

Phytotoxic effects of essential oil from *Eucalyptus cinerea* and its physiological mechanisms



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A. GRICHI^{1,2*}, Z. NASR², M. L. KHOUJA²

¹ Faculty of Sciences of Bizerta, 7021 Jarzouna, Tunisia

² National Institute for Research in Rural Engineering, Water and Forests Street Hédi Elkarraï, Elmanzeh IV, BP 10, Ariana 2080, Tunisia

* Corresponding author: aida.grichi@gmail.com

Abstract-In this study, we investigated the chemical composition of volatile oil of *Eucalyptus cinerea* and its allelopathic effects on germination, seedling growth, chlorophyll content, net photosynthetic rate, transpiration and stomatal conductance of *Sinapis arvensis*, *Erica vesicaria*, *Scorpiurus muricatus*, *Triticum durum*, *Vicia faba* and *Phaseolus vulgaris*. Dose-response studies were conducted under laboratory and field conditions. Germination, seedling growth and net photosynthetic rates were greatly affected by *E.cinerea* essential oil. The reduction increased with increasing of essential oil concentration. Relative to the control, greatest reduction in net photosynthetic rate were observed at highest concentrations. The trend for transpiration in response to *E.cinerea* essential oil was similar to that exhibited by net photosynthetic rate except that the effects were greater in magnitude as compared to leaf photosynthesis. Stomatal conductance followed patterns similar to those observed for transpiration and leaf photosynthesis, however the effects were more apparent suggesting that closure of stomata might be an important factor for the decreases in photosynthesis and transpiration in this study. The test plants responded differently to eucalypt oil exhibiting a differential species-specificity. Indeed, the weeds were affected more strongly than the crops. The results indicated that *E.cinerea* essential oil could inhibit the growth and many physiological processes of tested species. Thus, *E.cinerea* possesses weed-suppressing abilities. Hence, these could be a base for developing natural herbicides.

Keywords: Allelopathy, Seedling growth, Net photosynthetic rate, Transpiration, Stomatal conductance.

1. Introduction

Negative impacts of the continuous use of synthetic herbicides have consequences on human health and environment, and lead to increasing herbicidal resistance in many weed species (Vyvyan, 2002; Batish et al., 2004). Therefore, efforts to develop alternative means of weed control, which are not only eco-friendly, but also cost effective and bioefficacious are needed (Duke et al., 2002). Allelopathy offers potentially selective biological weed management through the production and release of allelochemicals from leaves, flowers, seeds, stems, and roots of living or decomposing plant materials (Weston, 1996). Allelopathy can describe any direct or indirect effect of plant chemical compounds on another plant or microbe by allelochemicals released through leaching from leaves or through volatile emissions (Weir, 2004). Earlier studies have documented that volatile oils and their constituents are potent seed germination inhibitors and retard plant growth (Muller et al., 1964; Barney et al., 2005; Batish et al., 2006; Ens et al., 2009). Kohli et al. (1998) reported that the volatile oil from lemon-scented eucalypt (*E.citriodora* Hook) and Tasmanian blue gum inhibits the germination and early seedling growth of *Parthenium hysterophorus* L. and pointed that these could be used for weed management. The phytotoxicity of some allelochemicals is attributed to their ability changing the normal metabolic processes in other plant, including respiration, photosynthesis, xylem element flux, membrane permeability, cell division and development, protein synthesis and enzyme activity alteration (Zeng et al., 2008). Especially, the allelochemicals could directly inhibit the PSII activity, interrupt dark respiration and ATP synthesis and induce ROS-mediated oxidant damage to other plants (Weir et al., 2004). The volatile organic compounds also affect the biosynthesis pathway of secondary



metabolites or specific steps of monoterpene metabolism (Santoro et al., 2011), inhibit photosynthesis and trigger the synthesis of reactive oxygen species (ROS) to disrupt the subcellular structure in *chlorella vulgaris* (Qjan et al., 2009). Volatile oil from *Artemisia ordosica* inhibits the growth and photosynthetic activity of *Palmellococcus miniatus* through the combined effects of components in volatile oil (Yang et al., 2012). Kaur et al. (2010) observed that the application of *Artemisia* oils on 6-week-old weed plants caused losses in chlorophyll concentrations in the leaves and injuries, ranging from chlorosis to necrosis. Singh et al. (2002) also found reduction in chlorophyll content of mature *C.occidentalis* and *E.cruss-galli* plants sprayed with eucalypt oils. Based on these reports, it is opined that since volatile of *E.cinerea* is phytotoxic to weeds, it should be explored for bioherbicidal activity. Thus, the present study aims to investigate the herbicidal activity of *E.cinerea* volatile oil in terms of effect on plant growth, and impacts on metabolic process such as chlorophyll content, photosynthesis, respiratory activity and stomatal conductance.

2. Materials and methods

2.1. Extraction of essential oil from *E.cinerea*

Plant material

Leaves were collected in April 2011 from *E.cinerea* trees acclimated in Souiniet arboreta (located in Ain Draham, north of Tunisia). The essential oils were extracted by hydrodistillation of 100g of boorishly crushed leaves for 4h, using a standard apparatus recommended by the European Pharmacopeia. Hydrodistillations were performed in triplicate. The yield in essential oil was expressed in % (v/w) of the dry material (Elaissi et al., 2010).

Chemical characterization of the oil

The chemical composition of the extracted essential oil was determined by gas chromatography-mass spectroscopy (GC-MS) (Elaissi et al., 2010).

GC Analysis: GC Analysis was carried out with a Hewlett-Packard 6890 apparatus equipped with FID and an intermediately polar Supelco SPB-20 cap. Column (30m×0.32 mm i.d., film thickness 0.25 µm). The oven temp. was programmed isothermal at 35°C for 1 min, rising from 35 to 250°C at 5°/min, and then held isothermal at 250°C for 3 min; injector temp., 250°C; detector temp., 280°C; carrier gas, N₂ (1.2 ml/min). The injected volume was 1 µl (10% essential oil in purified hexane). The relative concentration was calculated using the software HP Chemstation, which allowed assimilating the percentages of the various constituents. Retention indices (RI) were determined rel. to the retention times (t_R) of a series of n-alkanes (C₉-C₂₈) (Elaissi et al., 2010).

GC/MS Analysis: The essential oils were analyzed with a Hewlett-Packard 5890 series II apparatus equipped with a 5972 mass-selective detector and an intermediately polar Supelco SPB-20 cap. Column (30m*0.32mm i.d., film thickness 0.25 µm). He was used as the carrier gas. The mass spectrometer operating conditions were: ionization voltage, 70 eV; ion source, 230°C. The GC anal. Conditions were as described above (see GC Analysis) (Elaissi et al., 2010).

Compound Identification: The identification of the compounds was based on the comparison of their RI and mass spectra with those of authentic compounds by means of the NBS75K.L. and Wiley 275 databases and with literature data (Wiley, 1998).

Dose-response studies

Seeds of all test species: *Sinapis arvensis*, *Erica vesicaria*, *Scorpiurus muricatus*, *Triticum durum*, *Vicia faba* and *Phaseolus vulgaris* were collected locally from agricultural fields on Ousseltia (located in Kairouan, centrest of Tunisia, with arid bioclimatic stage). These were surface-sterilized with sodium hypochlorite (0.1%, w/v) for 2 min, washed under running tap water (for 5min) followed by distilled water and stored for further use.

Dose-response studies were conducted under laboratory conditions to determine the effect of *E.cinerea* essential oil on growth of test species. Briefly, 10 seeds of all these test species were germinated in Petri dishes (15cm diameter) on a filter paper wetted with 7ml of distilled water. To test the inhibitory effect of eucalypt oil, different amounts of oil were loaded on the inner side of cover of Petri dish (0.12, 0.25, 0.5 and 0.75µl/ml) after spacing the seeds on the base and then sealed immediately with tape. Control was kept without loading essential oil. For each concentration, five replicates were maintained. All the Petri dishes were kept in a growth chamber maintained at 16/18h light/dark period at 25±2°C temperature. After 7 days, the number of seeds that germinated was counted, and their root and shoot lengths were measured.

The percent of inhibition of germination, root and shoot lengths were calculated from the following equation: Inhibition (% of control) = $(100 - (\text{sample extracts}/\text{control}) \times 100)$ (Charoenying et al., 2010)

Greenhouse study

To test the herbicidal activity of the volatile oil from *E.cinerea* under field conditions, experiments were conducted in the greenhouse. Seeds of all test species were sown manually in 15cm pots. For this, 1200g of garden soil was taken in each pot and seeds of *Sinapis arvensis*, *Erica vesicaria*, *Scorpiurus muricatus*, *Triticum durum*, *Vicia faba* and *Phaseolus vulgaris* were sown. Pots were placed in experimental house with natural light conditions (Temperature 21°C, Humidity 32%, Sunshine 7h-1) and irrigated daily. When the plants were 4-week-old, they were spray treated with 25, 50, 75 and 100µl/ml solution of *E.cinerea* essential oil (or distilled water to serve as control) in such a manner that each plant received 6ml of treatment solution. For each treatment five replications were maintained. One- two and 3-days after spray (DAS), the treated test plants were examined for chlorophyll content levels, and the net photosynthesis, transpiration and stomatal conductance were measured.

Estimation of chlorophyll content

Chlorophyll content was determined using a chlorophyll content meter CCM-200 (SPAD).

Measure of net photosynthesis, transpiration and stomatal conductance

To measure net photosynthesis, transpiration and stomatal conductance we used the LI-6400XT Portable Photosynthesis and Fluorescence System (LI-COR).

2.2. Statistical analyses

All data obtained from seed germination, seedling growth, chlorophyll content net photosynthetic rate, transpiration and stomatal conductance assays of test species were expressed as mean values and were, on the condition of significant ANOVA, analyzed by means of multiple comparison SNK tests in order to investigate if significant differences existed between eucalypt oil concentrations and test species. Values of $p \leq 0.05$ were considered significantly different.

3. Results and discussion

3.1. Chemical characterization of the *E.cinerea* essential oil

The essential oil was obtained by boiling hydrodistillation of *E.cinerea* leaves, which gave an oil of 2.31% yield. The chromatogram obtained by gas chromatography coupled with mass spectrometry indicated that *E.cinerea* essential oil shared a high proportion of the 1,8-Cineole (48.3%), α -Pinene (14%), and a relatively high mean percentage of α -Terpineol (10.3%). The ρ -Cymene (hydrocarbons monoterpenes) represent (10%) of the total *E.cinerea* essential oil (Table.1). The presence of 1,8-cineole as the major compound in *E.cinerea*, α -Terpineol and α -pinene representing high proportion is in agreement with earlier studies (Elaissi et al., 2010).

3.2. Growth studies under laboratory conditions

The emergence of all the test species was significantly reduced. In general, a dose-response relationship was observed and the emergence declined with the increase in amount of *E.cinerea* essential oil. At 0.12µl/ml *E.cinerea* essential oil, there was no significant effect on emergence of test species, except in *P.vulgaris* and *S.murucatus*, where 62.5 and 56.25% respectively decrease was observed. However, at 0.75µl/ml *E.cinerea* essential oil, 0% emergence was observed in *S.arvensis*, 2.5% in *P.vulgaris* and 12% in *S.murucatus* (Figure1a). Not only emergence, even the seedling growth measured as root and shoot length was significantly reduced even at 0.12µl/ml *E.cinerea* essential oil. At 0.25µl/ml *E.cinerea* essential oil 61 to 95 % reduction was observed in root length of tested species. The reduction was greater with increasing amount of *E.cinerea* essential oil (Figure1b and c). Indeed, at highest concentration (0.75 µl/ml), the maximum inhibition in root length was observed in *S.arvensis* and *P.vulgaris* (100 and 99.64% respectively) (Figure1b). Likewise, the shoot length of test weeds was significantly reduced in response to *E.cinerea* essential oil, but with varying degrees of susceptibility. Also, the shoot growth was further reduced when eucalypt oil concentration increased. In general, the inhibitory effect was greater on weeds than on crops (Figure1a, b and c).

The observations made in the present study are parallel to earlier studies documenting the growth inhibitory activity of aromatic plants, including *Eucalyptus* species and their volatile oils. For example, volatile oil (0.12-0.30 mg/ml) from *Eucalyptus citriodora* reduced seedling growth and dry weight accumulation in *Cassia occidentalis*, *Amaranthus viridis* and *Echinochloa crus-galli* by $\geq 50\%$

(Batish et al., 2004). It was demonstrated that essential oils from tree Mediterranean Lamiaceae members *Rosmarinus officinalis*, *Thymus vulgaris* and *Satureja montana* (at 500ppm) severely reduced germination and seedling growth of weedy species such as *Chenopodium album*, *Portulaca oleracea* and *E.crus-galli* (Angelini, 2003). Later, it was reported that eucalypt oil (at 0.2-5.0 nl/ml) reduced seed germination and seedling growth of *P.hysterophorus* by 56-100% (Singh, 2005). The volatile oil from *Tagetes minuta* (at 100-1000ppm) was demonstrated that inhibited the germination of weed species such as *Taraxacum officinale*, *Mikania cordifolia* and *Cynodon dactylon* (Lopez, 2008). Recently, it was reported that volatile oil from *Artemisia scoparia* (at 0.14-0.35mg/ml) inhibited radical emergence and seedling growth in *Cyperus rotundus* and *Phalaris minor* (Singh, 2009). The observed growth inhibitory effects on test plants may either be due to synergistic or additive effect of compounds in *E.cinerea* oil. Allelopathy is the result of the simultaneous action of several compounds and often includes compounds whose chemistry is divergent (Einhellig, 2002). This ecological phenomenon is considered to be responsible for dominance and successful colonization of a particular exotic species in invaded plant community (Barney et al., 2005; Ens et al., 2009).

Table1. Chemical composition of the essential oils extracted from freshly collected mature leaves of *Eucalyptus cinerea*

Compound class and name	RI ^a	Composition (%)
<i>Monoterpene hydrocarbons</i>		28.6
α-Thujene	922	Tr
α-Pinene	930	14
β-Pinene	975	0.1
β-Myrcene	980	0.2
α-Terpinene	1006	0.3
ρ-Cymene	1015	10
γ-terpinene	1067	0.1
para-Cymenene	1071	3.5
α-Terpinolene	1089	0.4
<i>Oxygenated monoterpenes</i>		62.4
Camphor	1125	Tr
Neryloxide	1137	Tr
Borneol	1150	1.3
Table 1 (continued)		
Terpinene-4-ol	1163	1.1
α-Terpineol	1176	10.3
Fenchylacetate	1203	0.2
Carvacrylmethyloxide	1224	0.7
Carvacrolmethylether	1226	0.3
Linalylacetate	1240	0.1
Carvacrol	1279	0.1
1,8-Cineole	1282	48.3
<i>Sesquiterpene hydrocarbons</i>		2.1
Aromadendrene	1434	0.2
Germacrene D	1478	1.4
γ-Cadinene	1507	0.4
δ-Cadinene	1517	0.1
<i>Oxygenated sesquiterpenes</i>		0.3
β-Eudesmol	1362	0.2
β-Ionone	1462	0.1
Palustrol	1562	Tr
Caryophylleneoxide	1575	Tr
Ledol	1585	Tr
<i>Oxygenated diterpens</i>		0.1
(E)-Phytol	2102	0.1
<i>Aliphatics compound</i>		2.8
(z)-2-heptenal	926	1.1
1-Octen-3-ol	959	1
Decane	1000	0.2
Nonanal	1081	0.1
2-phenylethanol	1119	0.1
Decanal	1182	Tr
Octylacetate	1191	0.1
Decanol	1253	Tr
Viridiflorol	1579	0.1
Tricosene	2300	0.1
Total identified (%)		96.3

R.I^a: Retention Index

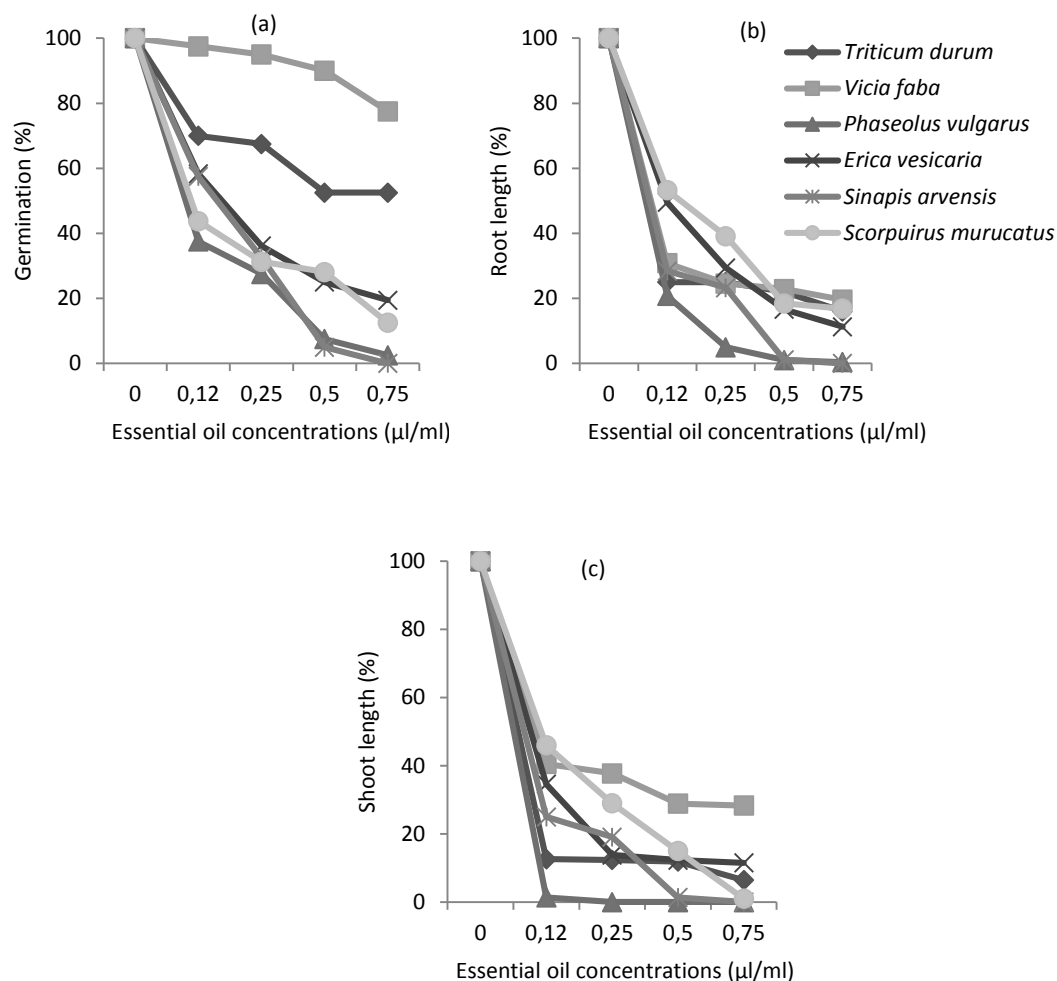


Figure1. The effect of *E. cinerea* essential oil on percent germination (a), root (b) and shoot length (c) of test species measured 7 days after treatment.

3.3. Eucalypt oil affects the chlorophyll content

A significant reduction was observed in chlorophyll content in leaves of weed plants sprayed with *E. cinerea* essential oil. Upon treatment with 25µl/ml, 1-DAS, the chlorophyll content was reduced for all weed species. The chlorophyll content declined further in response to treatment with higher concentrations of eucalypt oil (Figure2). The greatest inhibition in chlorophyll content was observed in *E. vesicaria*, 1-DAS, with 100µl/ml eucalypt oil. Moreover, in the crops, the reduction in chlorophyll content was greatest in *P. vulgaris* 1-DAS. In response to 100µl/ml eucalypt oil, 3-DAS, the inhibition in chlorophyll content was greatest in *E. vesicaria* (~99%) followed by *S. arvensis* (~96%), *S. murucatus* (~92%), *P. vulgaris* (~64%), *V. faba* (~63%) and *T. durum* (~30%) (Figure2). The observed loss in chlorophyll content is in agreement to earlier reports that volatile oils and monoterpenes reduce chlorophyll content and thus interferes with photosynthetic activity of the plants (Singh, 2002; Batish et al., 2004). However, whether the loss of chlorophyll is due to inhibition in chlorophyll synthesis or degradation of existing chlorophyll was not determined. The yellowing of weed leaves upon eucalypt oil spray may be the secondary effect due to decrease in chlorophyll content (Kaur, 2010).

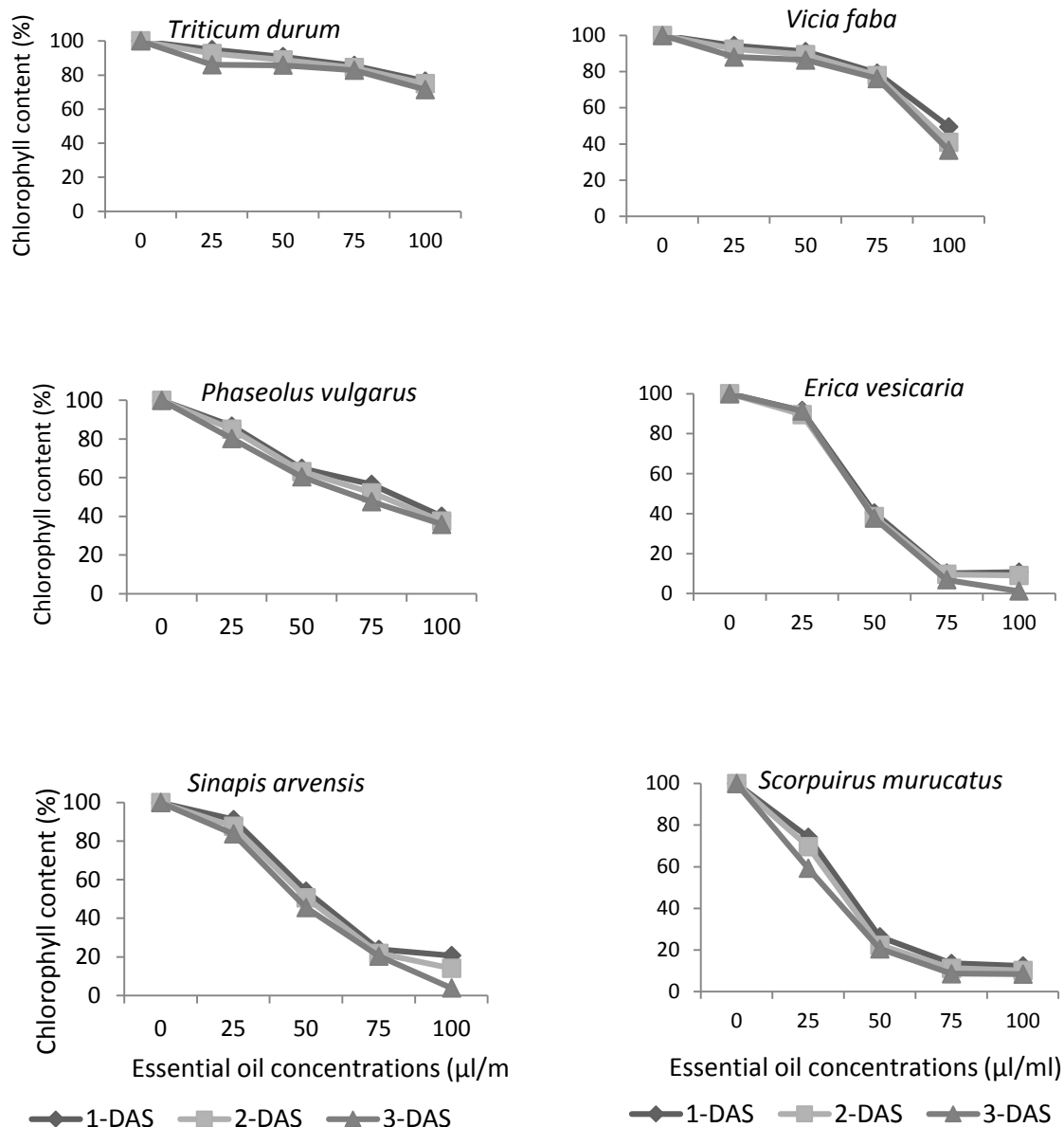


Figure 2. Effect of *E. cinerea* essential oil on chlorophyll content in 4-week-old plants of test weeds measured 1-2 and 3 days after spray (DAS).

3.4. Eucalypt oil affects the net photosynthesis, respiratory activity and stomatal conductance

Parallel to chlorophyll content, a significant decline in photosynthetic activity was observed in *E. cinerea* essential oil sprayed plants (Figure 3). In response to 25 µl/ml *Eucalyptus* oil treatment, net photosynthesis was reduced by 2.5-50%. It declined further with increasing of eucalypt oil concentration. At 100 µl/ml *E. cinerea* oil treatment, net photosynthesis decreased by 45% (*Vicia faba*) to 100% (*Erica vesicaria*, *Sinapis arvensis* and *Scorpiurus murucatus*) in test species. The same effects of *Eucalyptus* oil was observed in the case of respiratory activity and stomatal conductance (Figure 3). Indeed, at 100 µl/ml *Eucalyptus* oil treatment, the reduction was 38-100% and 46-100% for respiratory activity and stomatal conductance respectively. For all these parameters, weeds species were more affected than crops species. These observations are parallel to earlier reports that volatile oils affect the cellular respiration and thus interfere with energy metabolism of the plants resulting in reduced growth (Singh et al., 2006b, 2009). Earlier, Abraham et al. (2000) reported that monoterpenes, the constituents of volatile oils, due to high lipophilicity act as uncouplers of oxidative photophosphorylation, suppress respiration and thus imbalance cellular energy. Lorber and Muller

(1976) opined that volatile terpenes damage mitochondria and thus negatively impair respiration and energy metabolism.

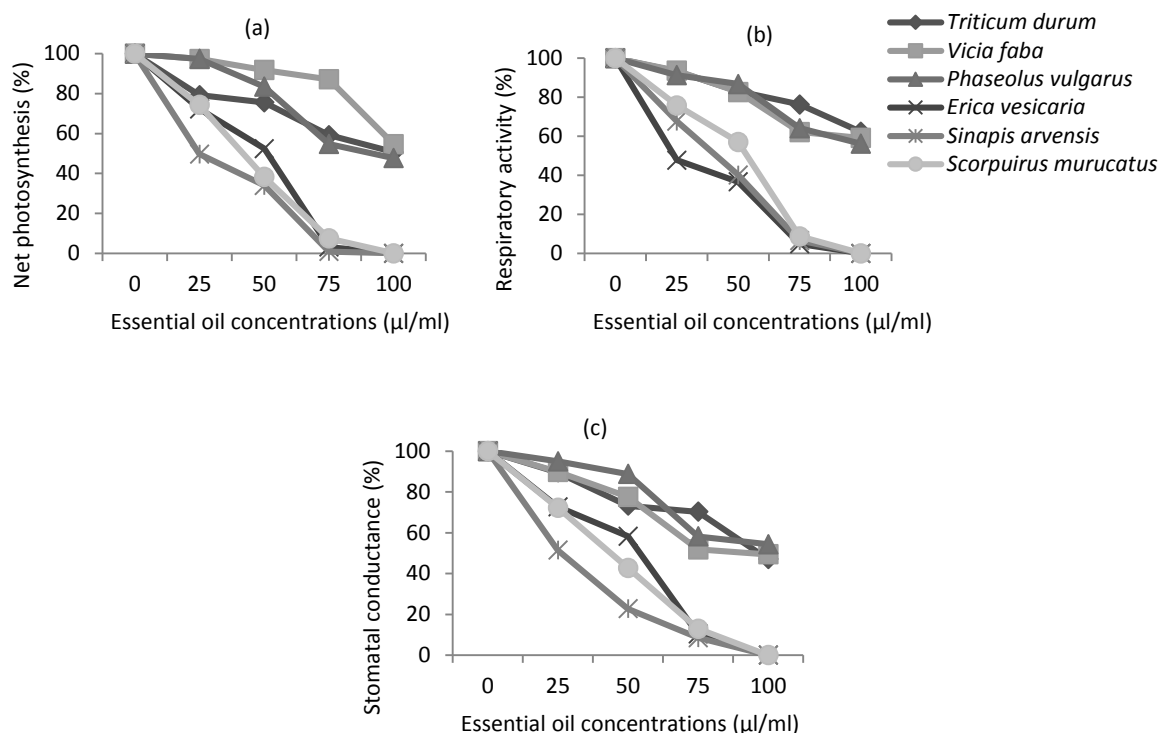


Figure 3. The effect of *E. cinerea* essential oil on net photosynthesis (a), respiratory activity (b) and stomatal conductance (c) of test species measured 3 days after treatment.

4. Conclusion

Based on the present study, we conclude that eucalypt oil possess a strong phytotoxicity against weeds by inhibition of seedling growth and alteration of photosynthetic machinery. Therefore, it could be useful for developing as a bioherbicide for weed management purposes. Indeed, use of *Eucalyptuscinerea* oil as allelopathic agent will be eco-friendly, cheaper, practical and efficacious mode of weed control. Nevertheless, further studies are required to explore the exact cascade of events that are responsible for growth inhibitory action of *E. cinerea* essential oil.

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