ISSN. 0972 - 8406
The NEHU Journal Vol. XVIII, No.2, July - December 2020, pp. 41-56

Phytotoxic assessment of some monoterpenes and their formulation with leaf extract of *Chenopodium ambrosioides*

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Abstract

The present study investigated the phytotoxicity of eight monoterpenes belonging to two major groups, i.e. oxygenated monoterpenes (linalool, citronellol, citronellal, 1, 8-cineole) and monoterpene hydrocarbons (limonene, β-pinene, p-cymene, α-terpinene) and their formulation with Chenopodium ambrosioides leaf extract against Cassia occidentalis. In a laboratory bioassay, monoterpenes (0.5, 1.0, 2.5 mM) and their combination with leaf extract (0.75%) of Chenopodium ambrosioides were tested on germination and seedling length of the weed. The inhibition was greatest with oxygenated monoterpenes, whereas leaf extract formulation improved phytotoxicity of hydrocarbon monoterpenes as they alone are not much effective. Post-emergence application of monoterpenes (1% and 2.5%, v/v) and their formulation with 2% leaf extract was evaluated on 4-week-old plant by two physiological parameters - photosynthetic efficiency and membrane integrity. Among all the monoterpenes, citronellol was found to be the most effective both in laboratory and green house bioassay, followed by citronellal. Post-emergent phytotoxicity of citronellal and linalool increased several fold by leaf extract formulation. These results were also confirmed by visible injury ranging from chlorosis to necrosis to complete wilting of plants. The study concludes that some monoterpenes have great scope for the development of new weed control strategies and their further formulation improves efficacy of active compounds, reduced dose usage and save costs of application.

Keywords: Membrane integrity, Photosynthetic efficiency, Phytotoxicity, Post-emergent, Visible injur

Introduction

In agriculture system the potential use of naturally derived products as new, effective, reduced-risk alternatives for the weed management is a contemporary issue because intensive use of synthetic herbicides resulted in soil and groundwater contamination, harm human health and increase herbicidal resistance in weed species (Dayan & Duke, 2014; Bhat *et al.*, 2019). With the rising demand of eco-friendly pest control options, public and private sector organizations have re-focused their search by looking back to natural sources for new biologically active compounds. So, worldwide efforts are being made to identify the new eco-friendly chemicals as a source of natural herbicides. In this direction, essential oils and their major bioactive compounds, *i.e.* monoterpenes, hold a promising potential. The essential oils/monoterpenes exhibit phytotoxic activity against several weeds (Vaughn & Spencer, 1993; Batish *et al.*, 2008; Mutlu *et al.*, 2011; Dayan & Duke, 2014; Isman, 2016; Fagodia *et al.*, 2017; Pouresmaeil *et al.*, 2020). These volatiles are suitable to prevent environmental poisoning because they possess little or no residual effect and are generally regarded as safe (GRAS) chemicals (Isman, 2000; Tworkoski, 2002; Dayan *et al.*, 2009). Various researchers have suggested that single compound may not be as effective as a combination of different allelochemicals which might act additively or synergistically towards the growth inhibition in plants (Vokou *et al.*, 2003; Jamil *et al.*, 2009; Vasilakoglou *et al.*, 2013; Chotsaeng *et al.*, 2017). These allelochemicals may prove beneficial in the weed management as they improve efficacy of active compound, reduce dose usage, save costs of application, allows the control of a diverse weed flora, delay development of herbicide resistant weeds and are safe unlike the chemicals being used in agriculture (Duke & Dayan, 2015).

Therefore, an attempt was made to improve the efficacy of eight most abundant monoterpenes present in the essential oils, including oxygenated monoterpenes and monoterpene hydrocarbons, through their formulations with water extract of an allelopathic plant, *Chenopodium ambrosioides* L. The choice of *C. ambrosioides* was based on its known phytotoxicity (Jimenez-osornio *et al.*, 1996; Hegazy & Farrag, 2007) and its rich photochemistry (Singh *et al.*, 2008). A great scope exists for the utilization of these compounds in combination with each other. As these formulations are free of toxic or contaminated products, therefore, they are suitable for use in agricultural and food industry. These liquid formulations are more dose and cost-effective bioherbicides due to their fast burn-down action even at lesser volume than their typical required quantity. To the best of our knowledge, there are no previous studies in the open literature on such possible integration of monoterpenes and aqueous leaf extract formulation. Thus, the objectives of this study were:

- To assess the phytotoxic potential of eight monoterpenes (linalool, citronellol, citronellal, 1,8-cineole, limonene, β-pinene, p-cymene and α-terpinene).
- To examine the impact of allelopathic leaf extract (*C. ambrosioides*) on phytotoxic efficacies of monoterpenes both under laboratory and green house conditions against widely growing urban weed - *Cassia occidentalis* L.
- To investigate the mechanisms by which monoterpenes and their formulation act on the weed.

Materials and methods

Chemicals and biological material

Technical grade linalool, citronellol, citronellal, 1,8-cineole, limonene, *β*-pinene, *p*-cymene and *α*-terpinene were purchased from Sigma Co., St. Louis, USA; Lancaster, UK, and Acros Organics, UK. Seeds of *Cassia occidentalis* L. and fresh leaves of *Chenopodium ambrosioides* L. were collected locally from plants growing wild in the campus of Panjab University, Chandigarh, India. Before use, *C. occidentalis* seeds were scarified with sulphuric acid and imbibed overnight in water.

Preparation of C. ambrosioides leaf extract

C. ambrosioides aqueous leaf extract were prepared by soaking required amounts of air dried leaf powder, 0.75g (0.75% for pre-emergent assay) and 2g (2% for post emergent assay) per 100 ml of distilled water at room temperature for 12 h. Thereafter, extracts were filtered through Whatman no. 1 filter paper and used.

Laboratory bioassay

Phytotoxicity of all the eight monoterpenes and their formulation with leaf extract of *C. ambrosioides* was studied on the germination and early growth of *C. occidentalis* under laboratory conditions. Monoterpene solutions (0.5, 1.0 and 2.5 mM) were prepared using Tween-20 (final concentration <0.01%). Distilled water with the same amount of Tween-20 served as a parallel control. Monoterpenes formulations were prepared by making their emulsions (0.5, 1.0 and 2.5 mM) in 0.75% leaf extract of *C. ambrosioides* with the help of Tween-20. 0.75% *C. ambrosioides* leaf extract along with same amount of Tween-20 served as a positive control. Pre-imbibed *C. occidentalis* seeds (15) were placed in Petri dishes (15 cm in diameter) lined with a thin layer of cotton wad and Whatman no.1 filter paper. Each Petri dish was moistened with 10 ml of respective treatment solution. The Petri dishes were then sealed with cello-tape® to avoid loss of the monoterpenes due to volatilization. For each treatment concentration, including controls, five independent Petri dishes were maintained as replicates. All the Petri dishes were kept in a growth chamber set at 25±2 °C and 16/8 h light/dark photoperiod of 240 μ mol photons m^{-2} s^{-1} photon flux density provided with fluorescent tubes and lamps. After 1 week, germination percent and length of the emerged seedlings was measured.

Greenhouse bioassay

To determine the post-emergent activity of monoterpenes and their formulation, plants of *C. occidentalis* were raised from seeds in 15 cm diameter polypropylene pots in a greenhouse. For this, 1500 g of garden soil mixed with sand in a ratio of 3:1 (*w/w*) was filled in each pot and ten seeds of *C. occidentalis* were sown per pot. One week after emergence, these were thinned to 5 plants per pot. Four week old *C. occidentalis* plants were spray treated with 1 and 2.5% solution of all the eight monoterpenes (or distilled water in case of control) and their formulations: 1% monoterpene formulation (1% Monoterpenes + 2% *C. ambrosioides* leaf extract) and 2.5% monoterpene formulation (2.5% Monoterpenes + 2% *C. ambrosioides* leaf extract). Spray treatment with 2% *C. ambrosioides* leaf extract served as a positive control. Plants were spray-treated with respective solutions at a volume of 140 ml/m^2. For each treatment, five replications were maintained. After 3-days of treatment, the photosynthetic efficiency and membrane integrity were measured from *C. occidentalis* leaves. Further, the test plants were observed for visual injury levels on the basis of chlorotic and necrotic areas developed after the treatment. The injury levels of the plant were rated on a scale of 0 (with no injury) to 4 (with complete mortality no recovery, expressed as ++++).

Estimation of Photosynthetic efficiency (Chlorophyll Fluorescence)

The maximum potential quantum efficiency of PSII of treated as well as control *C. occidentalis* leaves were measured using the OS-30p Chlorophyll Fluorometer (Opti Sciences, USA). For this, a leaf was attached on the leaf holder of the plant efficiency analyser equipment and subjected to dark conditions for about 10 min. Thereafter, its photosynthetic efficiency was calculated from the ratio of F_v/F_m, where F_v is variable chlorophyll fluorescence and F_m is maximum chlorophyll fluorescence in an illuminated leaf. This was repeated five times for each treatment.

Determination of Membrane integrity (REL)

Membrane integrity in terms of relative electrolyte leakage from *C. occidentalis* leaves was studied as per the method of Singh *et al.* (2007). For this, leaves (100 mg) were incubated in 10 ml of distilled water at 25°C for 1 hour in the test tubes and initial conductivity (E_1) of the bathing medium was measured. Thereafter, the test tubes containing leaf tissues were boiled for 15 min to release all the ions. These were then cooled to 25°C and the conductivity (E_2) was measured again. The relative electrolyte leakage was calculated using following formula and expressed in percentage.

$$\% \text{ REL} = (E_1/E_2) \times 100$$

Statistical analysis

All the experiments were conducted in a completely randomised design and were repeated twice. Values are presented as the mean ± SE (standard error) of the repeated experiments. Data was subjected to one-way ANOVA followed by comparison of mean values using post hoc Tukey's test at $P \leq 0.05$ significance level using software programme SPSS (version 16). Graphical representations were made on software programme Sigma plot (Version 8.0).

Results and discussion

Growth studies under laboratory conditions

In response to 0.5 mM and 1.0 mM of monoterpene and its formulation, there was no effect on seed germination of the test weed (data not presented), whereas at 2.5 mM, monoterpenes and their formulation showed a significant effect (Table 1). Monoterpenes when applied alone, germination was inhibited in the order of potency: citronellol > citronellal > 1, 8-cineole > limonene > *β*-pinene > linalool > *p*-cymene > *α*-terpinene. Among the prepared formulations, a positive and significant synergy was observed for *p*-cymene and *α*-terpene. Other monoterpene formulations showed either antagonistic or insignificant effect on the percent germination. Regarding the growth of emerged seedlings, application of monoterpenes showed following order of inhibition: citronellol > citronellal > 1, 8-cineole > linalool > β-pinene > limonene > p-cymene > α-terpinene (Figure 1a and 1b). At 2.5 mM, among all the tested monoterpenes, citronellol was found to be the most effective as it caused 95% reduction in the seedling length over the control, followed by citronellal with 74% inhibition and 1,8-cineole and linalool with 40% inhibition (Figure 1a). On the other hand, the hydrocarbon monoterpenes, α-terpinene, p-cymene, limonene and β-pinene, inhibited the seedling length by 3-34% (Figure 1b). On the whole, oxygenated monoterpenes exhibited high inhibitory potential in comparison to the hydrocarbon monoterpenes. Our observation is corroborated by studies of Vaughn & Spencer (1993), Kordali *et al.* (2007), De Martino *et al.* (2010) and de Oliveira *et al.*, (2018), who also reported the oxygenated monoterpenes are more phytotoxic than hydrocarbon monoterpenes. At ≥ 0.5 mM all monoterpenes formulations caused a significant reduction in the seedling length of *C. occidentalis*. Further, two different types of phenomenon, *i.e.* synergy/antagonism, was observed between monoterpenes and their formulation with *C. ambrosioides* leaf extract on the seedling length of *C. occidentalis*. Monoterpene hydrocarbons showed synergy with *C. ambrosioides* leaf extract as reflected by the improved efficacy. Limonene showed the highest synergy and caused 54% reduction in the seedling length at 2.5 mM concentration. Formulation of *β*-pinene, *p*-cymene and *α*-terpinene also showed improvement in their activity. In case of oxygenated monoterpenes, citronellal and citronellol showed antagonistic effects when formulated with *C. ambrosioides* leaf extract, whereas the activity of linalool and 1,8-cineole remains unaffected by formulation (Figure 1a).

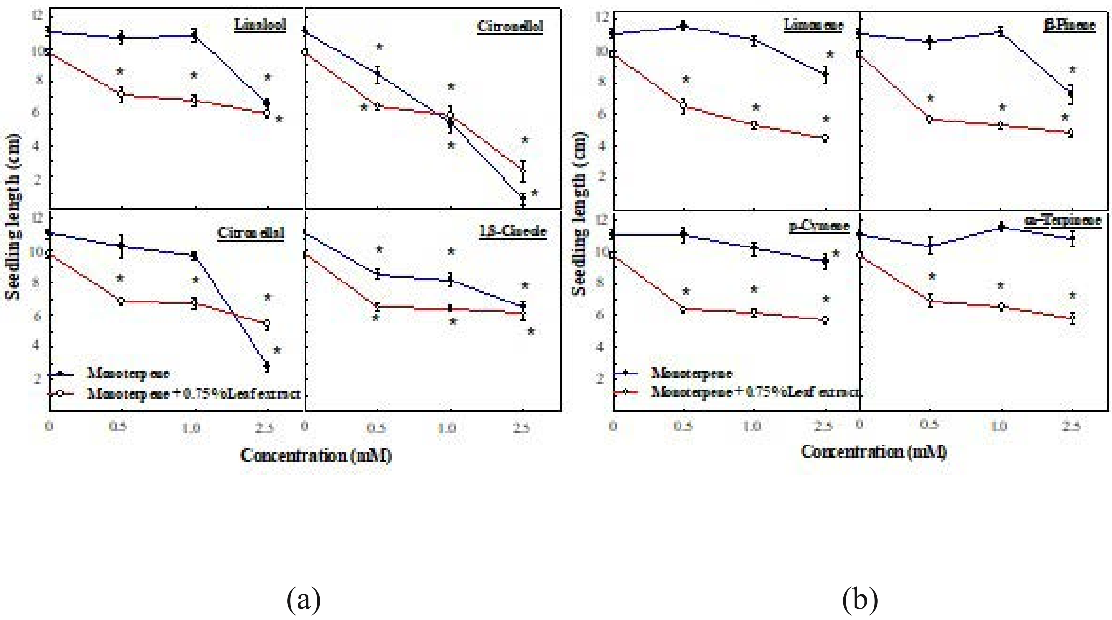

Figure 1. Effect of monoterpenes and their formulation with 0.75% leaf extract on seedling length of *C. occidentalis* measured 7 days after the treatment. Vertical bars along each data point represent the standard error of the mean. * represents significant difference from their respective controls (Distilled water and 0.75% leaf extract). (a) Oxygenated monoterpenes (b) Hydrocarbon monoterpenes

Table 1. Effect of different monoterpenes and their formulation (0.75% *C. ambrosioides* leaf extract) on percent germination of *C. occidentalis* measured after 7 days. Data presented as mean (%) ± SE. Different alphabets represent significant difference from their respective controls at $P \leq 0.05$. Values within parenthesis indicate percent decrease over + control (only leaf extract).

Treatment (2.5 mM)	Monoterpene	Monoterpene + Leaf extract
Control	100.0 ± 5.4 a	77.0 ± 2.9 a (100 %)
Linalool	76.5 ± 4.3 b	62.4 ± 1.8 b (81.0 %)
Citronellol	5.8 ± 3.1 b	21.2 ± 5.8 b (27.5 %)
Citronellal	25.2 ± 2.7 b	48.9 ± 2.6 b (63.5 %)
1,8-Cineole	58.6 ± 2.8 b	55.0 ± 3.7 b (71.4 %)
Limonene	59.4 ± 3.0 b	62.4 ± 1.8 b (81.0 %)
β-Pinene	65.3 ± 4.9 b	55.0 ± 3.0 b (71.4 %)
p-Cymene	84.9 ± 4.4 a	54.3 ± 2.9 b (70.5 %)
α-Terpinene	97.4 ± 4.6 a	52.4 ± 3.0 b (68.5%)

The synergism between monoterpenes has been evaluated by some workers to prove their better efficacy as potential bioherbicides over individual monoterpenes (Vokou *et al.*, 2003; He *et al.*, 2009; Vasilakoglou *et al.*, 2013). Jamil *et al.* (2009) studied the herbicidal potential of sorghum water extracts in combination with the leaf extracts of some allelopathic plants and concluded a better potential of these formulations. Besides, some synthetic herbicides have also been tried in combination with the aqueous extracts of allelopathic plants which have resulted in the reduction of doses of the applied herbicide for weed control (Cheema *et al.,* 2003; Ihsan *et al.,* 2015; Alsaadawi *et al.*, 2020). However, monoterpenes offer a better eco-friendly replacement option over synthetic herbicides, especially in combination with the aqueous extracts of allelopathic plants. The allelopathic effect of *C. ambrosioides* leaf extract has been attributed to the presence of ascaridole (Jimenez-osornio *et al.*, 1996; Hegazy & Farrag, 2007). However, we did not estimate the nature of these phytotoxins in the present study. Nevertheless, the findings of the present study suggested a positive synergy between monoterpene hydrocarbons and ascaridole as phytotoxicity of the monoterpene hydrocarbons was improved by their formulations with the leaf extract. The mechanism by which these compounds inhibit seed germination and growth remains unclear. However, loss/disruption of mitotic activity might be responsible for the reduction/inhibition of germination and seedling growth of tested plant (Romagni *et al.,* 2000). Koitabashi *et al.* (1997) demonstrated that the essential oils caused accumulation of lipid globules in the cytoplasm and reduced the size of cell organelles possibly due to the inhibition of DNA synthesis or membrane disruption resulting in anatomical and physiological changes.

Effect of Monoterpenes and their formulation on C. occidentalis under greenhouse conditions

In addition to the laboratory bioassay, green house bioassay was also performed to assess the post-emergent activity of monoterpenes and their formulation. Visible injury and physiological parameters, *viz.* photosynthetic efficiency and membrane integrity, was taken into consideration 3 days after treatment. Weed injury in the greenhouse was evaluated on the basis of visual estimates. Injury ratings included four categories: 0 indicated no injury, + minor injury, ++ moderate injury, +++ severe injury but recovery possible, and ++++ severe injury with no possibility of recovery. Injury symptoms increased with increasing concentrations of the volatiles (Table 2). The mature plants of the test weed were severely damaged upon spray of citronellol alone while other monoterpenes resulted in a minor or no injury. After formulation, citronellol exhibited antagonistic relation with the leaf extract, whereas citronellal and linalool act synergistically with the leaf extract. Formulated citronellal became most phytotoxic as it severely damaged the plant to a level where recovery was not possible (Figure 2). Slightly improved phytotoxicity was also shown by 1% limonene formulation. Chlorosis, necrosis, wilting and senescence was observed due to advanced intoxication, which reflects biochemical, physiological and structural changes, appropriately explained by the impairment in photosynthetic activity and increased ion leakage.

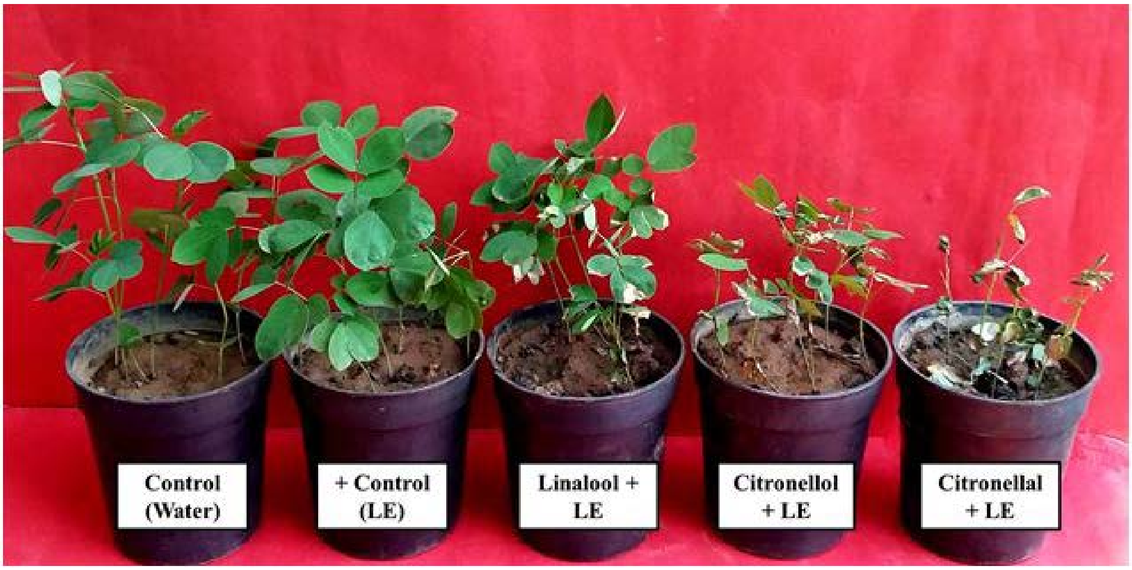

Figure 2. Photograph showing the effect of monoterpenes formulation (2.5% monoterpene + 2% LE) on 4 week old *C. occidentalis* plant 3-days after spray treatment. LE represents C. *ambrosiodies* leaf extract.

Chlorophyll fluorescence or photosynthetic efficiency is a good biomarker to identify mode of action of these phytotoxins. Photosynthetic efficiency in terms of F_v/F_m ratio gives clear evidence about the effect of treatments on chlorophyll content. In the present study, among all the tested monoterpenes only citronellol showed significant decrease in F_v/F_m ratio by 57% at a concentration of 1% and by 84% at 2.5% spray treatment with respect to control. However, in response to other monoterpenes, no significant change was observed (Figure 3A). In the monoterpenes formulation, both synergistic and antagonistic interaction was evidenced. Citronellal and linalool formulation revealed greater inhibition of photosynthetic efficiency as compared to the individual monoterpenes and this suggests a synergistic interaction of citronellal and linalool with 2% *C. ambrosioides* leaf extract.

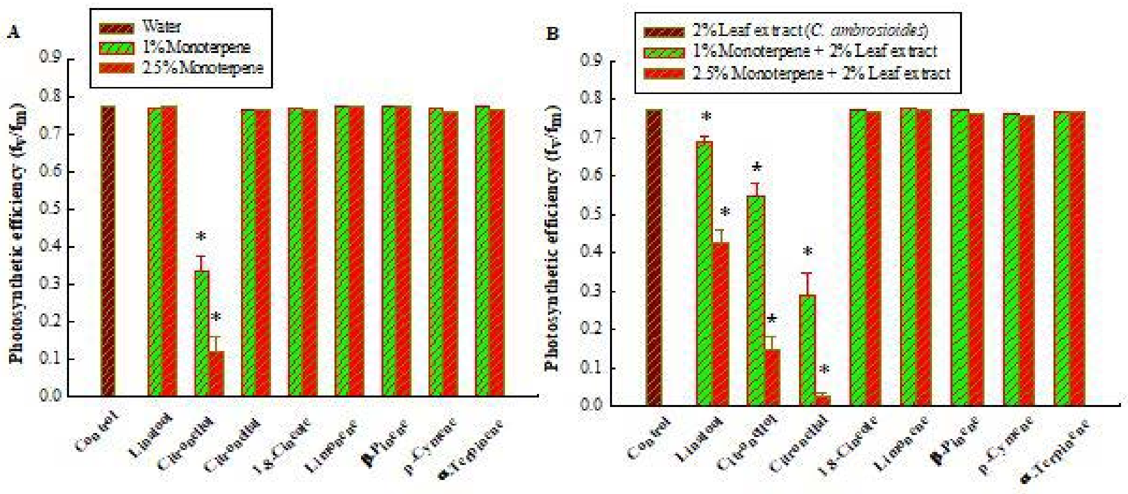

Figure 3. Effect of monoterpenes and their formulation on photosynthetic efficiency (represented as F_v/F_m) of *C. occidentalis* leaves recorded 3 days after spray treatment. Vertical bars represent the standard error of the mean. * represents significant difference from their respective controls at $p \leq 0.05$.

By the spray treatment of citronellal formulation, F_v/F_m ratio was significantly declined by 62% and 97%, respectively, at 1% and 2.5% compared to that of positive control. For linalool formulation, reduction was observed up to 45% at 2.5% spray treatment. On the contrary, 1% citronellol showed antagonism with *C. ambrosioides* leaf extract, reducing its effectivity by 28% in comparison to citronellol alone. However, 2.5% citronellol acted in an independent manner as its activity remained unchanged before and after formulation, and both the treatments showed significant reduction with respect to positive control. However, rest of the monoterpenes formulation were non-effective and caused no significant changes in test plant's photosynthetic efficiency (Figure 3B). Previously, studies have demonstrated post emergent herbicidal potential of essential oils/monoterpenes (Tworkoski, 2002; Kaur *et al*., 2010; Gouda *et al*., 2016). Chlorophyll fluorescence is often measured to determine the effect of various compounds on the light reaction of photosynthesis and the decrease in F_v/F_m ratio in response to monoterpenes has been reported earlier by some investigators (Dayan *et al*., 2000; Ibrahim *et al*., 2004; Grana *et al*., 2013). Singh *et al*. (2002) reported that monoterpenes reduce/inhibit chlorophyll content in the *C. occidentalis* plant. Loss of chlorophyll might affect photosynthetic machinery as it interferes with chloroplast functioning, membrane stability and stomatal behaviour (Kabanova & Chaika, 2001; Rai *et al*., 2003; Batish *et al*., 2007). Thus, from this study it is clear that among all tested monoterpenes and their formulations only three spray treatments, *i.e.* citronellol, citronellal formulation and linalool formulation, are able to interfere with photosynthetic machinery of the test plant. The reason for improved activity of citronellal and linalool after formulation is possibly due to presence of double bond in their structure, which offers a potential site for the attachment of functional groups resulting in chemical modifications that could improve the physiochemical and biological properties of the molecule.

Table 2: Symptoms of visible injury (rated on 0-4 scale) on test weed 3-days after treatement with different concentrations (%) of monoterpenes and their formulation with 2% *C. abrosioidies* leaf. Hear 0 stand for no injury, + minor injury, ++ moderate injury, +++ severe injury but recovery possible, ++++ severe injury with no recovery.

Treatment	Monoterpene		Monoterpene + Leaf extract	
	1 %	2.5 %	1 %	2.5 %
Linalool	+	+	++	++
Citronellol	+++	+++	++	+++
Citronellal	+	++	++	++++
1,8-Cineole	+	+	+	+
Limonene	0	+	+	+
β-Pinene	0	+	0	+
p-Cymene	0	+	0	+
α-Terpinene	0	+	0	+
Control	0		0	

Post-emergent application of monoterpenes and their formulations caused damage in the cuticle and cell membrane which lead to the wilting/desiccation of aerial parts. So, in addition to photosynthetic efficiency, membrane permeability was also taken into account to measure the stress induced inside the test plant. All oxygenated monoterpenes at 1% and 2.5% caused a significant ion leakage, except for 1,8-cineole (significant leakage only at 2.5%) (Table 3). All monoterpene hydrocarbons exhibited non-significant effect at 1% and 2.5% concentration except *p*-cymene and *α*-terpene (significant at 2.5%). However, all monoterpenes formulations exhibited greater membrane damage than their respective individual monoterpenes, except citronellol. At 1% monoterpenes formulation, synergy was observed in citronellal, linalool *β*-pinene and *p*-cymene. Likewise, at 2.5% concentration formulation of all monoterpenes except citronellol greater ion leakage was observed, with the most effective and improved synergy being in citronellal followed by linalool (Table 3). Changes in membrane permeability affects all other physiological and biochemical processes linked to membrane functioning. Thus, to study mode of action of herbicide, monitoring membrane integrity is a good physiological parameter (Dayan & Watson, 2011). The observations revealed in present study are in agreement with earlier reports that essential oils and monoterpenes induce damage and cause cell death in plants (Tworkoski, 2002; Mutlu *et al*., 2011; Kaur *et al*., 2012). Allelopathic compounds are known to depolarize and disrupt cell membranes thereby enhancing their permeability, inducing lipid peroxidation and finally leading to cell death due to production of reactive oxygen species (Singh *et al*., 2006; Mutlu *et al*., 2011). Although the complete action mechanism of these compounds is still not clear.

Table 3. Effect of monoterpenes and their formulation with 2% leaf extract on the relative electrolyte leakage (% REL) in *C. occidentalis* leaves recorded 3 days after treatment. Data presented as mean (%) ± SE. Different alphabets represent significant difference from their respective controls at $P \leq 0.05$

Treatments	**Monoterpene**		**Monoterpene + Leaf extract**	
	1%	**2.5%**	**1% + L**	**2.5% + L**
Linalool	8.17 ± 0.08 b	11.94 ± 0.55 c	21.93 ± 0.72 b	32.50 ± 0.94 c
Citronellol	41.54 ± 1.54 b	50.58 ± 0.58 c	26.50 ± 0.50 b	49.29 ± 0.51 c
Citronellal	12.89 ± 0.34 b	18.34 ± 0.21 c	26.50 ± 1.52 b	69.21 ± 1.12 c
1,8-Cineole	3.50 ± 0.75 a	6.45 ± 0.22 b	3.82 ± 0.38 a	8.77 ± 0.22 b
Limonene	2.27 ± 0.07 a	2.95 ± 0.44 a	5.99 ± 0.39 ab	7.54 ± 0.97 b
β-Pinene	2.34 ± 0.21 a	2.44 ± 0.05 a	6.20 ± 0.27 b	10.43 ± 0.24 c
p-Cymene	3.26 ± 0.07 ab	3.82 ± 0.14 b	7.48 ± 0.21 b	9.07 ± 0.40 b
α-Terpinene	3.09 ± 0.67 a	5.52 ± 0.23 b	3.30 ± 0.18 a	10.59 ± 0.22 b
Control	2.65 ± 0.15 a		3.00 ± 0.40 a	

Conclusion

The present study concludes that some monoterpenes and their formulation possesses phytotoxicity, affects germination and seedling growth, causes ion leakage and reduce the photosynthetic activity in test weed. These compounds have great potential to be used as leading chemicals for synthesis of new herbicides for sustainable weed management programmes. According to our knowledge, this is the first report regarding the monoterpenes formulation with allelopathic leaf extract. These liquid formulations are going to be more dose and cost effective bioherbicides. Furthermore, it is meaningful to continue studies with different combinations of essential oils/monoterpenes and allelopathic plant extracts in search of novel bioherbicides.

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