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RESEARCH PAPERS

Essential oils: an alternative approach to management of powdery mildew diseases

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Summary. In recent years there has been growing interest in the application of plant-derived substances in agriculture as alternatives to the use of pesticides, in order to obtain healthy crops and more environmentally sustainable crop production systems. The properties of some essential oils as natural fungicides were evaluated, to promote their use in alternative agriculture. Potentially detrimental effects caused by essential oil residues in soil were also assessed by mutagenicity assays to avoid possible adverse effects related to the use of these materials. Trials in a controlled environment were set up, using 'Romanesco' zucchini treated with essential oils, either exclusively or alternated with a synthetic fungicide. The treatments were applied when natural infection by *Podosphaera xanthii* appeared on test plants, and powdery mildew incidence and severity were assessed after six weeks. Preliminary results indicated that the alternation of natural materials with effective synthetic fungicide maintained effective disease control, and may also assist with management of pesticide resistance in *P. xanthii*. No relevant mutagenic effects of essential oil residues in soil were revealed, although an appropriate formulation useful under field conditions is required for effective application.

Key words: *Podosphaera xanthii*, mutagenicity tests, integrated disease management.

Introduction

Podosphaera xanthii (Castagne) U. Braun & Shishkoff, is an obligate biotrophic pathogen that rapidly colonizes green tissues of zucchini, causing powdery mildew which negatively affects host physiology and can lead to severe harvest losses (Gengotti and Brunelli, 2007; Galletti *et al.*, 2008; Giampaolo *et al.*, 2010). The disease can be severe, and cause premature defoliation of older leaves if this disease is not readily controlled when environmental conditions are favourable to fungal infection and sporulation (Sitterly, 1978; Zitter *et al.*, 1996).

Powdery mildew control using synthetic fungicides currently maintains a prominent role in plant protection, but these chemicals can cause development of the pathogen resistance towards chemical agents (McGrath, 2001; Pérez-García *et al.*, 2009) and risks for the environment.

The Directive 2009/128/EU, "establishing a framework for Community action to achieve the sustainable use of pesticides", proposes to reduce risks and impacts on human health and on the environment by the development of alternative agriculture techniques, including integrated pest management. Alternative or complementary plant protection practices are necessary to reduce the use of pesticides in order to obtain healthy crops and environmentally sustainable production, as well as to safeguard the health of farm workers and consumers. Recently

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published literature highlights the antimicrobial activities of plant extracts, essential oils and other materials (Rai and Carpinella 2006; Gilardi *et al.*, 2012), but only a few natural products are used in crop protection.

A natural substance proposed for pest management in agriculture should have the following properties: efficacy against the target organism, safety and biological selectivity, standardized composition and formulation and ready availability. Natural compounds generally have lower persistence and toxicity than synthetic compounds, reducing their potential environmental impact. The essential oils are complex mixtures of several chemical compounds including terpenes, alcohols, aldehydes and phenols, and these materials exhibit potential herbicidal and fungicidal properties (Tworkoski *et al.*, 2002). Some essential oils have antimicrobial properties (Shelef, 1983), are antiviral (Bishop, 1995), or antimycotic (Azzouz and Bullerman, 1982; Akgül and Kivanc, 1988; Mari *et al.*, 2003), and can have roles in weed control (Zanellato *et al.*, 2009).

Tea tree oil, derived from *Melaleuca alternifolia*, was found to be effective against a broad spectrum of plant pathogenic fungi (Vardi and Reuveni, 2009). The antimicrobial properties of clove oil (from *Syzygium aromaticum* L.) and rosemary oil (from *Rosmarinus officinalis* L.) are also well known (Daferera *et al.*, 2000; Angioni *et al.*, 2004; Jirovetz *et al.*, 2006). A formulation of Clove oil, whose major constituent is eugenol, is used for weed control, electively inhibiting plant germination, suggesting its potential use as a bioherbicide (Dudai *et al.*, 1999). Eugenol has also been demonstrated to be active against plant pathogenic nematodes (Sangwan *et al.*, 1990) and to have antifungal activity (de Oliveira Pereira *et al.* 2013). This product has been approved for use in organic food production (Mohan *et al.* 2011).

In recent years, there has been an increased interest in the use of essential oil combinations to improve their natural antimicrobial and antifungal activities. Fu *et al.* (2007) observed increased antifungal effects caused by combinations of essential oils from *S. aromaticum* and *R. officinalis*.

Previous studies of Annesi *et al.* (2011), carried out to evaluate the efficacy of a strengthener and an essential oil used alternately with a synthetic fungicide for control of powdery mildew, suggested that an integrated control management could reduce the use of pesticides on zucchini crops.

Our research focused on the evaluation of the antifungal properties of some essential oils as natural fungicides against *Podosphaera xanthii*, as well as the assessment of the potential genotoxicity of these materials, in order to promote their use in alternative agriculture. Moreover, a preliminary study of the presence of main components in essential oils was carried out, because the concentration of some active molecules may be crucial for the evaluation of their biological activity. It was also necessary to assess whether the use of these alternative methods of disease management may have detrimental effects on the crops.

Materials and methods

GREENHOUSE TRIALS

Pathogen identification

Samples of four infected leaves were collected in order to carry out fungal identification by molecular analysis. Mycelium scraped from diseased leaf tissues was transferred into a 2 mL tube and then ground to a powder with a plastic pestle after freezing in liquid nitrogen. The DNA was extracted by kit Nucleospin Plant® II (Macherey-Nagel GmbH & Co. KG). The Internal Transcribed Spacer (ITS) region of rDNA was amplified according to Chen *et al.* (2008) using the primer pairs G1/G2 and S1/S2. The PCR products were separated by electrophoresis through a 2% agarose gel and extracted with NucleoSpin® Extract II (Macherey-Nagel GmbH & Co. KG). The purified DNA was sequenced and analysed through Blast sequence analysis (Altschul *et al.*, 1990).

Plant material and experimental conditions

Experiments to evaluate the efficacy of essential oil treatments on zucchini crops naturally infected by *Podosphaera xanthii* were performed in a controlled environment. Two trials were carried out in different years. They consisted of treatments with natural compounds used alone or in alternation with the fungicide quinoxifen (Arius SC, Dow AgroSciences). Seeds of zucchini (cv. 'Romanesco' sel. Tullio) were sown in individual pots (diam. 20 cm, depth 14.2 cm) filled with soil (Zeoliter, Agricola2000 s.a.s) and were fertilized every 2 weeks by mineral-organic fertilizer NPK with microelements (ONE, Valagro s.p.a.). The first treatment was performed when natural powdery mildew appeared in the greenhouse. The trials

were carried out in a controlled environment, with 12 h photoperiod and temperature of 20°C (\pm 4°C). For each treatment four replicates were prepared, each consisting of three pots with two plants in each pot.

Analytical determination of essential oils

Tea tree oil, clove essential oil and rosemary essential oil were collected from the local market in Rome, Italy. 1,8-cineole, γ -terpinene, terpinen-4-ol, eugenol, sodium sulphate anhydrous and Tween 20 were supplied by Sigma Aldrich S.r.l. Hexane was acquired from Carlo Erba. Water was previously purified in a Milli-Q system (Millipore). The presence of main components, according to literature (Flamini *et al.*, 2000; Hammer *et al.*, 2004; Carson *et al.*, 2006; Jirovetz *et al.*, 2006), was confirmed by gas chromatography analyses coupled with a mass detector. The analyses were performed using a Thermo Scientific gas chromatographer Focus GC, equipped with a fused silica capillary TG-SQC (30 m \times 0.25 mm, film thickness 0.25 μ m Thermo Scientific) and a mass spectrometer 700 ITQ MS as detector. The analyses were carried out according to chromatographic and mass conditions described by Daferera *et al.* (2000).

Treatments

The oils were emulsified with 0.05% Tween 20 (Reuveni *et al.*, 1996; Terzi *et al.*, 2007) before application. In both trials, seven different treatments were applied (Table 1). The first applications of oils were

made after the first signs of powdery mildew were observed on plants, from natural infections. Thereafter, the treatments were repeated at 7 d intervals for 5 weeks according to the protection program. For each treatment, the plants were sprayed with a hand sprayer until run-off. The treatments were performed, for each product, with doses indicated on the respective labels or in literature (Galletti *et al.*, 2008; Copping, 2009; Vardi and Reuveni, 2009).

Disease assessments

Disease incidence (percentage of infected leaves on each treated plant) and disease severity were assessed after 6 weeks by observing each plant. All leaves were individually observed. Disease severity was assessed by evaluating the percentage of infected area, using a scale of four classes: 1 = no powdery mildew; 2 = 1–5% of leaf area affected; (3) = 5–30%; and 4 = >30% leaf area affected. The data obtained were processed by the McKinney formula (McKinney, 1923), which generates a numeric disease severity index (DI) as:

$$DI = (\sum vn) / (NV) \times 100$$

where v represents the numeric value of the class; n is the number of plants assigned to the class; N is the total number of the plants in the replication and V is the numeric value of the greatest severity class.

The effect of each treatment was estimated by analysis of variance (ANOVA) and LSD tests for $P \leq 0.05$.

Table 1. Treatment programmes and doses applied, in trials on essential oils for control of powdery mildew on zucchini.

Treatment	Applications (No.)	Application interval (days)	Dose (mL/L)
Water Control	5	7	
Tween 20	5	7	
Tea tree oil	5	7	1.2
Tea tree oil alternated with quinoxifen	3 2	14 14	1.2 0.25
Clove oil+ rosemary oil	5	7	0.8 (C) + 1.6 (R)
Clove oil+ rosemary oil alternated with quinoxifen	3 2	14 14	0.8 (C) + 1.6 (R) 0.25
Quinoxifen	2	14	0.25

MUTAGENICITY TEST***In vivo* and in greenhouse tests**

Two types of experiment were performed in different experimental conditions, the first *in vivo* and the second in a greenhouse. *Vicia faba* was chosen as a bioindicator plant, because it has large chromosomes amenable to the study of chromosome aberrations in somatic cells during mitotic cell division and of micronuclei in root tip cells. This plant is widely used in cytological and genotoxicity studies as well as in physiological experiments (Kanaya *et al.*, 1994). Short term tests (comet assays and micronuclei tests) were performed on *Vicia faba* root meristems *in vivo* in order to identify the appropriate dose for the treatments, and in greenhouse for the evaluation of residual effects of oils in the soil. The comet assay on plants has become a useful method for the assessment of the environmental genotoxic impacts. The assay is ideal to detect DNA damage because of its high sensitivity and specificity, and because it is a non-invasive technique. This assay may complement other test systems measuring different endpoints of genotoxicity.

Phytotoxicity and mutagenicity tests

In vivo tests were performed on *Vicia faba* roots as a preliminary study to evaluate the potential mutagenicity due to essential oils. *Vicia faba* seeds were allowed to germinate in quartz sandy soil treated with different concentrations of essential oils in order to perform phytotoxicity and genotoxicity tests (comet assay and micronuclei analyses). Aluminium basins,

each containing 250 g of sandy soil and 25 *Vicia faba* seeds, were each treated with 50 mL of one of the different essential oils. Basins wetted only with deionised H₂O were used as negative controls. Each basin was sealed with Parafilm and incubated at 20°C ± 1°C for 5 d to allow germination.

The essential oils tested consisted in tea tree oil and clove oil plus rosemary oil mixture. The oils were emulsified with 0.05% Tween 20 (Reuveni *et al.*, 1996; Terzi *et al.*, 2007) before application. The concentrations of essential oil mixtures for *in vivo* tests were chosen after a preliminary experiment (data not shown), where we examined the effects of essential oils on *Vicia faba* germination, and we identified the range of doses under the which boundary there is no effect on growth inhibition of the test plants (Table 2). Phytotoxicity was calculated by measuring the reduction in primary root lengths of *Vicia faba* seedlings exposed to different oil formulations.

Total germination percentage (Gt) was calculated as $Gt = (n/N \times 100)$, where n = total number of germinated seeds at the end of the experiment and N = total number of seeds used for the germination tests (Correa *et al.*, 2000).

Germination index (GI), evaluated as $[(Gt \times Lt) / (G_{H_2O} \times L_{H_2O})] \times 100$ is a maturity test based on seed germination and initial plant growth (Zucconi *et al.*, 1981), where G t is the total number of germinated seeds, L t is the median root length of the sample, G H₂O is the number of germinated seeds, and L H₂O is median root length of the water control. This index reflected the phytotoxicity of the essential oils, accounting for low toxicity, which affects root

Table 2. Treatment programmes and doses applied to *in vivo* mutagenicity tests.

Treatment	Dose mL/L
H ₂ O: Water control	
Tween 20: Control with emulsifier 0.05%	
Tea tree oil	0.3
Tea tree oil	0.6
Tea tree oil	0.9
Clove oil + rosemary oil	0.2(C) + 0.4 (R)
Clove oil + rosemary oil	0.4(C) + 0.8 (R)
Clove oil + rosemary oil	0.8 (C)+ 1.6 (R)

growth, and high toxicity, which affects germination.

The comet test was performed using an alkaline unwinding/alkaline electrophoresis (A/A) protocol as described by Sturchio *et al.* (2011). Olive Tail Moment has been used as a parameter of DNA damage (μm). Three slides were evaluated for each treatment.

Micronuclei tests evaluated the frequency of micronucleated cells in root meristems of *Vicia faba*, analyzing 15,000 cells (15 root tips, 1,000 cells for each root tip).

Each experiment was repeated three times (in three biological replicates). Analysis of variance (ANOVA) was performed with Dunnett's test to compare the differences in the means and standard errors of the data, at $P \leq 0.05$, using the software package SPSS.

The potential phytotoxicity and genotoxicity of essential oils were determined on *Vicia faba* seedlings grown in soil sampled from the treated zucchini crops, as above mentioned, in order to evaluate the residual effects of essential oils in the soil. At the end of the treatment programmes, surface soil samples were collected from zucchini pots and placed in aluminum basins. For each treatment, three replicates were performed. Each basin, containing approx. 250 g of soil and 25 seeds of *Vicia faba*, was moistened with 50 mL of deionised H_2O . Each aluminum basin was sealed with Parafilm and incubated at 20°C ($\pm 1^\circ\text{C}$) for 5 d to allow the seed to germinate, in order to evaluate any potential residual effects of essential oils on germination. Phytotoxicity, genotoxicity (comet and micronuclei tests), total germination percentage (Gt) and Germination index (GI) assays were performed following the protocols outline above.

Results

GREENHOUSE TRIALS

Fungal identification

The powdery mildew fungus was identified as *Podosphaera xanthii*: the sequence obtained has been deposited in GenBank with accession number KC609002. The sequence showed nucleotide similarity of 99–100% with other sequences of *P. xanthii* available in GenBank.

Main essential oil compounds

The proportions of major molecules of the essential oils used in this study are indicated in Table 3.

Effects of treatments

The first trial showed that powdery mildew had spread progressively reaching a mean incidence of 61.3% and a mean severity index of 48.8% in untreated plants. Six weeks after the first treatment, a general reduction of disease incidence was observed from all of the treatments compared to the untreated controls (Table 4). In particular, the greatest disease reduction (disease incidence $< 10\%$) occurred in plants treated with essential oils (both tea tree oil and clove plus rosemary oils) alternating with quinoxifen. These treatments differed significantly, both from the untreated plants and from the treatment with fungicide applied every 14 d. The treatments with clove plus rosemary oils applied at 1 week intervals also significantly reduced disease incidence compared to untreated controls. Plants sprayed with 0.05% Tween 20 and those sprayed with water did not show significant differences in disease incidence.

Disease severity indices followed a similar trend. Treatments with oils alternating with fungicide resulted in a mean severity index of 2.0% and 6.0% versus 48.8% for control plants. Tea tree oil applied at weekly intervals did not give any protection.

The second trial (Table 5) showed that powdery mildew reached an incidence of 82% and a severity index of 63% in untreated plants. This trial confirmed the results of the first trial, demonstrating the efficacy of treatments with clove plus rosemary oils, and clove plus rosemary oils alternating with quinoxifen. Treatments of tea tree oil alternating with quinoxifen did not produce results significantly different from the treatment with quinoxifen applied every 14 d. Disease severity indices for all the treatments were significantly different from the water controls. Only clove plus rosemary oils alternating with quinoxifen significantly differed from the fungicide applied alone.

MUTAGENICITY TESTS

In vivo tests

In vivo tests with *Vicia faba* showed phytotoxic effects (Figure 1A), particularly from the clove plus rosemary oil mixture, beside a corresponding decrease of the total germination percentage and of the germination index (Figures 1B-1C). Phenotypic analysis on *Vicia faba* primary roots after the treatment showed development of callose as an early

Table 3. Proportions (percent) of major components in the three essential oil preparations tested in this study.

Oil	γ -terpinene %	1,8-cineole %	Terpinen-4-ol %	Eugenol %
Tea tree oil	17.1		36.7	
Clove oil			5.8	69.3
Rosemary oil		36.9		

Table 4. First trial: treatments, mean disease incidence and indices of disease severity. Within columns, values followed by common letters do not differ according to LSD tests ($P < 0.05$).

Treatment	Disease Incidence (%)	Index of Disease Severity (%)
Water Control	61.3 a	48.8 a
Tween 20	59.1 a	48.5 a
Tea tree oil	56.4 ab	41.4 ab
Tea tree oil alternated with quinoxifen	4.4 d	2.1 d
Clove oil + rosemary oil	45.2 b	33.0 b
Clove oil + rosemary oil alternated with quinoxifen	8.1 d	6.1 d
Quinoxifen	20.8 c	16.9 c

Table 5. Second trial: treatments, mean disease incidence and indices of disease severity. Within columns, values followed by common letters do not differ according to LSD tests ($P < 0.05$).

Treatment	Disease Incidence (%)	Index of Disease Severity (%)
Water control	81.9 a	62.9 a
Tween 20	83.3 a	63.3 a
Tea tree oil	61.8 b	39.1 b
Tea tree oil alternated with quinoxifen	47.8 bc	23.9 bc
Clove oil + rosemary oil	58.5 bc	30.9 c
Clove oil + rosemary oil alternated with quinoxifen	30.3 d	16.9 d
Quinoxifen	45.5 c	22.9 c

symptom caused by lipid peroxidation, as reported by Yamamoto *et al.* (2001) (Figure 2). Comet assays showed DNA damage from all the concentrations tested (Figure 1D), while the micronucleus test revealed an increased frequency only with the formulation of essential oil mixture at high concentration (clove oil 0.8 mL/L + rosemary oil 1.6 mL/L) (Figure 1E).

Greenhouse tests

Vicia faba plants grown in soils treated with the essential oils did not produce any significant phytotoxic effects (Figure 3A), or effects on seed germination (Figures 3B and 3C). The comet assay detected DNA damage in all the samples tested (Figure 3D) and the micronuclei test highlighted a genotoxic effect only for the tea tree oil treatments (Figure 3E).

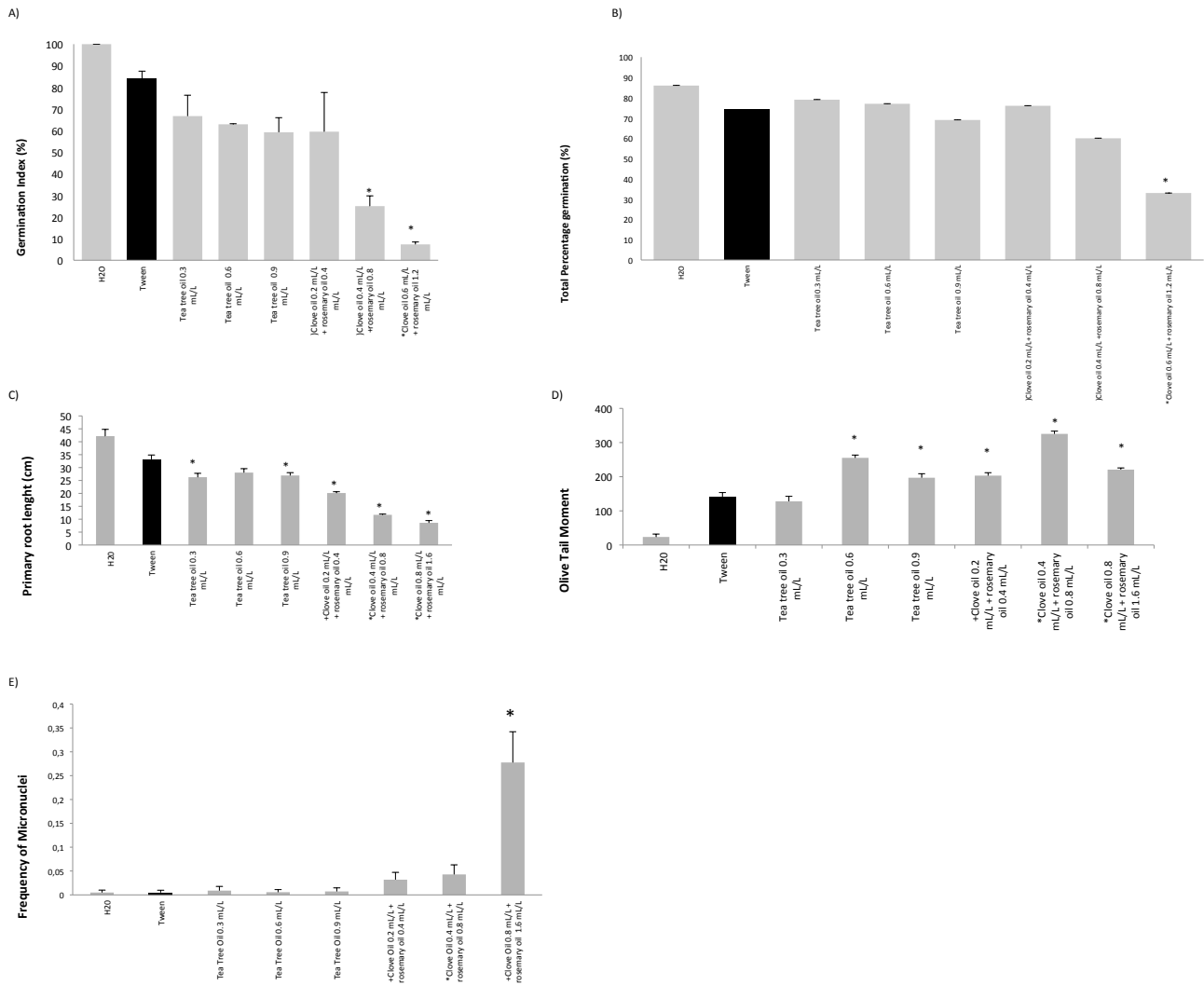


Figure 1. *In vivo* tests. A) Phytotoxicity tests; B) Mean germination indices; C) Mean total percentage germination (Gt); D) Comet assay: Olive Tail Moment is used as parameter to measure DNA damage; E) test (MN); the frequency of micronuclei is expressed as a percentage. Asterisks indicate significant differences compared to TWEEN, indicated by ANOVA with Dunnett tests at $P < 0.05$.



Figure 2. *In vivo* tests on *Vicia faba* roots treated with essential oil. Meristems showed a callose production after the treatment as an early symptom caused by lipid peroxidation.

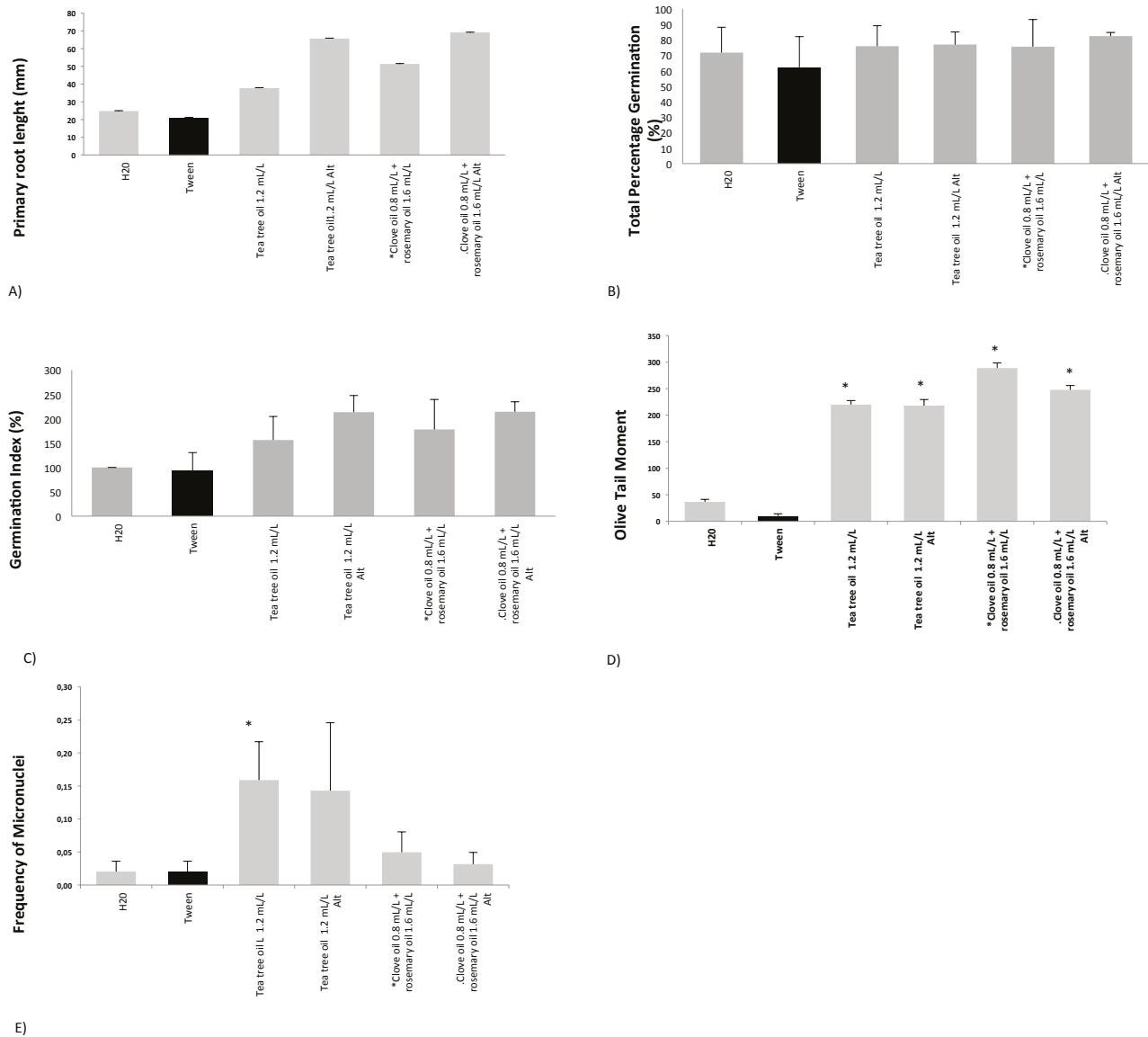


Figure 3. Greenhouse experiments. A) Phytotoxicity tests; B) Germination index; C) Total percentage germination (Gt); D) Comet assay: Olive Tail Moment is used as parameter to measure DNA damage; E) test (MN); the frequency of micronuclei is expressed as a percentage. Asterisk indicates significant differences compared to TWEEN by ANOVA with Dunnett test at $P < 0.05$.

Discussion

The trials described here have demonstrated the potential use of natural substances for management of powdery mildew in zucchini. The levels of disease control achieved were very satisfactory from treatments with clove oil plus rosemary oil applications,

both for reducing disease incidence and disease severity. In particular the application of clove oil and rosemary oil alone significantly reduced the disease in both trials. Clove oil plus rosemary oil treatments alternating with the fungicide provided the best disease control, despite the high pressure disease recorded in the second trial. Regarding TTO applica-

tion programs, the results obtained in the first trial were not confirmed in the second one, where disease control from these treatments were not satisfactory. Integrated pest management (IPM), if carried out with selected low environmental impact products in association with fungicide, could be a useful approach to achieve good disease control. In addition, the chemical diversity of the active ingredients probably minimize the occurrence of resistance development in the target pathogen (Brown *et al.*, 2002; Gilardi *et al.*, 2012). Moreover, because cucurbits have different harvest times, it is difficult to have a good disease control with synthetic chemicals and to maintain adherence to maximum pesticide residue limits for these crops.

The results of the present study demonstrated the efficacy of candidate products (clove oil plus rosemary oil) in greenhouse trials, although compounds identified as useful in-laboratory assays can be sometimes ineffective when applied on plants. Moreover phytotoxicity was not observed in treated plants after the compounds were applied. This is an important aspect in the evaluation of natural products, because some essential oils have been shown to cause considerable phytotoxicity at the concentrations useful for control of plant pathogenic fungi (Isman and Machial, 2006).

We evaluated the mutagenicity of tea tree oil and clove plus rosemary oils. *Vicia faba* roots showed that residual oils in the soil had no phytotoxic effects, supported also by the high germination index. Nevertheless, the use of tea tree oil could induce a genotoxic effect in *Vicia faba* meristems. Tea tree oil probably persists in the soil and is not easily degraded by soil bacteria at the same rates as of rosemary and clove essential oils. Eugenol, the major component of the clove oil, is considered non-persistent in soil because it is completely broken down to common organic acids by soil-inhabiting *Pseudomonas* bacteria (Rabenhorst, 1996). Conversely, the monoterpene hydrocarbon constituents of the tea tree oil are not likely to be mobile in soil (Misra *et al.*, 1996).

In vivo tests demonstrated phytotoxic effects on *Vicia faba* roots when seeds were directly exposed to the essential oils, particularly for the clove plus rosemary oil mixture. All the treatments induced significant increases in DNA migration, and this is confirmed by recent literature (Maistro *et al.*, 2010). A mutagenic effect was observed in the micronuclei

assay only with the formulation of essential oil mixture at the higher concentration tested (clove oil 0.8 mL/L + rosemary oil 1.6 mL/L). *In vivo* bioassays are likely to greatly overestimate the effects of plant derived compounds compared to what is likely to occur in field conditions (Keeley, 1988; Wardle *et al.*, 1998). The explanation of the different toxic effects between *in vivo* and greenhouse trials can be due to the transformation of the chemicals by organic and inorganic compounds in soil, adsorption by colloids and/or dilution by the precipitation (Brückner and Szabó, 2001).

In conclusion, we emphasize that the clove plus rosemary oil mixture tested in this study has provided a promising product, as indicated by efficacy and toxicity results. Additional research under open field condition is required, as well as it's important to determine appropriate formulations for these materials. Reducing the use of pesticides in favour of natural compounds such as those tested here is likely to have worthwhile environmental and human health benefits.

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