Phytotoxic Activity of Clove Oil, Its Constituents, and Its Modification by Light Intensity in Broccoli and Common Lambsquarters (Chenopodium album)

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Phytotoxic Activity of Clove Oil, Its Constituents, and Its Modification by Light Intensity in Broccoli and Common Lambsquarters (*Chenopodium album*)

Agnieszka Stokłosa, Renata Matraszek, Murray B. Isman, and Mahesh K. Upadhyaya*

Herbicidal activity of clove oil and its main constituents eugenol, β-caryophyllene, and α-humulene was studied by measuring their effects on cell membrane integrity in broccoli and common lambsquarters plants at the three- and nine-leaf stage, respectively. Roles of essential oil constituents in the overall phytotoxicity of clove oil, dose-response (10 to 160 mM) relationships of their phytotoxicity, and the effect of light intensity on phytotoxicity of clove oil and eugenol were studied. Most of the phytotoxicity of clove oil (2.5% solution) was due to eugenol, its largest constituent. β-caryophyllene and α-humulene played little or no role. Dose-response relationships showed that at equimolar concentration, eugenol was the most phytotoxic essential oil constituent of the clove oil. On a per unit biomass basis, membrane damage in response to clove oil and eugenol sprays decreased with increasing light intensity. This suggests that efficacy of essential oil in causing plant damage could be affected by light intensity experienced by plants prior to the oil spray.

Nomenclature: Common lambsquarters, *Chenopodium album* L. CHEAL, Broccoli, *Brassica oleracea* L. var. *italica* Plenck, purple sprouting broccoli.

Key words: Eugenol, β-caryophyllene, α-humulene, membrane integrity.

Organic agriculture has surged in popularity in recent years because of health and environmental concerns associated with synthetic herbicides (Dayan et al. 2009). Because synthetic herbicides are not permitted in organic production systems, the search for natural products has intensified (Duke et al. 2003; Isman 2000; Shahi et al. 2007). Chemicals derived from plants, including essential oils, that possess insecticidal, bactericidal and/or fungicidal properties, could be valuable tools for integrated pest management in organic agriculture (Daferera et al. 2003; Duke et al. 2003; Kalemba and Kunicka 2003; Kobaisy et al. 2001). Because of their low toxicity and rapid breakdown in the environment, these oils are safe to both the environment and the consumers (Isman 2000).

Clove oil, extracted from clove tree [*Syzygium aromaticum* (L.) Merr. & L. M. Perry], has strong fungicidal, insecticidal, and herbicidal properties (Duke et al. 2003; Isman 2000; Shahi et al. 2007) and offers a useful pest management option in organic crop production systems. Clove oil is available as a commercial nonselective foliar herbicide (Matratec [Brandt Consolidated Ltd., Springfield, IL]) for organic weed management. Main constituents of clove oil include phenylpropanoid eugenol and eugenol acetate, and sesquiterpenes β-caryophyllene, α-humulene, and humulene epoxide (Bauer et al. 1997; Raina et al. 2001; Srivastava et al. 2005). Little information is available on the herbicidal activity of various essential oil constituents of clove oil. Clove oil and its major constituent, eugenol, damage plant tissue by damaging its cellular membranes (Bainard et al. 2006; Srivastava et al. 2005; Tworkoski 2002). Presence of leaf epicuticular wax has been reported to influence their efficacy in damaging broccoli and common lambsquarters leaf tissue (Bainard et al. 2006). Solar irradiance intensity is known to influence the thickness of the epicuticular wax layer on the adaxial surface of leaves (Fondom et al. 2009), and species have been reported to differ in this regard. Decreasing light intensity has been reported to reduce leaf epicuticular wax (LEW) content in giant foxtail (*Setaria faberi* Herrm.) (Hatterman-Valenti et al. 2006), but no such effect was observed in case of velvetleaf (*Abutilon theophrasti* Medik.) (Hatterman-Valenti et al. 2011). However, the composition of LEW (viz., proportion of hydrophilic to hydrophobic components), which is known to influence leaf-wetting properties, was influenced by light intensity in both species (Hatterman-Valenti et al. 2006, 2011). Thus, light intensity exposure prior to clove oil spray could influence its herbicidal efficacy. Plant plasma membranes play a critical role in maintaining cellular integrity and destabilization of this lipid bilayer leads to cell death. As a result, monitoring membrane integrity, is a good biological marker for a number of herbicide mechanisms of action (Dayan and Watson 2011). Little information on the contribution of constituents of clove oil to cell membrane damage, herbicidal potential of these constituent oils, and effect of light intensity on their efficacy is available. This study therefore was conducted to determine: (1) relative contribution of constituents of clove oil to its overall phytotoxicity, (2) phytotoxic potency of clove oil constituents at equimolar concentrations, and (3) the effect of light intensity on efficacy of clove oil and its main constituent eugenol.

Materials and Methods

Plant Culture. Broccoli (purple sprouting) cv. 'Red Arrow' (West Coast Seeds, Delta, BC, Canada) and common lambsquarters (collected from Scott Research Farm in 2002) seeds were sown 1-cm-deep in a mixture of 75% peat and 25% perlite in 300-ml plastic pots in either a glasshouse or outdoors at the University of British Columbia Totem Field Laboratory. Greenhouse conditions during plant growth (December 2009 to April 2010) were: 300 μmol m^{-2} s^{-1} average photosynthetically active radiation (PAR) around noon (natural light supplemented with ~15 μmol m^{-2} s^{-1} PAR using high pressure sodium lamps), 14 h photoperiod, and 19.9 to 22.3 C mean night and 25.8 to 27.4 C mean day temperatures. Plants (1 per pot) were grown to three-leaf stage for broccoli and eight- to nine-leaf stage for common lambsquarters. In all experiments described below, measurements were taken on the

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second leaf for broccoli and the sixth leaf for common lambsquarters.

Membrane Integrity Measurement. In all experiments the effect of clove bud oil (Ecosafe Natural Products Inc., Saanichton, BC, Canada) and its three major constituents: eugenol, β-caryophyllene, and α-humulene (Sigma Aldrich, St. Louis, MO) (Jirovetz et al. 2006) on membrane integrity was measured to assess tissue damage. Clove oil used in all experiments of this study contained 63.96% eugenol, 13.99% β-caryophyllene, and 6.06% α-humulene (unpublished GC-MS data, R. Bradbury, Ecosafe Natural Products, Inc.). Essential oil solutions containing 0.2% Tween 20 (Fisher Scientific Co., Fair Lawn, NJ) were sprayed on the adaxial leaf surface of seedlings to the point of drip using 50 ml spray bottles. Two h after spraying, five discs (10-mm-diam) were excised from the treated leaves and incubated in 25 by 100 mm culture tubes in 10 ml distilled water in darkness on a rotary shaker at 90 rpm for 3 h. Electrolyte concentration of the bathing medium was measured using a conductivity meter (Hanna Instruments Inc., Laval, PQ, Canada) to assess the loss of membrane integrity.

Contribution of Essential Oil Constituents of Clove Oil to Its Toxicity. In order to assess the contribution of eugenol, β-caryophyllene, and α-humulene to the toxicity of clove oil, effects of their concentrations in proportion to their relative presence in the clove oil were studied as described above. A 2.5% solution of clove oil, which has been shown to be phytotoxic (Bainard et al. 2006), was used. Experiments were conducted in a glasshouse using a completely randomized experimental design (n = 7) and were repeated.

Dose-Response Relationships of Phytotoxicity of Clove Oil and Its Major Constituents. In order to compare relative phytotoxicty of major constituents of clove oil, leaf tissue damage caused by eugenol, β-caryophyllene, and α-humulene at equimolar concentrations was studied. Broccoli and common lambsquarters leaves were exposed to 10, 20, 40, 80, and 160 mM concentrations of these essential oils and their effects on membrane integrity were compared. The millimolar concentrations of the main constituents in 2.5% solution of clove oil were approximately as follows: 102 mM for eugenol, 15 mM for β-caryophyllene, and 7 mM for α-humulene. Experiments were conducted in a glasshouse using a completely randomized experimental design ($n = 7$) and were repeated twice.

Effect of Light Intensity on Efficacy of Clove Oil and Eugenol. In order to determine the effect of light intensity on efficacy of clove oil and its main constituent eugenol, broccoli and common lambsquarters seedlings were grown (mid-July to the end of August, 2010) under 0, 1, 2, or 3 layers of charcoal fiberglass insect screen mesh (Wire Products Inc, Tuscaloosa, AL), both on the top and the sides, in 60 cm by 60 cm by 45 cm high frames made of polyvinyl carbonate pipes at the Totem Field Laboratory. On the top of each frame, a layer of cellulose acetate film (diacetate type, 0.127 mm) (McMaster-Carr, New Brunswick, NJ) was placed to exclude the rainfall. On a sunny day, average light intensities at noon, measured using a LI-185B photometer (LI-COR Inc, Lincoln, NE) were 1,500; 800; 500 or 250 μE m^{-2} s^{-1} under 0, 1, 2, or 3 screens, respectively.

The plants were sprayed with either 2.5% clove oil or 1.6% eugenol in water containing 0.2% Tween 20. The electrolyte leakage from leaf discs excised from these plants was measured as described above. The area of sprayed leaves was measured using a Portable Area Meter LI-3000 (LI-COR Inc), the leaves were dried at 50 C for 72 h, and weighed. Specific leaf weight (SLW: leaf dry weight, [g]/leaf area [cm]) was calculated. A randomized complete block design with four blocks (frames) per light treatment was used. There were two plants in each block per light treatment. The experiment was repeated twice.

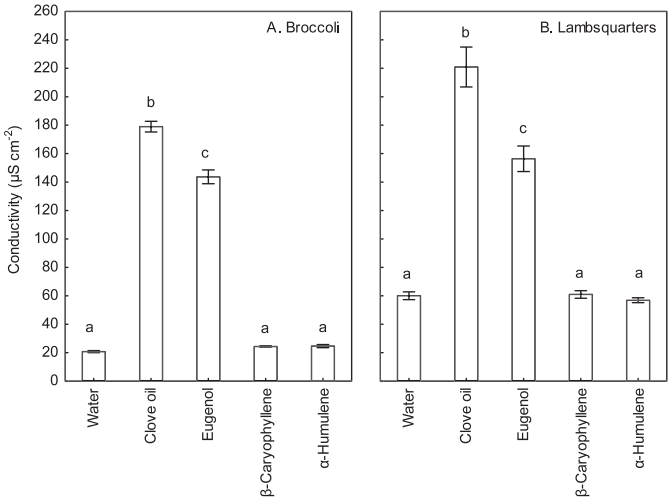

Figure 1. Effect of clove oil (2.5%), eugenol (1.6%), β-caryophyllene (0.35%), and α-humulene (0.15%) on leakage of electrolytes from broccoli and common lambsquarter leaf discs. Values are means ± SE of two experiments with seven replications per experiment. Bars with different letters indicate a significant ($P \leq 0.05$) difference.

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA) using SAS (ver. 9.2, SAS Institute Inc., Cary, NC). Because there was no significant experiment by treatment interaction, data from different experiments were pooled. To meet assumptions of ANOVA, data from the contribution of essential oil constituents of clove oil to its toxicity study were log transformed, whereas SLW data were root transformed; however, untransformed data are presented

Table 1. Influence of light intensity on leaf area and specific leaf weight (SLW) in broccoli and common lambsquarters.

	Leaf area (cm^2)		SLW (g m^{-2})	
Light intensity (μE m^{-2} s^{-1})	Broccoli	Lambsquarters	Broccoli	Lambsquarters
1,500	20.1 ± 1.1a[a]	6.9 ± 1.6a	46.1 ± 1.2a	42.1 ± 1.2a
800	17.3 ± 0.9b	6.8 ± 1.3a	37.3 ± 0.8b	34.2 ± 0.9b
500	14.4 ± 0.6c	7.0 ± 1.3a	29.2 ± 1.1c	26.7 ± 0.6c
250	11.7 ± 0.5d	6.0 ± 1.1b	26.1 ± 0.9d	22.4 ± 0.4d

[a] Values (mean ± SE) followed by different letters are significantly ($P \leq 0.01$) different.

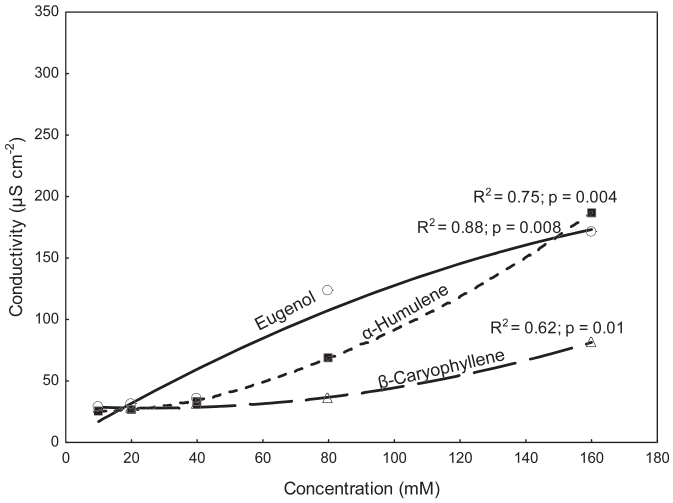

Figure 2. Dose-response curves for effects of eugenol, β-caryophyllene, and α-humulene on leakage of electrolytes from broccoli leaf discs. Values are means of three experiments with seven replications per experiment.

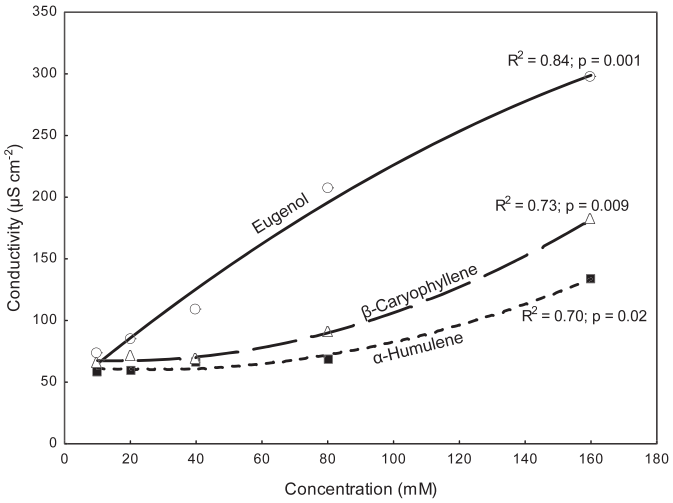

Figure 3. Dose-response curves for effects of eugenol, β-caryophyllene, and α-humulene on leakage of electrolytes from common lambsquarter leaf discs. Values are means of three experiments with seven replications per experiment.

in Figure 1 and Table 1. A completely randomized design was used in all, except in the light intensity, experiments. A two-factorial randomized block design was used for the light intensity experiments. Means were separated using Tukey's test ($P = 0.05$).

Results and Discussion

Toxicity of Clove Oil and Its Constituents. Clove oil and its major constituent eugenol have been reported to injure leaf tissue, which results in electrolyte leakage from discs excised from treated leaves (Bainard et al. 2006; Srivastava et al. 2005; Tworkoski 2002). Eugenol also has been reported to suppress some weedy species (Vaid et al. 2010). In order to determine relative contribution of eugenol, β-caryophyllene, and α-humulene, the major constituents of clove oil (Bauer et al. 1997; Raina et al. 2001; Srivastava et al. 2005), their phytotoxicities were studied at concentrations relative to their proportion in the clove oil. Clove oil used in our experiments has been reported to contain 63.96% eugenol, 13.99% β-caryophyllene, and 6.06% α-humulene (unpublished GC-MS data, R. Bradbury, Ecosafe Natural Products Inc.). Eugenol, which constitutes 63.96% of clove oil, accounted for 81% and 70% of the phytotoxic activity of clove oil on broccoli and common lambsquarters, respectively (Figure 1). Decadienal monocyclic sesquiterpenes β-caryophyllene and α-humulene, which constitute 13.99 and 6.06% of clove oil, respectively, showed little or no phytotoxic activity compared to the control (Figure 1). Ratsch (2004) and Small and Catling (1999) also reported that β-caryophyllene and α-humulene used at natural concentrations are not toxic to plants. The difference between overall phytotoxicity of clove oil, and that accounted for by eugenol, β-caryophyllene and α-humulune in this study, might be due to other constituents of the clove oil not included in this study, and their possible interaction(s).

Toxicity of the Equimolar Concentrations of Clove Oil Constituents. Since β-caryophyllene and α-humulene showed little or no phytotoxicity at concentrations present in the clove oil (Figure 1), their dose-response relationships were compared with eugenol to determine their phytotoxicity over a wide range of concentrations. The concentration of eugenol in 2.5% solution of clove oil used in this study was 102 mM; the 160 mM concentration used in this experiment was therefore 57% greater. In both broccoli and common lambsquarters, eugenol showed an inverse exponential dose-response relationship and was the most phytotoxic compound over a wide range of concentrations (Figures 2 and 3). Phytotoxicity of clove oil largely is due to eugenol, not only because of its presence in the largest amount but also because of its higher potency compared to β-caryophyllene and α-humulene on an equimolar basis; even at 10 to 80 mM concentrations (well below those that occur in 2.5% clove oil solution) eugenol was more toxic compared to β-caryophyllene and α-humulene.

Concentrations of β-caryophyllene and α-humulene used in this experiment were higher compared to those present in a 2.5% solution of clove oil. β-caryophyllene and α-humulene showed exponential dose-response relationships, being more phytotoxic at higher concentrations. α-Humulene was more phytotoxic than β-caryophyllene in broccoli (Figure 2) and less phytotoxic in common lambsquarters (Figure 3). At 160 mM, α-humulene was almost as phytotoxic as eugenol in broccoli. Wang et al (2009) reported that ≥ 3 mg L^{-1} ($\sim$0.02 mM) of β-caryophyllene, isolated from mile-a-minute (*Mikania micrantha* Kunth), significantly inhibited the germination rate and seedling growth of field mustard (*Brassica campestris* L.) and radish (*Raphanus sativus* L.). Tellez et al. (2000) reported only mild phytotoxicity of essential oil extracted from American beautyberry (*Callicarpa americana* L.), with humulene epoxide II (13.9%) and α-humulene (10.0%) as main components, and Tworkoski (2002) found no toxicity of α-humulene in dandelion (*Taraxacum officinale* F. H. Wigg.).

Light Intensity and Toxicity of Clove Oil and Eugenol. Light intensity is known to influence leaf area and SLW (fresh biomass per unit leaf area) (Lombardini et al. 2009; Senevirathna et al. 2003). The effect on SLW has been attributed to changes in the number and density of palisade and spongy mesophyll cells (Chabot and Chabot 1977). Effects of light intensity on leaf area and SLW might influence the area exposed to solar irradiance, CO_2 absorption and transport, and/

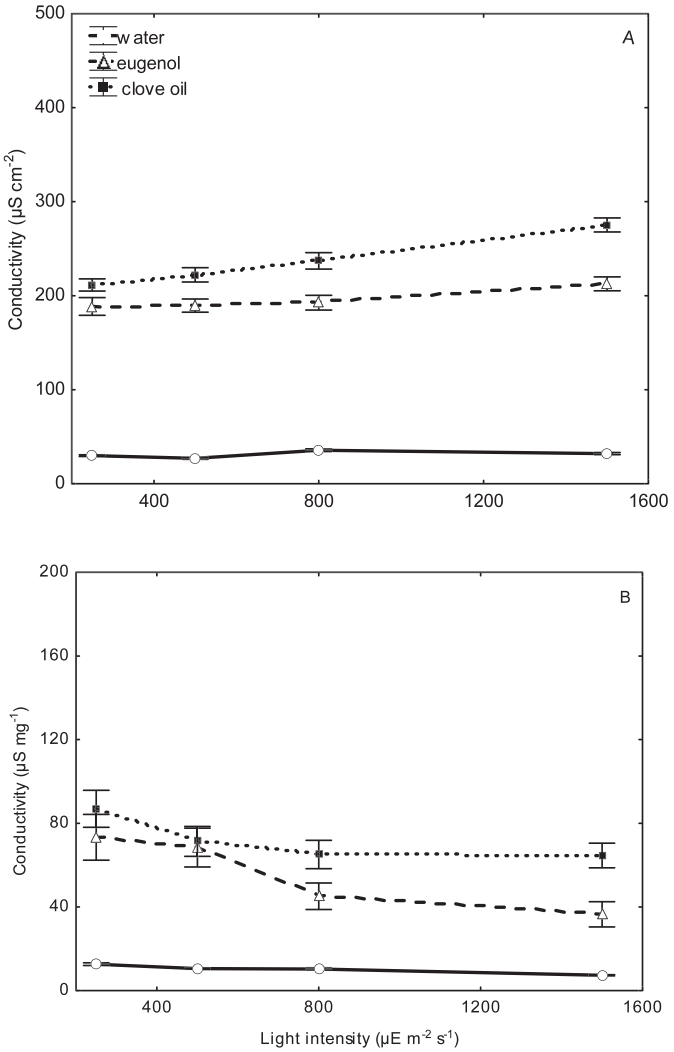

Figure 4. Effect of light intensity on leakage of electrolytes from leaf discs of broccoli after spraying with eugenol and clove oil. (a) Leakage from broccoli discs (µS cm^{-2}) ($P \leq 0.01$, LSD = 21), and (b) leakage per mg biomass (µS mg^{-1}) ($P \leq 0.01$, LSD = 10). Values are means ± SE of three experiments.

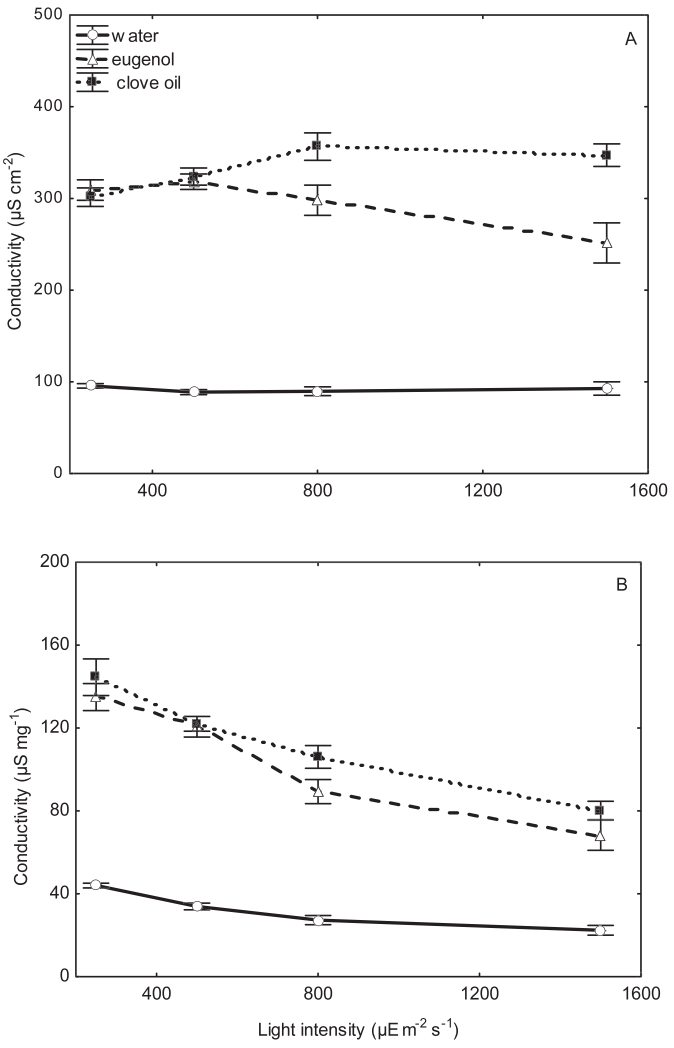

Figure 5. Effect of light intensity on leakage of electrolytes from common lambsquarter leaf discs sprayed with eugenol and clove oil. (a) Leakage from common lambsquarters discs (µS cm^{-2}) ($P \leq 0.01$, LSD = 39), and (b) leakage per mg biomass (µS mg^{-1}) ($P \leq 0.01$, LSD = 24). Values are means ± SE of three experiments.

or transpiration (Terashima et al. 2006). In this study, the leaf area of both broccoli and common lambsquarters seedlings increased significantly ($P \leq 0.01$) at high light intensities (viz., 500, 800, 1,500 µE m^{-2} s^{-1}), compared to the lowest (250 µE m^{-2} s^{-1}) light intensity (Table 1). The increase was progressive with increasing light intensity in broccoli but not common lambsquarters. The magnitude of increase also was greater in broccoli compared to common lambsquarters. The leaf area of seedlings developed at 1,500 µE m^{-2} s^{-1}, compared to those at the lowest light intensity, increased by 71.8% in broccoli but by only 15% in common lambsquarters (Table 1).

The specific leaf weight increased significantly with increasing light intensity in both species (Table 1). Compared to the SLW of leaves that developed at 250 µE m^{-2} s^{-1}, SLW at 1,500 µE m^{-2} s^{-1} light intensity were 76.6 and 87.9% greater in the case of broccoli and common lambsquarters, respectively. Thus, the magnitude of effect of light intensity on SLW was similar for both species, but that for the leaf area was very different.

Leakage of electrolytes per leaf disc increased significantly with increasing light intensity during leaf development in broccoli seedlings sprayed with clove oil or eugenol (Figure 4a). In common lambsquarters, it increased up to 800 µE m^{-2} s^{-1} and then leveled off in seedlings sprayed with clove oil, and significantly decreased after 500 µE m^{-2} s^{-1} light intensity in seedlings sprayed with eugenol (Figure 5a). When the leaf tissue damage in response to clove oil and eugenol treatments was expressed on a per unit leaf biomass basis, the electrolyte leakage decreased significantly with increasing light intensity in both broccoli and common lambsquarters (Figures 4b and 5b).

Increases in leaf area, as well as SLW in response to increasing light intensity during seedling development, are expected to increase solute leakage upon tissue damage following essential oil treatment—the higher leaf area by increasing the interception of spray and the higher SLW (more biomass per unit leaf area) by making more solute available for the leakage. However, for discs of equal area, as SLW increased with increasing light intensity exposure, the leaf area intercepting the spray per unit biomass should decline. Furthermore, increasing light intensity is known to increase the thickness as well as influence the chemistry of epicuticular wax on the adaxial surface of leaves (Hatterman-Valenti et al.

2006; Paiva et al. 2003). Hatterman-Valenti et al. (2006) reported that decreasing light intensity increased epicuticular wax fatty acids and aldehyde contents and decreased primary alcohols and esters. A change in amount of epicuticular wax on the adaxial surface as well as its chemistry in response to increasing light intensity could decrease retention of spray solution as well as the penetration of foliar-applied chemicals, which in turn could reduce their efficacy. Interestingly, a slight, but nonsignificant, decrease in electrolyte leakage per unit biomass with exposure to increasing light intensity prior to spray was also observed in common lambsquarters leaves sprayed with water (Figure 5b), which might be due to a direct effect of solar irradiance on these leaves.

In conclusion, eugenol contributes most to the phytotoxic activity of clove oil because of its presence in the largest amount, as well its greater phytoxicity compared to β-caryophyllene and α-humulene, which played a relatively minor role, if any. Additionally, light intensity influences the efficacy of both clove oil and eugenol in broccoli and common lambsquarters in terms of electrolyte leakage.

Acknowledgments

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