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Research article

Phytotoxicity Stress Induced by Allelochemicals from Foliar Spray of *Sida cordifolia* Methanol Leaf Extract on *Ageratum conyzoides* and *Oryza sativa*

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Abstract

Allelochemicals are key inhibitors that induce chemical stress in plants. Their mechanisms as agents of oxidative stress are not well understood. A field study was conducted to evaluate herbicidal potential of *Sida cordifolia* methanol leaf extract (SCLE) on *Ageratum conyzoides* and *Oryza sativa* (weedy rice). SCLE concentrations of 0, 3, 6 and 9 g L^{-1} were prepared and sprayed twice at 7 days interval. The results showed that the SCLE significantly ($p<0.05$) affected growth attributes, chlorophyll pigments, and proline, and catalase, superoxide dismutase and peroxidase enzyme activities in a concentration-dependent pattern. It was found that high concentrations of SCLE induced greater phytotoxicity against *A. conyzoides* compared to *O. sativa*. SCLE spraying stimulated production of reactive oxygen species, boosting their ability to cause damage and inhibit growth. The allelochemicals in the extract stimulated an increased in the level of proline, which is an indicator of oxidative stress. Understanding the physiological and biochemical responses to Sida extract can improve our knowledge on allelochemical target sites and help us to explicate the mechanisms of action of such compounds.

Keywords: allelochemical; allelopathy; antioxidant; methanol extract; Sida; inhibition

1. Introduction

Due to the incessant application of synthetic herbicides in the agricultural system coupled with a rapid increase in herbicide-resistant weed species across the globe, attention has been focused on alternative weed management including allelopathy which refers to plant interference to inhibit the growth of other plants. Allelopathy is a relatively new discipline but has already been used to solve a range of practical problems in agriculture, and has contributed to our understanding of interactions between plant species (Niakan & Saberi, 2009). Certain plant species can influence the growth, yield and distribution of neighboring plants. This approach has been successfully employed in pest biocontrol the

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programs (Inderjit & Keating, 1999). A potential allelopathic phenomenon was noticed in the invasive weed specie Sida cordifolia (Ahmed et al., 2017). The plant invaded a mass of land and colonized other species probably via leaves or root exudation of phytotoxins as one of the suggested mechanisms to gain dominance advantage. *Sida cordifolia* is used extensively in traditional medicine (Ahmed et al., 2018). Nonetheless, research on its herbicidal potential is lacking in the literature. Visual observation of site invaded by *S. cordifolia* displays a drastic decrease in the preponderance of indigenous plants species. Hence, this study was attempted to examine the allelopathic activity of *S. cordifolia*.

Allelopathy has been considered to play a vital role in successfully controlling the invasion of alien species by secreting allelochemicals. Allelochemicals are released usually via foliage leaching, root exudation, volatilization, leaf litter, and residue decomposition (Cheema et al., 2013). Biotic and abiotic components affect physiological functions and plant stability such as excessive generation of reactive oxygen species (ROS), inflict damage on carbohydrates, lipids, proteins, DNA and trigger oxidative stress (Gill & Tuteja, 2010). ROS are regarded as the main source of damage and indicator of phytotoxicity stress in plants. They play a signaling role to numerous environmental responses and developmental processes in plant interactions such as cell division, growth and programmed cell death (Bais et al., 2003; Apel & Hirt, 2004). We evaluate allelopathic effect of foliar spray of *S. cordifolia* leaf extract on biochemical activity and growth of *Ageratum conyzoides* and *O. sativa* under field conditions.

2. Materials and Methods

The vegetative stage of fresh healthy and uniform leaves of *S. cordifolia* were collected at Sokoto, Nigeria and identified by Mr. Abdulaziz Mafara. A voucher (UDUH/ANS/0296) was deposited at the herberium of Department of Biological Sciences, Usmanu Danfodiyo University. The leaves were washed with tap water, dried under shade, pulverized (1 mm sieve) and preserved at 2^{o}C for further analysis. *Ageratum conyzoides* and weedy rice seeds were obtained from the seed bank at Weed Science and Seed Technology Laboratories, Department of Crop Science, Universiti Putra Malaysia. Seedlings were raised on plastic germination trays of 30 cm x 37 cm x 5.5 cm with peat moss in a glasshouse under day/night temperatures of 34/22^{o}C. The weedy rice and *A. conyzoides* seedlings at 2-3 and 4-6 leaves stages were transferred to the field at Ladang 10.

2.1 Experimental layout

A field experiment was conducted in Ladang 10, Faculty of Agriculture, Universiti Putra Malaysia. The study was carried out from April-June 2017. Thirty-two plots of 2 m x 2 m were prepared and covered with black polythene sheets to impede the growth of weeds and minimize the effects of competition. Plants were planted at one plant per hole and 30 cm apart. The physicochemical properties of the soil were determined.

2.2 Preparation of Sida leaf extract

A total of 25 g dried powder of *S. cordifolia* leaf was mixed with 1 L methanol (80 %), shaken on an orbital shaker for 12 h and then kept for another 12 h. The mixture was filtered through a 2 mm sieve and centrifuged at 3000 rpm for 15 min. The leaf residual was re-washed and the two filtrates were combined. The mixture was subjected to rotary

evaporation (RE-2L, Labfreez, China) at 40^{o}C and lyophilized (Labconco Freezone 2.5, USA). From the crude powder, a series of 0, 3.0, 6.0, and 9.0 g L^{-1} of distilled water and 0.05 % of surfactant (Silwit 614) was added to facilitate penetration and absorption of the extract.

2.2.1 Spraying method

Different treatment levels (3, 6 and 9 g L^{-1}) of Sida leaf extract were sprayed on *A. conyzoides* and weedy rice at the 8 and 4 leaf growth stages using a hand atomizer at a water carrier volume of 400 mL^{-1} 4 m^2. Spraying was repeated twice at 7 days interval. Distilled water was sprayed on the control. Irrigation was conducted when needed.

2.3 Plant growth evaluation

After 21 days post foliar spraying of *S. cordifolia* leaf extract, data on morphological, physiological and biochemical indices were evaluated. Shoot height and shoot and root dry weights were measured and weighed.

2.3.1 Leaf area

Leaves from the weed species were collected three weeks after spray, separated and leaf area measured with LI-3000 Li-COR USA leaf area meter.

2.3.2 Chlorophyll fluorescence

The fluorescence of new leaf was measured at 10-11 am using a portable chlorophyll fluorescence system (Fluorometer, Model FMS 2, Hansatech Instruments Ltd, UK) following the method of (Schreiber et al., 1995). Briefly, leaves were darkened by attaching light exclusion leaf clips. The intensity of pulses to determine maximum fluorescence emissions (delivered for 3 s) was approximately 1300 µmol m^{-2} s^{-1}, and the fluorescence responses were induced by emitting diodes. The minimum (F_0) and maximum (F_m) fluorescence were determined.

2.3.3 Chlorophyll pigments

Chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoids were estimated according to Lichenthaler & Buschmann (2001). Fresh leaves (0.2 g) were extracted with acetone (10 mL) in a glass bottle covered with aluminum foil under dark condition for 48 h. Chlorophyll determination was performed with a spectrophotometer (UV- 3101P, Labomed Inc, USA) at 661.6 nm, 644.8 nm, 470 nm for the Chl*a*, Chl*b* and carotenoids. The pigment contents were calculated and expressed as micrograms per gram of fresh weight.

2.3.4 Determination of proline

Proline was determined according to Singh et al. (2006). Fresh leaf (100 mg) was pulverized with 3 mL of 3% sulfo-salicylic acid and centrifuged at 2000 rpm at 25^{o}C for 5 min. A reagent mixture (2 mL) consisting of 0.5 g ninhydrin, 20 mL distilled water and 30 mL glacial acetic acid was added to 0.2 mL supernatant and then 0.4 mL of distilled water was added to the mixture. The mixture was boiled for 1 h before being cooled and

extracted with toluene (6 mL). Absorbance was then determined using spectrophotometer (UV- 3101P, Labomed Inc, USA) at 520 nm and the proline was evaluated using standard curve and expressed as mg g^{-1}fresh weight.

2.3.5 Enzymes extraction

A total of 0.5f g of leaf was ground with 1 % polyvinyl poly pyrrolidone (8 mL) and potassium phosphate buffer (50 mL). The homogenate mixture was centrifuged at 15000 g for 30 min and the supernatant was used for enzyme assays as described by Hakiman & Maizah (2009). Catalase (CAT) activity was analyzed according to Niakan & Saberi (2009). One mL of the reaction mixture containing potassium phosphate buffer (pH 7.0), 250 µL of enzyme extract and 60 mM hydrogen peroxide was added. The resultant reaction was evaluated at 240 nm for 3 min and H_2O_2 consumption was determined using an extraction coefficient (39.4 mM^{-1} cm^{-1}). Superoxide dismutase (SOD) was assayed following the method of Esfandiari et al. (2007). A reaction mixture of 0.01 mL of 2.25 mM nitro-blue tetrazolium, 1.5 mL of 100 mL potassium phosphate buffer, 0.1 mL of 200 mM methionine, 0.1 mL of 3 mL EDTA, 0.05 mL of enzyme extract and 1.0 mL distilled water was prepared. The reaction was initiated by adding 0.1 mL riboflavin (60 µM) and absorbance was measured at 560 nm. Peroxidase (POD) was determined using the guaiacol oxidation method (Hakiman & Maizah, 2009). A total of 3 mL of reaction mixture containing 0.1 mL enzyme extract, 2.9 mL potassium phosphate buffer (pH 7.0) and 8 mM guaiacol was prepared. The reaction was initiated by adding 2.75 mM H_2O_2 and the absorbance was recorded using a spectrophotometer (UV- 3101P, Labomed Inc, USA) at 470 nm after 3 min.

2.4 Statistical analysis

The experiment was performed according to a randomizedcomplete block design (RCBD) with four replications. Data relating to morphological and physiological analysis were evaluated for normality and conducted with the Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was performed, and data analyzed using SAS software 9.4 version. Comparison of means was conducted using Tukey' test at (HSD) $p \leq 0.05$.

3. Results and Discussion

The plant height, and the shoot and root dry weights of *A. conyzoides* and *O. sativa* were measured and the results are shown in Table 1. From the results, it is clear that foliar spray of SCLE significantly ($P<0.05$) reduced biomass at all concentrations tested while the maximum decrease (14.46%) in shoot dry weight was observed in *O. sativa* at 9 g L^{-1} (w/v). Many studies have found that reduction in plant growth is proportional to the increase in plant extract concentrations. Increasing concentrations of saffron extract reduced shoot length, root length and seedling dry weights of *Gypsophilla pillosa* and *Rapistrum rogosum* (Alimoradi et al., 2008). Aqueous leachates of the leaves of *Populous deltoids* reduced seedling growth of *Lens culinaris, Trifolium alexandrinum, Vigna mungo, Helianthus annuus* and *Brassica juncea* by 10 to 30% at 30 and 60 days after sowing (Ladhari et al., 2020). The results of the present study were in agreement with Maqbool (2010), who reported that foliar application of sorghum extract at high

Table 1. Effect of methanol leaf extract of *S. cordifolia* on plant height cm (PH), shoot (SDW) and root dry weights (RDW) and total biomass (TW) of tested species

Leaf Extract (g/L)	PH (cm)	SDW (g)	RDW (g)	TW (g)
A. conyzoides				
9	47.33±0.73^{b}	2.57±0.10^{b}	0.40±0.10^{b}	3.12±0.11^{a}
6	49.70±2.69^{ab}	3.38±0.31^{ab}	0.55±0.14^{ab}	3.98±0.38^{ab}
3	52.88±1.64^{ab}	3.59±0.41^{ab}	0.64±0.12^{ab}	4.01±0.28^{ab}
0	54.70±1.36^{a}	4.83±0.75^{a}	0.96±0.20^{a}	5.89±0.88^{b}
O. sativa (weedy rice)				
9	36.08±2.32^{a}	2.83±0.14^{b}	1.00±0.08^{b}	3.83±0.18^{a}
6	40.55±1.59^{a}	3.29±0.24^{ab}	1.72±0.31^{ab}	5.01±0.54^{b}
3	41.60±2.46^{a}	4.12±0.45^{ab}	1.84±0.23^{ab}	5.63±0.82^{b}
0	43.35±2.07^{a}	4.57±0.54^{a}	1.88±0.06^{a}	5.97±0.69^{b}

Data represent mean±standard error of four replicates. Superscript letters with the same letters are not significantly different, using Tukey's mean comparison ($P<0.05$).

concentrations on maize hindered the morphological and biochemical characters of the crop at the vegetative stage. In the present study, biomass decreased significantly ($P<0.05$) at maximum concentration of 9 g L^{-1} by 47.03% and 35.84% in *A. conyzoides* and *O. sativa*. Water extracts of *Acacia nilotica* bark, *Eucalyptus camaldulensis* and *Prosopis juliflora* significantly reduced seedling growth and biomass yield of *Asphodelus tenuifolius* and *Ipomea* sp (Khan et al., 2005). Castor beans leaf aqueous extract significantly affected the radicle length and dry weight of soy bean (Faria et al., 2009; da Silva et al., 2016).

As a good indicator of resource capture and conversion, leaf area had shown decrease in both species (*A. conyzoides* and *O. sativa*) and these decreases were associated with decreases in plant height. Leaf area was decreased significantly ($P<0.05$) by 34.56% and 31.28% in *O. sativa* and *A. conyzoides* at the maximum concentration of foliar spray of *S. cordifolia* leaf extract (Figure 1B and A). The SCLE concentrations reduced leaf area in *A. conyzoide* but no significant difference was recorded. Similar findings were observed by Nekonam et al. (2014), who found that the foliar spraying of the aerial parts of *Crocus sativa, Datura inoxia, Nicotiana tabacum, Nerium oleader, Ricinus communis* and *Sorghum vulgare* decreased pigweed leaf area but no significant difference between the concentration levels were observed.

Chlorophyll is a determinant factor in photosynthesis. The chlorophyll and carotenoids contents decreased significantly ($p<0.05$) in response to foliar spray with SCLE. After spraying, the leaves displayed furled margins and yellowing, a typical sign of stress condition. The decrease in the pigments varied among the species with *A. conyzoides* showing high percentages of inhibition (compared to the control) of chlorophyll *a*, chlorophyll *b* and total chlorophyll (78.4%, 76.8%, and 72.0%) at maximum concentration (Figure 2A), which were greater than *O. sativa* (62.9%, 64.5%, and 63.9%) (Figure 2B). The decreases in the pigment content occurred in a concentration dependent pattern. The reduction might be due to the interference of allelochemicals with thylakoid membranes, or due to inhibition of enzymes involved in the biosynthesis of the chlorophyll. Plant growth, chlorophyll content and photosynthesis were inhibited by high concentrations of allelochemicals (Ding et al., 2016). *Artemisia judaica* shoot extract reduced the chlorophyll

content of lettuce in a dose-dependent manner (Zhang et al., 2008). The chlorophyll-*b* and carotenoid content of *Cucurbita pepo* decreased (81.4% and 77.8%) in concentration-dependent when treated with *Portulaca oleiracea* root extract, and this caused degradation of chlorophyll and reduction in chlorophyll biosynthesis (Ahmed & Hamid, 2015). In our study, total chlorophyll in *A. conyzoides* and *O. sativa* declined by 72.0% and 63.9% at 9 g L^{-1} against the control while the chlorophyll ratio (CHL*a*/*b*) in both species reached a maximum at 6 g L^{-1} and a minimum at 9 g L^{-1} and 3 g L^{-1} in *A. conyzoides* and *O. sativa*, respectively (Figure 2A and B). The SCLE treatment significantly inhibited carotenoids by 68.39% and 39.02% in *A. conyzoides* and *O. sativa* compared to the control (Figure 3A and B).

The maximum quantum efficiency of a photosystem (Fv/Fm) is a photo-inhibition index widely used in light utilization analysis (Netondo et al., 2004; Santos, 2004). His index, known as the quantum yield ratio (Fv/Fm), provides valuable information of photosynthetic activity and stress indicators in plants. In the present study, the maximum quantum yield for both sunny and cloudy conditions indicated the occurrence of significant ($P<0.05$) photoinhibition at all the concentrations of SCLE, with decreased in photosystem II under sunny conditions (Figure 4) that corresponded with an increase in antioxidant enzymes and this activity may lead to the inhibition of the primary reactions in the antenna complex and the hindering of photosynthate products. Thylakoid membrane damage and inhibition of energy transfer from reaction centers to antenna molecules can lead to photo-inhibition and lower Fm/Fv ratios (Krause & Wies, 1984; Colom & Vazzana, 2003). Allelochemicals can significantly affect the performance of thylakoid electron transport during the light reactions and stomatal control of carbon dioxide and the carbon cycle in the dark reaction (Reigosa et al., 2006). Similarly, allelochemical compounds (hydroxybenzoic, *p*- hydroxybenzoicand ferulic acids) significantly decreased the quantum yield of photosystem II while ferulic acid exhibited severe reduction in quantum efficiency of dark-adapted leaves (Hussain et al., 2010).

Five days after foliar spraying of SCLE, chlorosis, leaf furling and firing symptoms were noticed on *A. conyzoides* which caused yellowish leaves and slow growth (Figure 5). But after 10 days, the plants had initiated recovery and displayed compensatory growth. A feasible cause of the initial yellowing could be the presence of various allelochemicals (stearic and palmitic acids, 10E, 12Z–9-hydroxyoctadeca-10,12-dienoic acids; flavonoids and alkaloids ephedrine, hypaphorine, vasicinone and vasicinol) in the SCLE (Nunes et al., 2006; Ahmed et al., 2017). Moreover, maximum concentrations of the extract acted as herbicide by disturbing plant processes like membrane permeability, reducing chlorophyll content and inhibiting photosynthesis. Several reports showed that allelochemicals caused decreases in chlorophyll content and inhibited photosynthesis processes (Liu et al., 2009; Abu-Romman et al., 2010; Ding et al., 2016). The chlorophyll pigment of maize, wheat and *Pinus tobuliformis* were inhibited by *Cinnamomum septentrionale* and *Jiuniperus rigida* extracts (Liu et al., 2017). Enzymes activities involved in chlorophyll biosynthesis pathway and photosynthetic efficiency were frequently distorted in the presence of allelochemicals (Romagni, 2000; Batoul et al., 2014; Yang et al., 2017).

Proline is amino acid associated with plant stress. SCLE had significant ($P<0.05$) stimulatory effect on proline synthesis, with substantial increases of 94.4% and 111.3% at maximum concentration (9 g L^{-1}) observed in *A. conyzoides* and *O. sativa* compared to the control (Figure 6A and B). High accumulation of proline in the leaves in response to stress may help to stabilize protein molecules and cellular membranes against allelochemical stress. Chlorophyll molecular stability depends essentially on the integrity of membrane structures, which proline usually maintains (Aggarwal et al., 2011; Ahmed

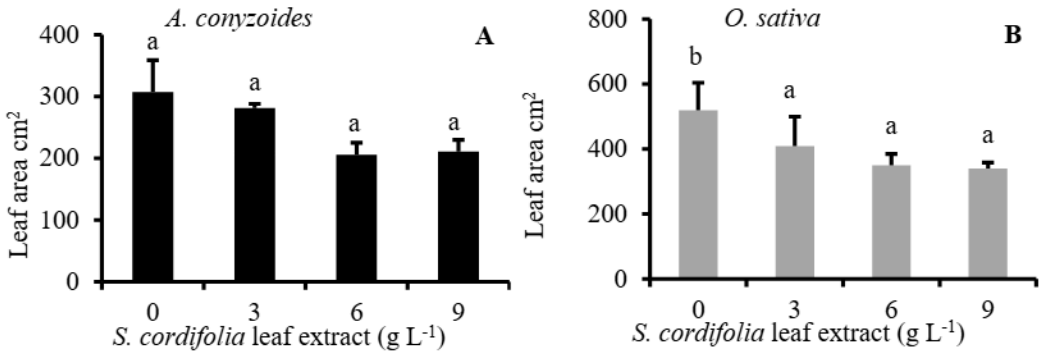

Figure 1. Effect of foliar spray of *S. cordifolia* leaf extract on leaf area (cm^2) of *A. conyzoides* (A) and *O. sativa* (B). Different letters along the bars indicate significant differences at P<0.05 (Tukey's test).

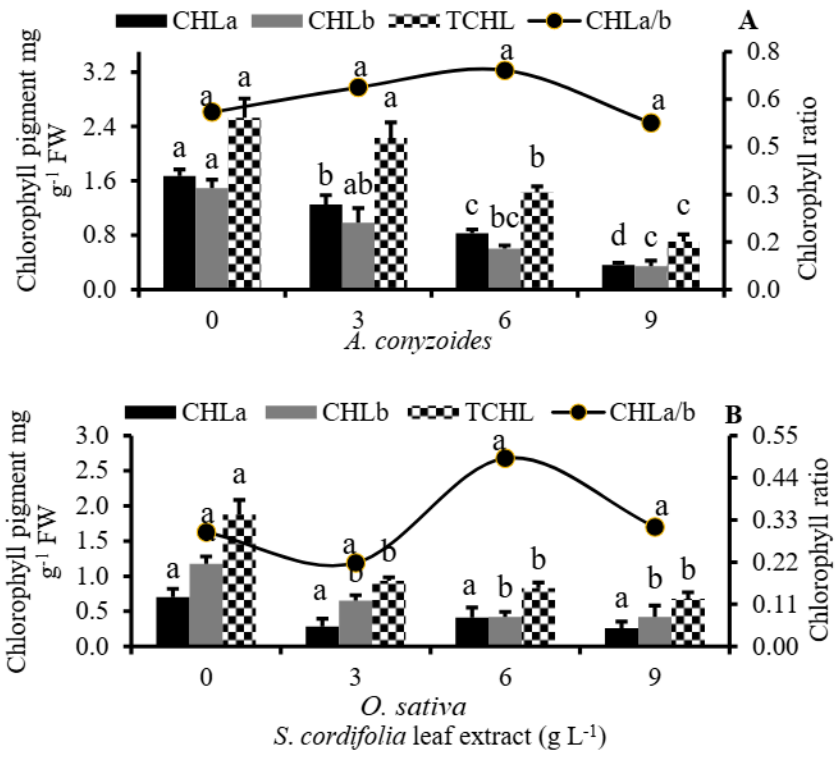

Figure 2. Effect of foliar spray of SCLE at different concentrations on chlorophyll*a*, chlorophyll*b*, total chlorophyll and chlorophyll ration of *A. conyzoides* (A) and *O. sativa* (B). Different letters along the bars indicate significant difference at P<0.05 (Tukey's test).

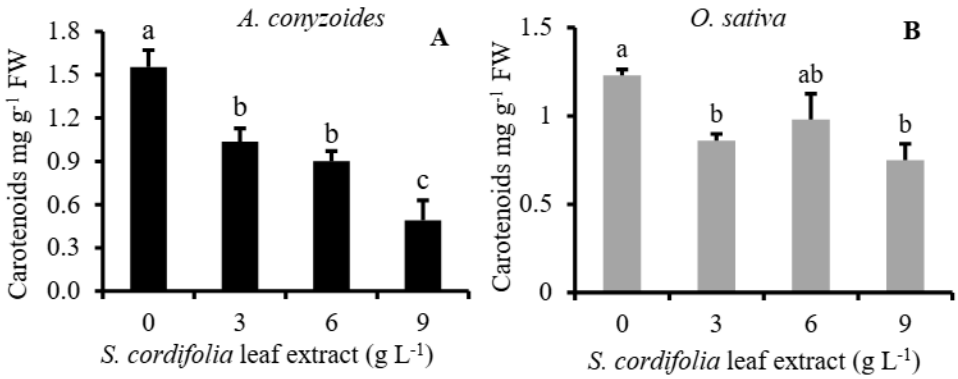

Figure 3. Effect of foliar spray of SCLE at different concentrations on carotenoid content of *A. conyzoides* (A) and *O. sativa* (B). Different letters along the bars indicate significant differences at P<0.05 (Tukey's test).

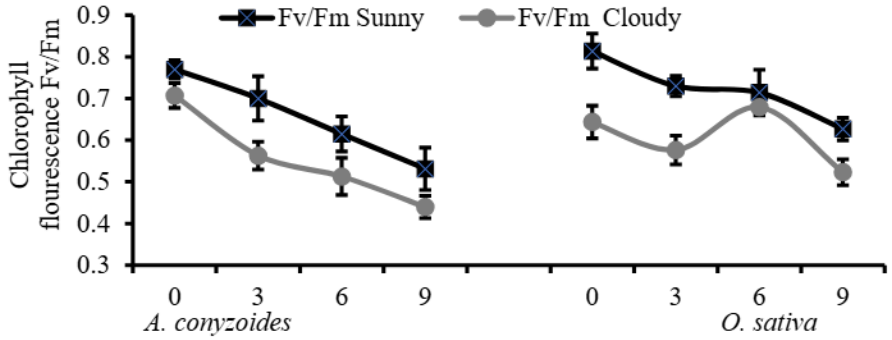

Figure 4. Quantum efficiency of PSII reaction center in the sunny and cloudy adapted states in the leaves of *A. conyzoides* and *O. sativa* at 7 days after second foliar spraying of SCLE. Data represent mean±standard error of four replicates.

et al., 2011). Foliar spray of *Medicago sativa* extract increased the proline contents of three wheat varieties (Perveen et al., 2016). Leachate extracts of *Acacia auriculiformis*, *Anacardium occidentale*, *Albizia lebbeck*, *Eucalyptus citriodora*, *Emblica officinalis*, *Shorea robusta* and *Tectona grandis* produced significant increases in the proline content in *Cicera rietinum* (Das et al., 2012). In contrast, *Heliotropium bacciferum* extract decreased the proline content in *Oryza sativa* and *Teucrium polium* (Al-Taisan, 2014).

In this study, the antioxidant enzymes (POD, CAT and SOD) showed elevated activities following exposure to SCLE at all concentrations compared to the control. The increases in the POD activity of *A. conyzoides* and *O. sativa* were 44.09% and 43.67% which were more than the control treatment at 9 g L^{-1} (Figure 7A and B) while CAT (Figure 7C and D) and SOD (Figure 7E and F) increased activities of 61.07% and 88.0% for *A. conyzoides*, and 24.4% and 48.9% for *O. sativa*. The allelochemicals in the SCLE more than likely induced peroxidation since the leaves were the first organ to be exposed to the allelochemicals in the SCLE, and this could be an important factor that regulated the occurrence of phytotoxicity in the leaves. The SCLE treatment induced accumulation of

Figure 5. *Ageratum conyzoides* seedlings showing the beginning of chlorotic condition 5 days after second foliar spray of SCLE

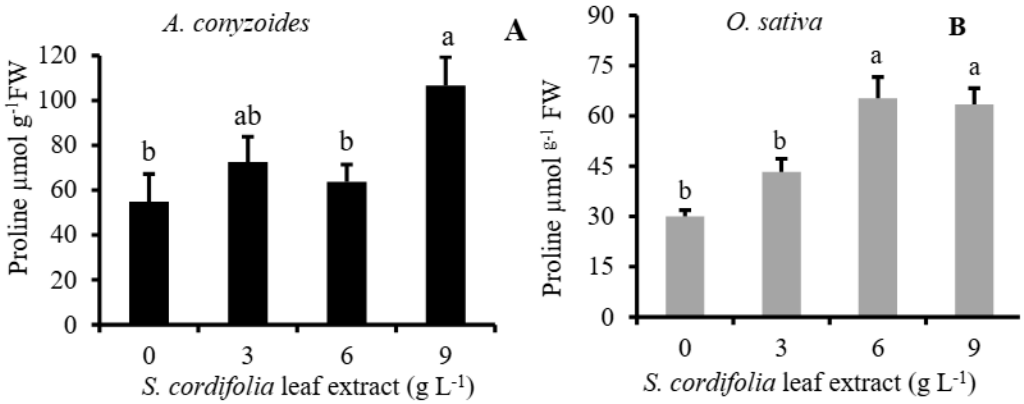

Figure 6. Effect of foliar spray of SCLE at different concentrations on proline contents of *A. conyzoides* (A) and *O. sativa* (B). Different letters along the bars indicate significant differences at P<0.05 (Tukey's test).

ROS in the test species but more effects were observed in *A. conyzoides* probably due to its broader leaf surface area. The significant decrease in chlorophyll pigments and large increase in proline content indicate changes in protein levels and markers to phytotoxicity or senescence. *Microcystis aeruginosa* antioxidant enzyme activities increased at low concentrations of *Solidago canadensis* extracts but activities were hindered when the extract concentration was increased (Huang et al., 2017). The activities of CAT, POD and SOD enzymes in *Pinus tabuliformis* and *Triticum aestivum* were significantly inhibited at higher concentrations but stimulated at the lower concentration (Huang et al., 2013; Liu et al., 2017). This showed that the activities of protective enzymes were stimulated by exposure to allelochemicals and the overwhelming increases in the enzymes suggested an abundance of ROS. Though ROS are highly oxidative in nature, they also play an important role in the regulation of many cellular processes including the production of plant stress

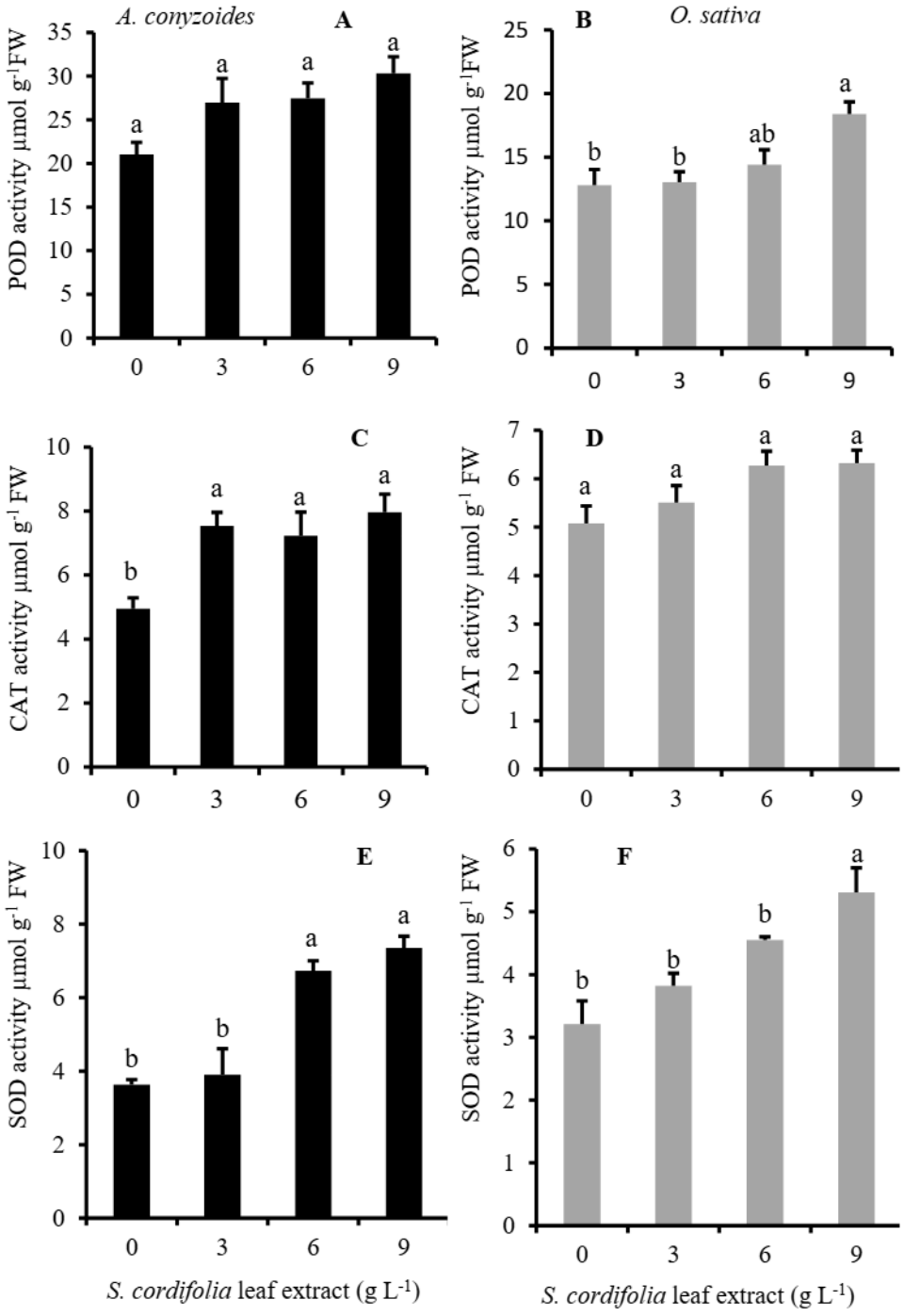

Figure 7. Effect of foliar spray of SCLE at different concentrations on enzymes activities (POD, CAT and SOD) in *A. conyzoides* (A, C and E) and *O. sativa* (B, D and F). Different letters along the bars indicate significant differences at P<0.05 (Tukey's test).

hormones (salicylic acid, jasmonic, ethylene, and nitric oxide), programmed cell death, stomatal behavior and hormonal signaling (Mittler, 2002). Generation of ROS enzymes increases plant tolerance to oxidative stress. Several mechanisms of allelopathy have remained elusive and the mode of activity through chemical signaling, direct inhibition of photosystem II components, interruption of ATP synthesis, and genetic programming have been suggested. Many allelochemicals hindered the incorporation of certain amino acids into proteins hence reducing the rate of protein synthesis (Hussain & Reigosa, 2014). Ferulic acid decreased *Zea mays* seedlings growth, and protease, phospholipase, maltase and hydrolase activities (Devi & Prasad, 1992). Allelochemicals such as rutin, gallic and fagomine acids were reported to upregulate stress-related genes producing heat-shock protein (Ambika, 2013).

4. Conclusions

From the present study, it can be concluded that the foliar spray of SCLE inhibited the growth-related activities of *A. conyzoides* and *O. sativa*. The extracts exerted phytotoxicity on the plant species and caused reduction in biomass, chlorophyll pigments and photosystem II. These responses coincide with the stimulated generation of antioxidant enzymes (POD, CAT and SOD), which are indicator of physiological and biochemical responses to stress. Enzymes stimulation by the extract indicated the importance of these enzymes in the protection from oxidative stress and oxidation of plasma membrane, effects that rare seen as leaf damage tissue and chlorosis. The use of SCLE induced phytotoxicity and early senescence, and this could be a primary mechanism of activity of the sida extract. Though the maximum concentration (9 gL^{-1}) of SCLE sprayed twice at 7-day interval was sufficient to induce damage, a much higher concentration may constitute a lethal and more efficient bioherbicide.

5. Acknowledgements

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6. Conflicts of Interest

The authors declare that they have no conflicts of interest.

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