The essential oils in agriculture as an alternative strategy to herbicides: a case study

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Abstract: In this study we investigated the allelopathic effect of some essential oils extracted from aromatic plants to inhibit weed seed germination. The study was performed to evaluate the potential genotoxic effect of lavender on *Vicia faba* root meristems by genotoxicity tests (comet assay and micronuclei test) and to evaluate microbial diversity fluctuation by Restriction Fragment Length Polymorphism (RFLP) molecular analysis on ecological, biological and conventional soil. The results showed a phytotoxicity effect of the essential oil for all the concentration tested, while there is not a dose-dependent relation between oil concentrations and the genotoxic effect. A fluctuation of microbial communities in relation to the release of essential oils by *Lavandula officinalis* was also observed. Then we suggest that the essential oils could be useful as potential bioherbicides as an alternative strategy to the chemical remedy, but further studies are necessary to evaluate their appropriate use in the field.

Keywords: lavender; bioherbicides; biological agriculture; phytotoxicity; genotoxicity; comet assay; micronuclei test; RFLP; microbial diversity.

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1 Introduction

Traditional agriculture, based on the local production and on the manual work, has been replaced by a more technological agriculture. The autochthonous varieties of cultivar have been replaced by good yield varieties with remarkable increment in agricultural production, but also with high environmental costs (abundant irrigations, fertilisers and pesticides use, increase soil working). The phytosanitary products ('pesticides' and 'herbicides') have been widely used to protect the crops from parasites (insects and acarus) and from pathogens (bacteria, viruses, fungi) and to eliminate weeds. The herbicides are constituted by toxic substances (in some cases carcinogenic) and their incorrect use can determine risks for animal and human health.

The European Directives have contributed to reduce the risks of phytochemical use, establishing their concentration in fruits, vegetables, cereals and animal products and according to the classification, packing, labelling, recording, commercialisation and use (Binetti, 2007). The limited use of phytochemicals permits the development for more sustainable agriculture at low environmental impact. During the last years there has been an increased interest in developing allelopathic phenomena application in organic agriculture, utilising agriculture species that produce allelopathic substances which are toxic for weeds, directly or after the soil microorganisms activity (Newman, 1982; Dudai et al., 1999). An allelopathic substance (allelochemical) is 'a chemical substance not nutritious produced from the secondary metabolism of an organism that acts to increase health, function and other species biological population'. The allelochemicals are secondary metabolites that plants produce like messages (Williamson, 1990). The allelopathic species alternation, with negative effects on seed germination and plant development, can be used in organic weed management. Allelopathic substances are also the essential oils produced from the aromatic plants (Willis, 1985; Dudai, 1999).

These essential oils are complex mixtures of several chemical compounds including terpenes, alcohols, aldehydes and phenols.

Many plant species have been studied for their allelopathic potential; in particular, aromatic plants have been reported being able to produce a large amount of allelochemicals compounds (Dudai, 1999). The ability to suppress weeds by means of essential oils extracted from aromatic plants could be a valuable tool for weed control (Tworkoski, 2002).

In previous works, the potential toxic effect of the essential oil of cinnamon (*Cinnamomum zeylanicum* L.), lavender (*Lavandula* spp.) and peppermint (*Mentha piperita* L.) on the seed germination of some weed species common in the Mediterranean environment (*Amatanthus retroflexus* L., *Solanum nigrum* L., *Portulaca oleracea* L., *Chenopodium album* L., *Sinapis arvensis* L., *Lolium* spp., *Vicia sativa* L.) were investigated (Dudai et al., 1999; Caporali et al., 2005).

The inhibition ability does not differ among types of oils, being essentially dependent on their concentrations. The higher concentrations are able to totally inhibit weed seed germination even when a different selectivity are observed (Putnam et al., 1986). However, the relation between oil concentrations and inhibition ability is not always dose-dependent for all these species (Campiglia et al., 2007).

Then, experiments in a greenhouse were carried out with the same essential oils and the same concentrations. The results showed an inhibition on weed seed germination to the lowest concentrations with an effect not always dose-dependent (Sturchio, 2006).

In this paper we analysed the phytotoxic and genotoxic effects of the lavender essential oil in vitro and the effects of lavender plant use in the field. The evaluation of the effects was performed in *Vicia faba* root meristems. *Vicia faba* was chosen as a test plant because it has large chromosomes suitable for the study of chromosome aberrations and micronuclei in root tip cells following the mitotic division (Sturchio et al., 2007).

2 Experimental analysis

The experimental set-up consisted of in vitro and in-field tests.

2.1 In vitro phytotoxicity and genotoxicity tests

50 *V. faba* seeds were sowed in 500 g of sterilised sandy control soil in aluminium basins. Each basin was treated with water emulsion of *Lavandula* spp. (120 ml of water emulsion) at different concentrations (0 mg/l, 0.2 mg/l, 0.4 mg/l, 0.6 mg/l, 1.8 mg/l, 5.4 mg/l) of oil and was allowed to germinate in a climatic chamber at 20 ºC ±1 for 5 days. A sandy soil basin, treated with 120 ml of water, was used as negative control.

Each basin was sealed with laboratory film (Parafilm® M) in order to permit the exchange of oxygen and CO_2 but not the volatilisation of the essential oil.

The seedlings were used in the phytotoxicity and genotoxicity tests.

2.1.1 Phytotoxicity

The seedlings were taken out and the phytotoxicity was calculated by measuring the primary roots length of *V. faba* seedlings exposed to the essential oil to study the eventual toxic effects. The comparison between the mean of primary roots length of each samples with the control one can provide indication about eventual toxic effects

2.1.2 Genotoxicity

The genotoxic effect of lavender essential oil was estimated using micronuclei test and comet assay.

Micronuclei test: Micronuclei are Feulgen positive corpuscles localised within the cell wall in the cytoplasmatic area surrounding the main nucleus. They are formed by chromosome or chromosome fragments that are not incorporated into daughter nuclei at the time of cell division (Ma, 1982).

The genotoxic effects were evaluated by following the frequency of micronucleated cells in *V. faba* root meristems (De Marco et al., 1990; De Simone et al., 1992). The micronucleated cells frequency was scored from 15,000 cells (12 root tips, 400–500 cells for tip).

The *V. faba* root tips were cut and fixed in glacial acetic acid and ethanol 3:1 (v/v) solution for 24 h, then treated with HCl 1N to 60 °C for 8 min, dyed to Schiff's reagent for 24 h and fixed on microscope cover glass. For each samples were observed six tips to the optical microscope. The resulting data were analysed by Analysis of Variance (One way ANOVA) with a Dunnett's multiple comparison versus the control group, using the statistical software SPSS 6.1 (SPSS Inc. Chicago, IL).

Comet assay: Single Cell Gel Electrophoresis (SCGE) is a cytogenetic test to evaluate the genetic damage in single cell induced by mutagenic agents. It is a simple, sensitive and rapid short-term genotoxicity test; basically, it can be applied to all type of cells, including plant cells. It is a method for measuring, under alkaline conditions pH ≥ 13.5, all the DNA structural conformation changes, such as ds/ss breaks, alkali-labile sites (abasic sites, oxidised bases, etc.) (Koppen et al., 1996; Menke et al., 2000).

The test was performed under alkaline unwinding/alkaline electrophoresis (A/A) protocol (Angelis et al., 2000) modified.

The *V. faba* seedlings root tips were chopped in 0.5 ml of PBS with 10 mM Na_2 EDTA. The suspension was filtered (20 μm filter) to remove most of the tissue debris. The filtrate was mixed with 200 μl of LMP agarose to 0.5% in PBS.

The microscope slides were pre-treated with a NMP agarose layer to 1% and a NMP second layer to 0.5–0.6%. The slides were dipped in lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris-HCl and Sarcosyl 1% and 10% of DMSO, pH = 10 added before to use) for 1 h to 4 °C.

The slides were dipped in electrophoresis buffer for 40 min and then electrophorised at 300 mA and 30 V for 50 min. After electrophoresis the slides were placed in a neutralisation buffer (0.4 M Tris-HCl, pH = 7.5) to RT for 15 min. The slides were therefore fixed (a first step in 70% ethanol and second step in absolute ethanol), rinsed in distilled water and dyed with 100 μl ethidium bromide (20 μg/ml). The comets were viewed by epifluorescence microscope with an excitation filter of 515–560 nm and barrier filter of 590 nm. The DNA migration was determined using an images analysis system (IAS 2000, Delta Sistemi, Italia). For each comet several parameters were acquired. The % DNA was used as a parameter of DNA damage (μm).

The resulting data were analysed by One way ANOVA with a Dunnett's multiple comparison versus the control group using the statistical software SPSS (Chicago, IL).

2.2 In-field tests

The experiments were carried out on two similar soils:

- *Ecological farm 'Tenuta dei ciclamini' in Terni*: The experimental area consisted of eight plots (Eco 1, Eco 2, Eco 3, Eco 4a, Eco 4b, and Eco 4c, Control a and Control b). Each plot was delimited with lavender plants (Figure 1). Ecological treatments experimental design was as follows:

  Eco 1: *L. esculentum* mulched with *L. officinalis* minced

  Eco 2: *L. esculentum* mulched with *L. officinalis* biomass

Eco 3: *L. esculentum* mulched with *L. officinalis* biomass

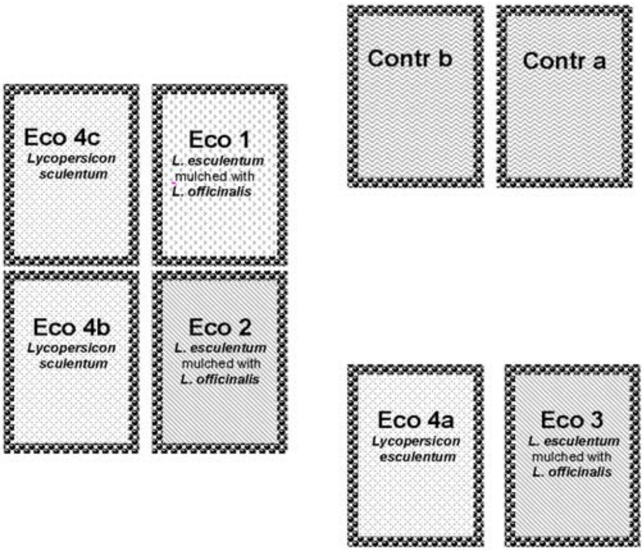
Eco 4a, Eco 4b, Eco 4c: *L. esculentum*

Control a, Control b: uncultivated.

- *Tuscia University Department Crop Productions experimental fields*: The experimental area consisted of biological (Bio samples) and conventional plots (Conv samples). Plots were disposed in alternate design (Figure 2). A second area designated for ecological treatment has been arranged with hedges of lavender (samples L1, L2, L3) and mint (samples M1, M2, M3).

The RFLP (Restriction Fragment Length Polymorphism) molecular analysis was utilised to evaluate lavender effects and biological/conventional agricultural treatments on soil microorganisms (Ranjard et al., 2000). The micronuclei and comet tests was utilised to estimate phytotoxicity and genotoxicity of lavander in the same soils.

Figure 1 Ecological treatment experimental design (ecological farm 'Tenuta dei ciclamini' in Terni)
Eco 1: *L. esculentum* mulched with *L. officinalis* minced;
Eco 2: *L. esculentum* mulched with *L. officinalis* biomass;
Eco 3: *L. esculentum* mulched with *L. officinalis* biomass;
Eco 4a, Eco 4b, Eco 4c: *L.esculentum* ; Contr a e Contr b: uncultivated

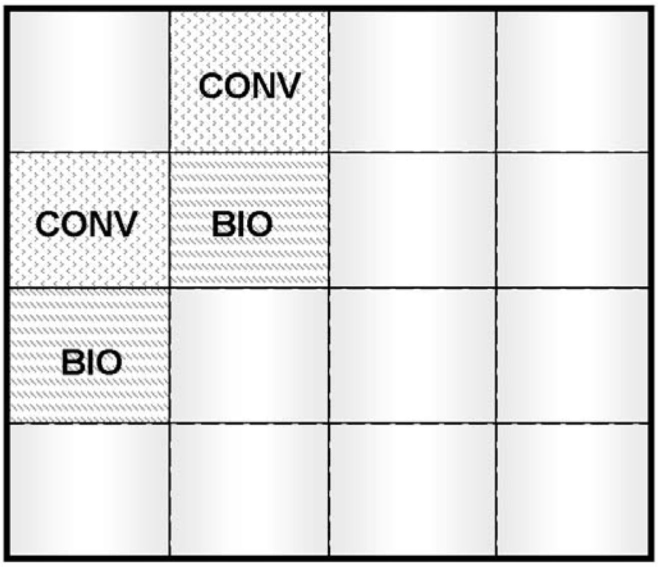


2.2.1 Phytotoxicity and genotoxicity tests

Sample collection: Soil samples (about 500 g each) were collected in plastic bags from surface soil (30 cm) and placed in aluminium basins. Each basin containing 50 seeds/basin, treated with 120 ml of H_2O, was allowed to germinate in a climatic chamber at 20°C ±1 for five days. Control sandy soil basin was used as negative control. For each sample were prepared three repetitions.

The seedlings were used in the phytotoxicity and genotoxicity tests as described above.

Figure 2 Biological and conventional treatments experimental design (Tuscia University farm) (Bio: Biological; Conv: conventional)



2.2.2 *Molecular analysis RFLP*

The genetic fingerprints provide complex band profile, a characteristic number of different bands for profile, which yield a representative of the genetic structure of the microbial community.

Each restriction fragment from the 28S rRNA gene amplified by PCR (Porteus et al., 1994) was treated as a unit character and scored as 1 (present) or 0 (absent) across all microbial samples. A tolerance value was applied to each fragment to compensate for misalignment of homologous fragments owing to technical imperfections: (1% for fragments 50–700 bp). From this binary matrix, the similarity of RFLP profile was calculated as a distance matrix with the Jaccard coefficient. The distance matrices were then used as data for multidimensional scaling analysis method (MDS) and cluster analysis.

MDS analysis was used to visualise the relationship among the samples based on results of RFLP analysis. The closer the points in the map were to each other, the more the microbial communities represented by the points were similar. Cluster analysis based on distance matrix revealed associations among lines which agree with expectations based on MDS information (Ramirez-Moreno et al., 2003; Takeshi et al., 2004).

DNA extraction: Soil samples (about 50 g each) were collected in sterile tubes from surface soil (10 cm), stored in plastic bags at 4 °C. The DNA extraction was performed with FastDNA SPIN kit for soil, Bio 101 – Systems (CA, USA).

Penicillium chrysogenum pure cultures were used as positive control.

DNA amplification: The amplification was performed in a reaction volume of 100 μl containing:

–10 mM Tris HCl (pH 8.3)

–50 mM KCl

–1.5 mM $MgCl_2$

–0.2 mM of dNTPs

–0.2 mM of each primer sense and antisense

–2.5 U of Taq Gold DNA Polymerase

To detect a wide range of eukaryotic DNA, the 28S rDNA primers, provided by Pharmacia Biotech, were used (Marshall et al. (2003):

sense primer:	5'GCA TAT CAA TAA GCG GAG GAA AAG
antisense primer:	5'GGT CCG TGT TTC AAG ACG G

Target sequence amplification of each sample was obtained after 35 cycles each consisting of DNA denaturation at 95 °C for 10 sec, primers annealing at 52 °C for 30 sec and extension at 72 °C for 30 sec, by using a tempcycler (GeneAmp PCR System 2400, Perkin Elmer Cetus, USA). After PCR reaction, an electrophoretic analysis was performed to control the DNA amplication.

Restriction digestion with BsuRI: Enzymatic digestion was performed by incubating 25 µl of each PCR product with 20 restriction enzyme units (BsuRI) for 6 h at 37 ^oC. Twenty-five enzyme units were added after the 3rd hour of digestion, according to manufactured instructions (Invitrogen Life Technologies).

The restriction fragments were analysed by polyacrylamide gel electrophoresis Gene Gel Excel 12.5 (Amersham) using GenePhore Electrophoresis Unit (Amersham). The plus One^{TM}DNA Silver Staining kit (Amersham) was used to reveal digested DNA.

*Image and statistical analysis:*The data analysis was performed taking into account the similarity values of the BsuRI fragments after image analysis by the software GelPro Analyser^{TM}Version 3.1 (Media Cybernetics). Restriction patterns were compared by scoring similarities using the Jaccard coefficient, then applying MDS and cluster analysis from the resulting similarities matrix. These similarity analysis were performed with PAST software (Ryan et al., 1995).

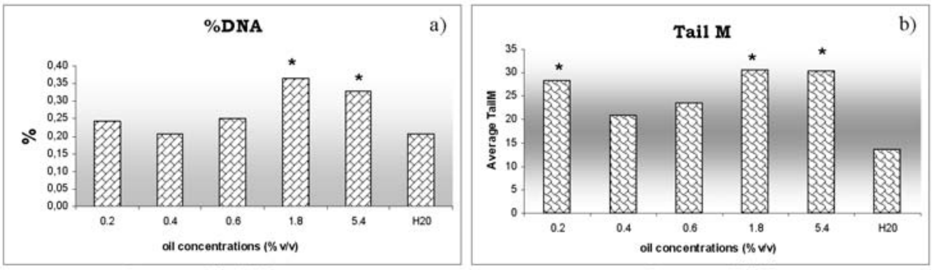
3 Results

3.1 *In vitro tests*

3.1.1 *Phytotoxicity and genotoxicity tests*

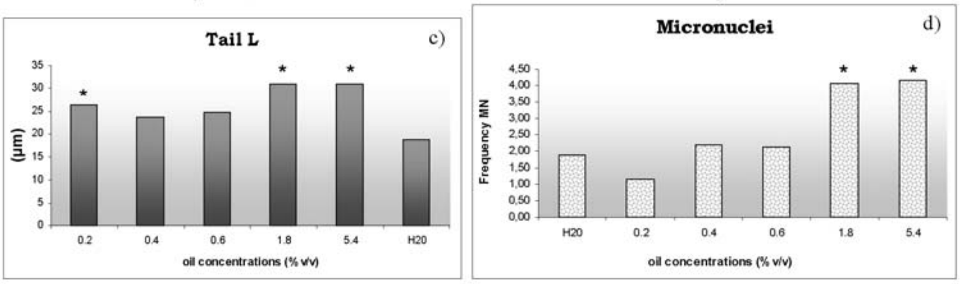
The results showed a phytotoxicity effect at all concentrations tested, with a decrement of mean value in all samples compared to the control sample. Both the two genotoxicity tests (comet assay and micronuclei test) showed that the concentration range between 1.8–5.4 mg/l of *Lavandula spp.* essential oil caused a significative DNA damage. At lower concentrations (between 0.2–0.6 mg/l) only comet assay results showed a DNA damage increment only at 0.2 mg/l concentration (Figure 3). These results show the absence of a dose-dependent relation between oil concentrations and genotoxic effect.

Figure 3 *In vitro* test set up with lavander essential oil at different concentration.



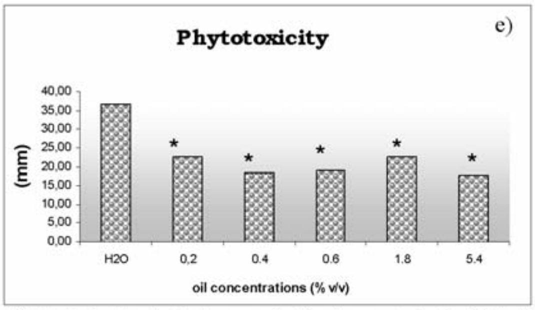
Comet assay: The %DNA

Comet assay: Tail M



c) Comet assay: Tail L

d) Micronuclei test: micronucleated cells frequency in root meristems



e) Phytotoxicity test: calculated by measuring the primary roots length of *Vicia faba*

The samples marked with an asterisk were resulted statistically significant (ANOVA; $p < 0.05$)

3.2 In-field tests

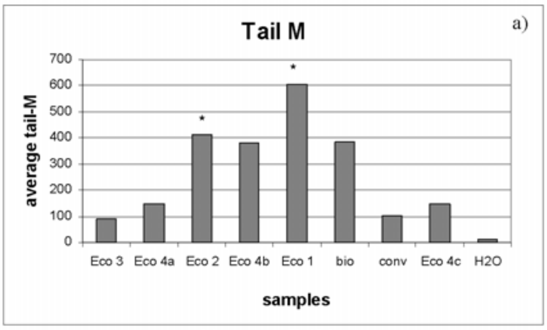
3.2.1 Phytotoxicity and Genotoxicity tests

Soil samples coming from biological treatment in the Tuscia University farm showed DNA damage with comet assay and phytotoxic effects, but not mutagenic effects with micronuclei test. Instead, conventional treatment soils showed an increase of the frequency of micronuclei even if not statistically significant.

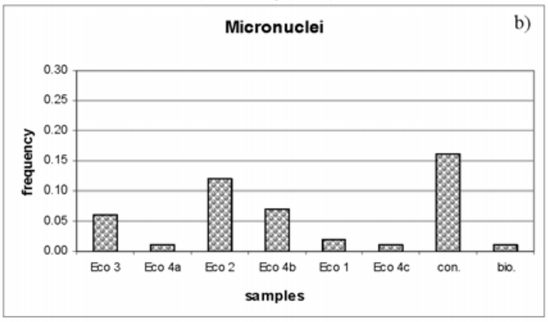
Among soil samples coming from ecological treatment in the ecological farm 'Tenuta dei ciclamini' in Terni, sample soil Eco1, mulched with *L. officinalis* minced, showed DNA damage with comet assay and primary roots length reduction with phytotoxicity test, but not micronucleated cells frequency increase (micronuclei test) (Figure 4).

The sample Eco2, mulched with *L. officinalis*, showed a significant DNA damage and a decrease in primary root length (Figure 4).

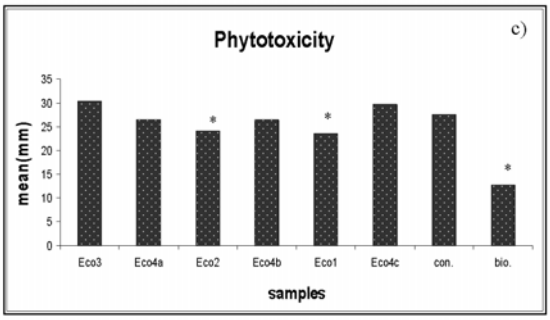
Figure 4 Test carried out in experimental field. In Terni experimental field,
Eco 1: *L. esculentum* mulched with *L. officinalis* minced;
Eco 2: *L. esculentum* mulched with *L. officinalis* biomass*;*
Eco 3*: L. esculentum* mulched with *L. officinalis* biomass*;*
Eco 4a, Eco 4b, Eco 4c: *L.esculentum*) and in Tuscia University experimental field, biological (Bio) and conventional (Conv) cultivated



a) Comet assay (Tail M)



b) Micronuclei test (micronuclei frequency)



c) Phytotoxicity test: calculated by measuring the primary roots length of *Vicia faba*

The samples marked with an asterisk were resulted statistically significant (ANOVA; $p< 0.05$)

3.2 Molecular analysis RFLP

The results obtained by 28S RFLP analysis show different clusters between the soil treated with conventional methods and biological ones (Tuscia University farm) at a level of similarity of about 30%. The sample soil Eco1 (ecological farm 'Tenuta dei ciclamini' in Terni) mulched with *L. officinalis* minced shows a different RFLP profile that reflects a fluctuation on microbial communities due to the release of essential oils by *L.*

officinalis. The ecological samples (L1, L2, L3, M1, M2, M3) from Tuscia University farm are clustered in a third group, separate from the others. The results obtained by the cluster analysis (Figure 6) were confirmed by the MDS analysis, where the sample Eco1 formed a distinct group in the MDS map (Figure 5).

Figure 5 Multidimentional analysis (MDS) of RFLP 28S analysis carried out in experimental field. In Terni experimental field,
Eco 1: *L. esculentum* mulched with *L. officinalis* minced;
Eco 2: *L. esculentum* mulched with *L. officinalis* biomass;
Eco 3: *L. esculentum* mulched with *L. officinalis* biomass;
Eco 4a, Eco 4b, Eco 4c: *L.esculentum*; Contr a e Contr b: uncultivated and in Tuscia University experimental field, biological and conventional.
Bio 1 and Bio 2: biological; Conv 1 and Conv 2: conventional and ecological
L1, L2, L3: lavander cultivated; M1, M2, M3: mint cultivated.

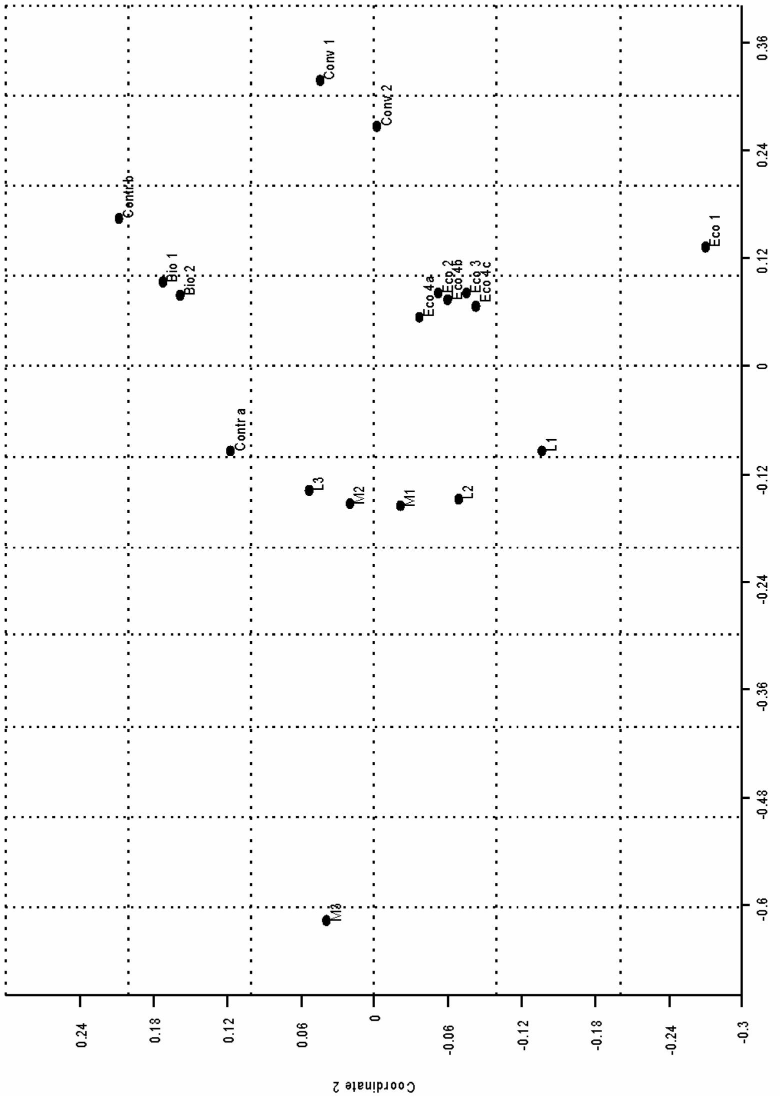
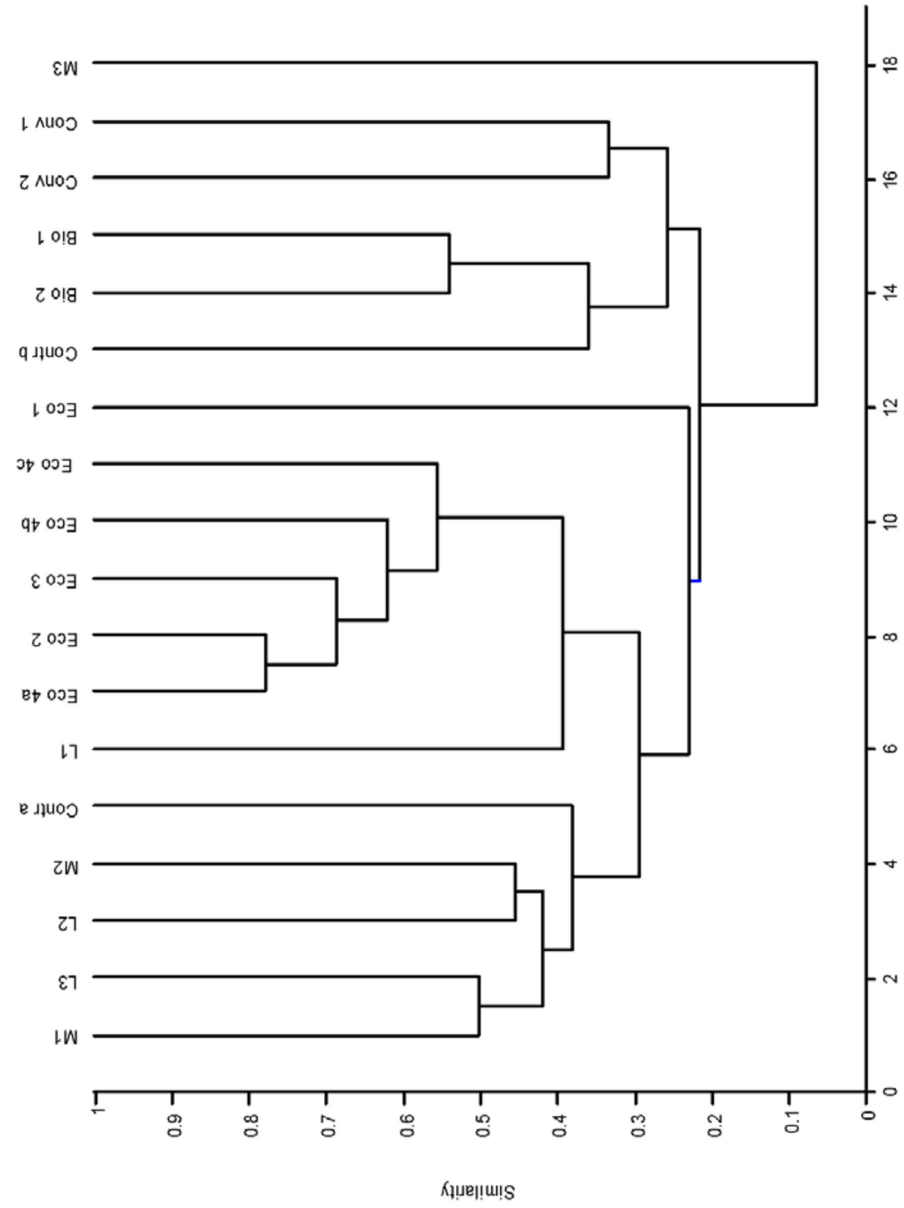


Figure 6 Cluster analysis of RFLP 28S analysis carried out in experimental field by scoring imilarities obtained with Jaccard coefficient. Resulting similarities matrix were performed with PAST software. In Terni experimental field,
Eco 1: *L. esculentum* mulched with *L. officinalis* minced;
Eco 2: *L. esculentum* mulched with *L. officinalis* biomass;
Eco 3: *L. esculentum* mulched with *L. officinalis* biomass;
Eco 4a, Eco 4b, Eco 4c: *L.esculentum*; Contr a e Contr b: uncultivated and in Tuscia University experimental field, biological and conventional.
Bio 1 and Bio 2: biological; Conv 1 and Conv 2: conventional and ecological L1, L2, L3: lavander cultivated; M1, M2, M3: mint cultivated.



4 Discussion

4.1 Preliminary screening

The evaluation of preliminary screening suggests that the essential oils could be useful as potential bioherbicides being alternative to conventional herbicides. The essential oils could avoid the chemical pollution caused by conventional agriculture, protecting the operating agricultural health and reducing or even eliminating toxic substance residuals in food. At last, they could improve the nutritional and organoleptic characteristics of the cultivated products (minor nitrate contained, greater vitamins contained, greater dried substance contained).

4.2 Mechanisms of herbicidal action

In any case, additional work is required to determine the mechanism of herbicidal action of essential oils so to develop a formulation that could be used under open field conditions. Essential oils are highly volatile and their persistence in the open field condition should be improved and it should be investigated the effect on crop species as well.

Further 'on field' research is necessary to develop an appropriate technology of essential oil application for inhibiting weed seed germination.

The reason why the essential oils inhibit seed germination remains largely unknown.

Probably the interference of essential oils on mitotic activity might also be responsible for the observed reduction or inhibition of weed seed germination. In more details, after the germination some biochemical process or mitotic interference activity inhibiting cotyledon growth have been reported (Dudai, 1999). Furthermore, the relation between oil concentrations and inhibition ability was not always dose-dependent for all the species.

Previous studies demonstrate that among the essential oils tested, the cinnamon exerted the stronger inhibitory effect followed by lavender and peppermint essential oils (Campiglia et al., 2007). The dicotyledonous species have been more susceptible to essential oils compared with the monocotyledons ones, even if a dose able to totally inhibit the seed germination has been recorded only for redroot pigweed.

Interestingly, the volatile components of lavender essential oils have also been found to display potent antifungal activity; however, no significant differences in activity have been reported between different *Lavandula* oil volatiles (Inouye et al., 2001; Moon et al., 2004). Vapour treatment would appear to have an advantage over solution treatment on microbial growth that could be inhibited by a smaller amount of essential oil, also acting as a potent inhibitor of sporulation. Previous studies suggest that the gaseous contact activity of the essential oils was determined mainly by the maximum vapour concentration at an early stage of incubation and that maintaining high vapour concentration for long periods of time was not necessary (Inouye et al., 2001).

4.3 'In vitro' tests

Genotoxicity and phytotoxicity tests revealed the toxic effect of essential oils at the highest concentrations (1.8, 5.4 mg/l) in the 'in vitro' tests, quantifying the upper limit values to set up a correct experimental design in ecological agriculture.

These results demonstrated that higher concentrations of essential oils can be useful for an efficient control of weed germination. At the same time the results obtained with comet assay, at the higher concentrations and also at the 0.2 mg/l, confirmed the results of previous studies on seed germination (Campiglia et al., 2007) in which the relationship between oil concentrations and inhibition ability was not always dose-dependent (Figure 3).

4.4 'In-field' tests

Results from genotoxicity and phytotoxicity tests about samples Eco1 (mulched with *L. officinalis*, minced) were statistically significant (Figures 4a, 4c). According to the results of the in vitro tests, this toxic effect is probably due to the use of lavender minced, probably because this treatment can cause a higher release of essential oil on soil.

The different results from the two genotoxicity tests, comet assay (Figure 4a) and Micronuclei test (Figure 4b) may be due to various reasons. The comet assay seemed to be more sensitive than the micronucleus test to assess the DNA damage induced by toxic substances, but the micronuclei test detects lesions which survived at least one mitotic cycle, while the comet assay identifies reparable DNA lesions or alkali-labile sites. There is a need to combine the comet assay with the use of other genotoxicity tests, such as the micronuclei test, to get a more comprehensive understanding of specific environments.

Vice versa the conventional treatment for soils from Tuscia University showed an increase of the frequency of micronuclei even if not statistically significant (Figure 4b); this result is probably due to the presence of substances that can interfere with the mitotic cycle.

Furthermore, in order to evaluated the impact of the use of the essential oils on the fungal community structures in the treated soils, molecular strategy based on the RFLP analysis of PCR fragments amplified from genomic 28S DNA has been used. Our study confirmed the hypothesis of the inhibition effect of the vapour concentration of essential oil, as showed in the RFLP analysis where the sample Eco1 (mulched with *L. officinalis* minced) formed a distinct group in the MDS map and in the cluster analysis (Figures 5 and 6). In fact, in the sample Eco 1, probably *L. officinalis* minced determining a major release of essential oil on soil, caused a different fluctuation of fungal communities and consequently clustered separately from the other samples.

5 Conclusion

The results of this study show that the use of essential oils in agriculture makes it advisable to assess their genotoxic potential, identifying their mutagenic components because plant species represent a great source of biologically active compounds whose effects on heritable material are mostly unknown. Research on investigating the safety and possible new activities of herbal products is a welcome advance that could lead to the development of new products with favourable risk-benefit profiles (Barnes, 2003).

Further researches are necessary to identify and isolate the chemical components of lavender oil, which will allow the identification of biologically active metabolites of the oil and determination of any synergistic effects of the 'mixed' components, in order to obtain a sufficient toxic response useful to inhibit weed germination (Heather et al., 2005).

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