, 450 & 1080 mL of OMW per pot) at different moments over mint cuttings plantation. OMW was spread 218 d, 186 d and 40 d before or 38 d after peppermint (*Mentha piperita* L.) cuttings implantation (Table I, Fig. 1).

Cuttings (20 cm) of peppermint were planted in pots as three cuttings spaced by 10 cm per pot. Seven repetitions (pots) were prepared for each essay. OMW spread 218 d and 186 d before implantation was purchased on March 2006 from the press unit Abou-fez. OMW spread before 40 d and after 38 d of implantation was purchased from the same press unit on December 2006. OMW was stored in plastic tanks of 30 L capacity at 4°C until use. Characteristics of OMW used in experiments are presented in Table II. Total phenols were determined according to the colorimetric method using Folin-Ciocalteu's reagent (Box, 1983). Chemical Oxygen Demand (COD) was determined with COD meter according to the micromethod (Rodier, 1996). Phytotoxicity test of germination was done using maize seeds according to Casa *et al.* (2003).

Determination of biomass yield. After 110 d from peppermint cuttings implantation, plants corresponding to the different spreading cases were cut along the ground to recover stems and leaves. Soil containing roots was suspended in water to recover roots. After washing with water and rinsing with distilled water, all separate organs were dried with Joseph paper then weighted to determine fresh weight. Dry weight was determined after drying at 70°C till constant weight of samples. Water content of different organs (stems, leaves & roots) was determined by difference between fresh and dry weight for three plants sampled at random from each lot.

Determination of chlorophylls. Leaves situated at 3 to 10 cm from the apex were used to chlorophylls determination. A 0.125 g of leaves freshly picked were crushed at cold and dry conditions after addition of a pinch of magnesium carbonate and 2 pinches of anhydrous sodium sulfate. The crush was then extracted 4 times in successive 5 mL of pure acetone. The recovered extract was then passed through Whatman filter paper and the filtrate was complemented to 25 mL with pure acetone. The acetonic extract diluted to $4/5^{th}$ with distilled water was used to determine the concentration of chlorophylls in the sample according to Mac Kinney (1941).

Determination of total proteins in roots. Roots were crushed at low temperature in the presence of a pinch of Fontainebleau sand, in 2 mL of buffer sodium phosphate: 100 mM, pH 6.4. The crush was centrifuged at 9.000 *g* for 10 min at 4°C. The total proteins were determined in the supernatant according to Bradford (1976) using the bovine serum albumin (BSA) as standard.

Determination of phenols in leaves. Phenols in the peppermint leaves were extracted according to Shobana and Akhilender Naidu (2000). One gram of fresh leaves was finely crushed while cool with 5 mL of ethanol/distilled water (1:1) in a mortar. The crush was centrifuged at 7.500 *g* during 15 min. Phenols were determined in the supernatant according Box *et al.* (1983) with Folin-Ciocalteu reagent.

Statistical analyses. Essays were done in triplicate then the mean and standard deviation were determined. Student test was used to compare means using prism Pad 4 software. Statistical test was performed at $P<0.05$.

RESULTS AND DISCUSSION

The results of biomass, hydrous state and chlorophylls were generally similar for all doses except for the dose 54 mL 100^{-1} g for which cuttings died when OMW application was close to implantation (spreading 40 d before & 38 d after implantation). We describe in this paper only the results of the dose 9 mL 100^{-1} g. For phenols contents of leaves, results of the three spread doses have been presented.

Peppermint plants (110 d old) subjected to different OMW spreading showed variations in all physiological parameters depending on the time of OMW spreading and plants growth stage. OMW spreading to soil significantly reduced the plant biomass in all spreading cases except for the spreading 218 d before implantation (Fig. 1). This reduction is more important when OMW is applied at the vegetative phase of growth (spreading 38 d after implantation) rather than when it's applied before implantation. Although OMW spreading altered the biomass of roots and shoots, the root/shoot ratio increased with diminution of duration between spreading and implantation. An increase in the root/shoot ratio after OMW spreading was also observed by Mekki *et al.* (2006b) for chickpea, beans and wheat. Our study showed that unlike tomato (Ouzounidou *et al.*, 2008), mint shoots were more sensitive to OMW phytotoxicity than roots though the roots were directly in contact with OMW. This showed that peppermint shoots suffer more than roots of nutrient deficiency and lipophylicity of phenols and fatty acids of OMW, which suggested a natural resistance of peppermint roots to OMW.

Changes in the dry weight of peppermint for all OMW spreadings (Fig. 2) were closely followed water content (Fig. 3). OMW spreading significantly reduced the water content of leaves and stems for all spreadings except for the

Table I. Timing of OMW spreading at 9, 22.5 and 54 mL 100^{-1} g of soil in pots, the control hasn't received any OMW

Spreadings	Time of OMW spreading	Duration between spreading and cutting implantation (days)	Time of cuttings implantation	Duration between cuttings implantation and plants cut (days)	Time of mint plants cut
Control	_		05/ 02/07	110	26/05/07
Spreading 1	02/07/06	+218	05/ 02/07	110	26/05/07
Spreading 2	03/08/06	+186	05/ 02/07	110	26/05/07
Spreading 3	27/12/06	+40	05/ 02/07	110	26/05/07
Spreading 4	15/03/07	-38	05/ 02/07	110	26/05/07

Table II. Characterization of OMW purchased on March 2006 spread 218 d and 186 d before implantation (a) and OMW purchased on December 2006 spread 40 d before and 38 d after implantation (b)

Characters	(a)	(b)
pH	4.7±0.11	4.74± 0.06
Total Phenols (g L^{-1})	18.92±0.98	15.33± 1.14
COD (g O_2 L^{-1})	130.6±2.46	97.42± 1.52
Phytotoxicity	61.95±5.43	57.37±5.60
Maize seeds germination (%)		

Fig. 1. Timing of OMW spreading in report to mint cuttings implantation (d=days). Control didn't receive any OMW before or after implantation

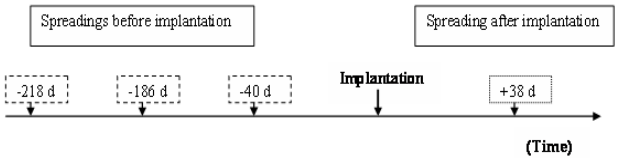
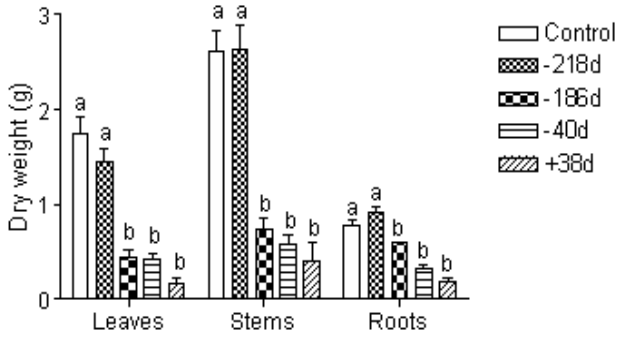
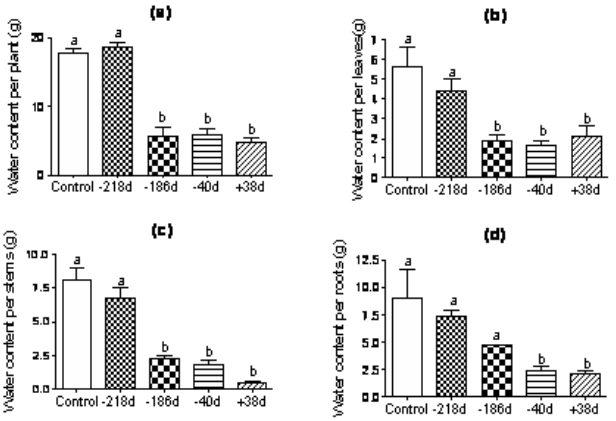


Fig. 2. Effect of OMW spreading as 9 mL 100^{-1} g at different times to peppermint cutting implantation on leaves stems and roots dry weight. Different small letters refer to significant differences (P<0.05) in comparison with control



spreading at 218 d before implantation (Fig. 3). For roots, water content was significantly reduced just for the spreading 40 d before and 38 d after implantation. Ouzounidou *et al*. (2008) reported that OMW spreading affects the hydrous state and reduces water use efficiency of leaves for tomato planted on soil and sand. They also reported a net decline of nutrients assimilation, transpiration and stomatal conductance just for plantations in sand culture. This observation suggests that in our study, where

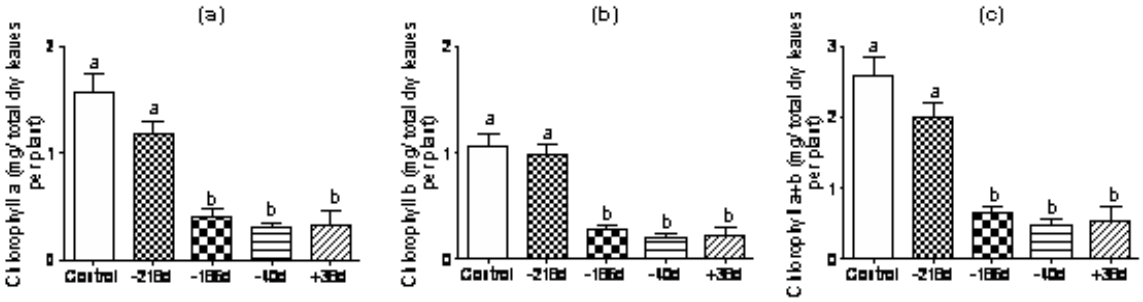
Fig. 3. Water content per total plant (a), leaves (b), stems (c) and roots (d) of peppermint planted in soil receiving OMW in different times before or after implantation. Different small letters refer to significant differences (P<0.05) in comparison with control



OMW is applied to soil, changes in peppermint water content might not be due to changes in transpiration and stomatal conductance but should be a consequence of an alteration of plasma membrane structures (El Hadrami *et al.*, 2004; Kistner *et al.,* 2004). Such changes show that OMW like other stresses (Creelman *et al.,* 1990; Surowy & Boyer, 1991) triggered structural and metabolic changes in growth and development of the plant.

OMW spreading to soil has significantly affected the amount of chlorophylls a and b in the peppermint leaves except for the spreading 218 d before implantation (Fig. 4). These data are consistent with those of Mekki *et al.* (2006b), who reported that OMW application at the vegetative phase of growth (15 d after sown) caused a significant reduction of chlorophylls a and b for tomato, chickpea, beans, wheat and barley. A sharp decrease in chlorophylls was also reported by Rinaldi *et al*. (2003) for wheat planted in a soil that had recently received OMW. According to Lichtenthaler (1987), chloroses in mainly due to Mg deficiency in soil. Piotrawska *et al.* (2006) showed an immediate increase of Mg in soil just after OMW spreading followed by an apparent decrease after only 14 days of spreading, mainly due to OMW acidity. Loss of chlorophylls can also be attributed to interference of toxic substances present in OMW during chlorophylls synthesis. Recently, Ouzounidou *et al.* (2008) reported that OMW application caused a significant restriction of absorption and translocation of K, Na, Fe, Ca

Fig. 4. Dosage of chlorophyll a (a), chlorophyll b (b) and chlorophyll (a+b) (c) in fresh leaves of peppermint planted in soil receiving OMW in different times before or after implantation. Different small letters refer to significant differences (P<0.05) in comparison with control



and Mg for tomato, which caused deficiencies of that element. The lack of K causes N deficiency and decreased chlorophyll synthesis, while reduction in concentration of Fe in the leaves may affect the electron transport in thylacoïds (Morales *et al*., 1998).

The load of proteins in roots was also reduced after OMW spreading to soil as reported for bean, wheat and barley but not for chickpea (Mekki *et al.*, 2006b), which showed an increase in total proteins after OMW application.

The test of the osmotic stress of OMW toward spearmint (*Mentha spicata*) showed that OMW acts just like the other stressful osmotic agents (mannitol, urea, NaCl) by causing a low water disponibility for cells, forcing them to osmoregulate (Fig. 5). Osmoregulation mechanism undergo accumulation of organic solute (Jones *et al.,* 2000; Sofo *et al.*, 2004), growth inhibition (Creelman *et al.,* 1990), modulation of gene regulation (Surowy & Boyer, 1991) especially those of lipoxygenase, which causes peroxidation (Sofo *et al.*, 2004) or changes membrane fat composition (Jones *et al.*, 2000).

The duration between OMW spreading and cuttings implantation is important, since peppermint resists to OMW toxicity and tends to control values (Fig. 2–6). This finding suggests that OMW phytotoxicity has gradually dissipated by biotic and abiotic constituents of soil (Piotrawska *et al.*, 2006; Mekki *et al.,* 2006a; Saadi *et al.,* 2007). The toxicity of OMW spread in pots as 9 mL 100^{-1} g (equivalent to 2 L m^{-2}) haven't disappeared until 218 d from spreading. In a field experiment, Saadi *et al*. (2007) report that 90 d are sufficient to reduce phytotoxicity of OMW spread at 7.2 L m^{-2}. Similarly, Di Giovacchino *et al*. (2001) recommended duration of 60 d for a spreading reaching 50 L m^{-2} and allowing a significant increase of grapevine and maize yields. In a field experiment conducted under soil and climatic conditions as in this experiment, we obtained a significant increase in spearmint yield when the spreading was done 60 d before cuttings implantation. These observations confronted with the results of this paper show that the container (pot) enclosing plant roots accentuated the impact of OMW on the plant.

Two types of effects are possible at the exhibition of a cell to a toxic substance: a direct effect on the rate of growth

Fig. 5. Variation of weight of stems cuts (2 mm) of spearmint (*Mentha spicata*) incubated in NaCl 10%, mannitol 0.8 M, Urea 1.4M and raw OMW (h=hours, M=Molar). Values are presented as mean ± SD

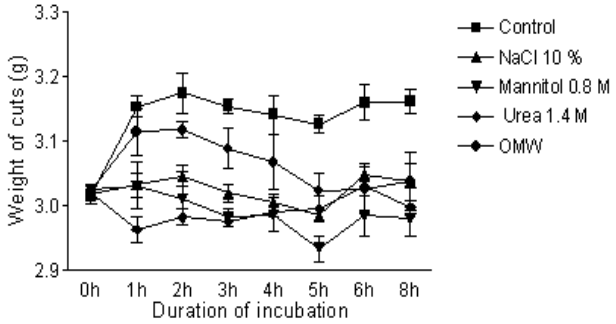
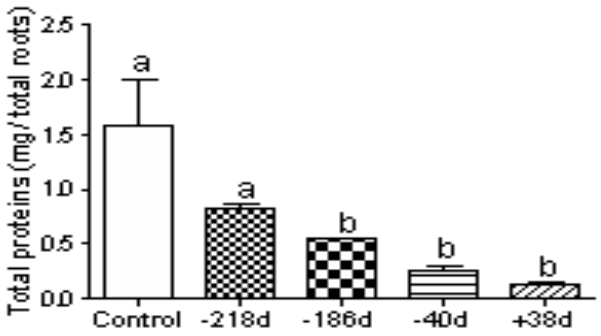
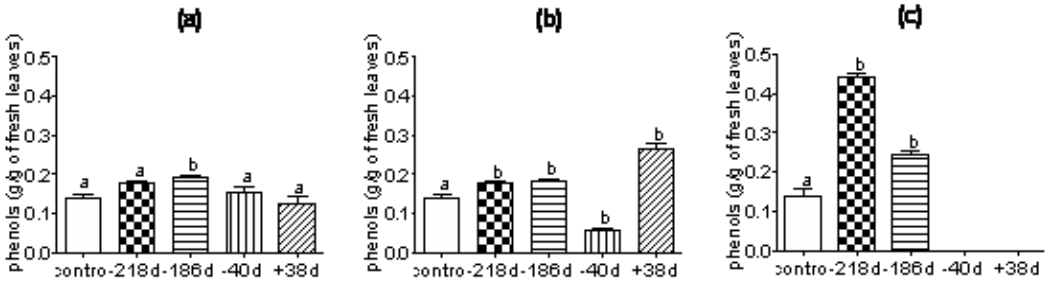


Fig. 6. Total proteins in roots of peppermint planted in soil receiving OMW in different times before or after mint implantation. Different small letters refer to significant differences (P<0.05) of different samples in comparison with control



(increase in the cost of synthesis of biomass) or indirect effects (reduction of absorption of substrates, disruption of energy processes, increasing costs of maintenance & repairing damages of membranes & DNA). Our results show that OMW toxicity could act indirectly on the synthesis of chlorophylls, photosynthesis and growth of peppermint through absorption and transport of Mg, mineral N and water. The lack of energy created by reduction of

Fig. 7. Effect of OMW spreading on mint leaves phenols. OMW is spread as 9 mL 100^{-1} g of soil (a), 22.5 mL 100^{-1} g of soil (b) and 54 mL 100^{-1} g of soil (c) 218 d, 186 d, 40 d before or 38 d after cuttings implantation. Different small letters refer to significant differences (P<0.05) in comparison with control



photosynthesis and increase of respiration stimulates the synthesis of traumatin and jasmonic acid analogue of absissic acid (Siedow, 1991; Creelman & Mullet, 1997; Ouzounidou *et al*., 2008). The increase load of jasmonic acid in the sap stimulates the closure of stomata (Horton, 1991), the degradation of chlorophylls (Weidhase *et al.,* 1987, Parthier, 1990) and respiration (Popova *et al*., 1988) and inhibits biosynthesis of RuBPCase (Weidhase *et al*., 1987) inducing a decrease in plant growth (Sembdner & Parthier, 1993).

The negative effect of OMW on growth of peppermint shoots becomes more important when OMW is spread on the vegetative phase of growth. This suggests that mint roots facilitate absorption and transport of toxic substances in the OMW (Fig. 2), which is supported by the work of Martin *et al*. (2002) who found that fungi with vesicles and arbuscules are infiltrated symbiotically in roots and increase their surface absorption and thus facilitate the transfer of OMW toxic substances to the plant. Dose of phenols in leaves confirmed Martin *et al.* (2002) results and showed that peppermint roots absorb and transfer OMW phenols to leaves accumulating them (Fig. 7). This accumulation is very low at 9 mL 100^{-1} g but tends to increase with the elevation of dose within the limits of mint tolerance to OMW. Peppermint leaves remarkably stored phenols when the dose of 22.5 mL 100^{-1} g is applied to rooted cuttings, while they lost phenols when the same dose is given 40 d before implantation (Fig. 7b). This observation is consistent with that of Martin *et al*. (2002), who found that the inoculation of plants with *Glomus mosseae* or *G deserticola* 30 d before OMW spreading was beneficial for all spread doses. When the application and inoculation are made at the same time plants do support just the low dose (10 g kg^{-1}). Roots play a similar role as fungi enhance plant resistance to OMW. Role of roots has a limit as these organs become ineffective as the dose increases from 22.5 to 54 mL 100^{-1} g (Fig. 7c). Indeed, for high dose of 54 mL/100g, the transport of phenols to leaves is considerable when OMW stay for a long time in soil before implantation, however, when spreading is close to plantation (spreading before 40 d or after 38 d of implantation) cuttings hardly tolerate the high dose (Fig. 7c.). For the dose of 9 mL 100^{-1} g, a length of 40 d of OMW in soil before or after implantation is sufficient to maintain phenols of leaves at the normal state (Fig. 7a). For the dose 22.5 mL 100^{-1}g, duration between spreading and implantation well above 40 d is necessary to avoid a degeneration of the metabolism of phenols in leaves.

Until now no study has accurately estimated the limit beyond, which phenols become phytotoxic to leaves and the limit of roots resistance to phenols. Monitoring changes in phenols and peroxidase activity in the entire plant, at short scale, is necessary if we want to evaluate this limit and the contribution of each organ in the response of phenols metabolism of the plant to OMW.

CONCLUSION

The model plant Peppermint used in this study showed phytotoxic effect of OMW on peppermint leaf growth and metabolism of phenols. As OMW spreading is close to mint implantation as phytotoxicity is more expressed concerning biomass, water content, proteins in roots and chlorophylls in leaves. Phenols accumulation in leaves was proportional to OMW spread at the vegetative phase of growth. Mint leaves would play a role in plant resistance to phenols within the limits of their ability to accumulate these products.

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