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Effects of *Eucalyptus* Allelopathy on Growth Characters and Antioxidant Enzymes Activity in Phalaris Weed

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Abstract: In this study, different amounts of aqueous extracts [0, 5, 15 and 30% (v/v)] and decompose of *Eucalyptus* leaves [(0, 3 and 6% (w/w)] were applied to Phalaris in pot culture and evaluated on growth parameters and antioxidant enzymes activity. The results showed that most of the growth parameters of phalaris were decreased when exposed to different amounts of decompose and water extracts of *Eucalyptus* leaves, especially in the leaf decompose. Also, antioxidant enzymes activity in root and shoot of Phalaris were affected by aqueous extracts and decompose of *Eucalyptus* leaves. The findings indicated that decompose of *Eucalyptus* leaves decreased catalase activity and increased ascorbate activity in root and shoot of Phalaris in comparison to control while aqueous extract of *Eucalyptus* leaves significant raised ascorbate peroxidase in Phalaris root. Activity of other enzymes such as polyphenol oxidase and peroxidase in Phalaris affected less aqueous extracts and decompose of *Eucalyptus* leaves.

Key words: Allelopathy, *Eucalyptus*, phalaris, growth, antioxidant

INTRODUCTION

In classic opinion, allelopathy refers to the chemical inhibition of one species by another. Modern research suggested that allelopathic effects can be both positive and negative, depending upon the doses and organisms (Bais *et al*., 2003). When plants are exposed to allelochemicals, their growth and development are affected (Putnam and Duke, 1978; Niakan *et al*., 2008). The readily visible effects include inhibited or retarded germination rate, seeds darkened and swollen, reduced root or radicle and shoot or coleoptile extension, swelling or necrosis of root tips, curling of the root axis, discoloration, lack of root hairs, increased number of seminal roots, reduced dry weight and lowered reproductive capacity. These gross morphological effects may be secondary manifestations of primary events, caused by a variety of more specific effects acting at the cellular or molecular level in the receiver plants (Singh *et al*., 2006; Peng *et al*., 2004; Prati and Bossdorf, 2004). The activity of enzymes was also affected by allelopathic compounds (Peng *et al*., 2004; Niakan *et al*., 2008). Environmental stress can create oxidative stress through increased production of Reactive Oxygen Species (ROS), e.g., singlet oxygen ($^1O_2$), hydroxyl radical (HO°) and hydrogen peroxide (H_2O_2) (Blokhina *et al*., 2003; Lee *et al*., 2007; Gechev and Hille, 2005; Apel and Hirt, 2004). Reactive oxygen species may cause lipid peroxidation whereas superoxide dismutase (SOD) and peroxidase (POD) are inhibiting (Blokhina *et al*., 2003; Singh *et al*., 2006). Under oxidative stress, plants respond by increasing antioxidant defenses, notably enzymes such as SOD and POD. However, excessive ROS may cause a decrease in SOD and POD activities (Mishra *et al*., 1993). Numerous studies have supported the signaling role of reactive oxygen species during different environmental., responses and developmental processes, including biotic and abiotic stress responses, allelopathic plant-plant interactions, cell division and elongation and programmed cell death (Apel and Hirt, 2004; Bais *et al*., 2003; Pei *et al*., 2000).

Eucalyptus is one of the most important tree species for wood production in the world. It is said that *Eucalyptus* is toxic, due to allelopathic properties, which serve to reduce germination of other plant (Khan *et al*., 2005; Shiming, 2005).

Batish *et al*. (2004) reported that *Eucalyptus citriodora* produces volatile materials that inhibit the germination, seedling length, chlorophyll content and respiratory ability in *Phalaris minor*, *Chenopodium album*, *Echinochola crus-gali*, *Ageratum conyzoide*, *Parthenium hysterphorus* and *Amaranthus* sp. Shiming (2005) showed the allelopathic effects of 4 species of *Eucalyptus* on radish seed germination and seedling growth.

Researches showed that *Eucalyptus* species released volatile compounds such as benzoic, cinnamic and phenolic acids, which inhibited growth of crops and

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weeds growing near it (Sasikumar *et al*., 2001; Kohli *et al*., 1998). Daizy *et al*. (2004) reported that volatile oil from leaves of *Eucalyptus citriodora* significantly reduced germination seedling length and chlorophyll content in *Triticum aestivum*, *Zea mays* and *Raphanus sativus*. Batish *et al*. (2004) reported that water extracts of *E. citriodora* significantly reduced weed establishment.

As a scientific discipline, allelopathy is still relatively new. But it has already contributed to the solution of practical problems in agriculture and provided explanations for observed plant-plant interactions. Allelochemicals might be directly used as natural product pesticides as well as models for developing new pesticides. By modifying these allelochemicals, the end product could be more active, selective, or persistent.

The aim of the present study was to determine the inhibitory effect of decompose and aqueous extract of *Eucalyptus* leaf on growth, generation of reactive oxygen species, as indicators of oxidative stress and changes in activities of antioxidant enzymes in Phalaris weed.

MATERIALS AND METHODS

In summer 2008, *Eucalyptus* leaves (*Eucalyptus camaldulensis*) were harvested from natural habitat located on Azad shahr city (37.053 longitude, 55.095 latitude and 34 m above sea level) in North Iran. Then leaves were dried in shade and grinded. Dried samples were mixed with soil (Si-Clay tissue) in ratio (0, control), 3 and 6% (w/w) and this mixture were placed in shade for 30 days.

Aqueous extract of *Eucalyptus* leaves preparation: Five grams of dried *Eucalyptus* leaves (*Eucalyptus camaldulensis*) were added to 150 mL distilled water and were shaked for 12 h. The mixture was passed through Wathman paper (number 2) and filtrated through microporous filter (0.2 μm pore side) (Narwal and Tauro, 1996) and prepared aqueous extract in concentrations 0, 5, 15 and 30% (v/v).

Phalaris planting

Phalaris planting in Decompose of *Eucalyptus* leaves: Phalaris seeds (*Phalaris minor*) were imbibed in distilled water for 48 h and then divided to 2 parts. The first part was planted in pots that include mixture of soil (Si-Clay tissue) and *Eucalyptus* Eucalypt leaves in ratio 0 (control), 3 and 6%. Pots maintained in a photoperiod of 10-14 (L-D) and controlled temperature (20±2°C) and irrigated 3 days interval with 200 mL water. After 60 days plants were harvested and used for growth parameters measurement and biochemical assays.

Phalaris planting for spray aqueous extract of *Eucalyptus* leaves: The Phalaris seeds were placed in water for 48 h and planted in pots soils with Si-Clay tissue. After 30 days, Phalaris plants were sprayed to aqueous extract of *Eucalyptus* leave in concentrations of 0 (control), 5, 15 and 30% (v/v) for 3 times in week during 30 days. Then, plants were brought out of soil and used for growth parameters measurement and biochemical assays.

Growth parameters measurement: After 60 days, root and shoot length of Phalaris plants that had been treated by decompose and aqueous extract of *Eucalyptus* leaves were measured. Root and shoot length, fresh and dry weights of root and shoot and leaf area of *Phalaris* plants under treatments of decompose and aqueous extract of *Eucalyptus* leaves also were determined. For dry weight, root and shoot of Phalaris were placed in hot-air oven at 90°C for 24 h.

Biochemical assays

Enzyme extraction: one gram of the roots or shoots of Phalaris plants treated with decompose or aqueous extract of *Eucalyptus* leaf were weighted and homogenized with 4 mL extract solution containing 1.2 g Tries, 2 g ascorbate, 3.8 g borax (Di-sodium tetra borate), 2g EDTA-Na_2, 50 g polyethylene glycol 2000 in 100 mL distilled water. The solution was placed in 4°C for 24 h and then was centrifuged for 30 min at 4000 g. The clear supernatant was taken as enzyme extract and used of them for catalase and peroxidase, poly phenol oxidase and ascorbate peroxidase activity assay.

Catalase activity assay: The catalase activity was assayed by Chance and Maehly (1955) method with the following modification: 5 mL of assay mixture for catalase activity contained: 300 μM of phosphate buffer (pH 6.8), 100 μM of H_2O_2 and 1 mL of the twice diluted enzyme extracted. After incubation at 25°C for 1 min, the reaction was stopped by adding 10 mL of 2% (v/v) H_2SO_4 and the residual H_2O_2 was titrated against 0.01 N of $KMnO_4$ until a faint purple color persisted for at least 15 sec. A control was run at the same time but the enzyme activity was stopped at zero time. One unit of catalase activity is defined as the amount of enzyme which breaks down 1 μmol of H_2O_2 min^{-1} under the described assay condition.

Peroxidase activity assay: The peroxidase activity was determined by Koroi (1989) method. The 0.1 mL of enzyme extract was added to assay mixture containing: 2 mL 0.2 M acetate buffers (pH 5.0), 0.4 mL of 3% H_2O_2 and 0.2 mL of

0.01 M benzidin solution in 50% alcohol. The absorbance was taken at 530 nm. In order to protect of enzyme activity, the experiment was done in chilled conditions.

Ascorbate peroxidase activity assay: Assay mixture of ascorbate peroxidase activity was consisted of: 2 mL phosphate buffer 0.05 M (pH 6.5) were mixed to 0.2 mL H_2O_2 3%, 0.2 mL ascorbic acid 50 μM and 0.1 mL enzyme extract was added to them. The enzyme activity was determined by taking the absorbance at 265 nm according (Arrigoni, 1994).

Poly phenol oxidase activity assay: The activity of poly phenol oxidase was assayed by Manoranjan and Mishra (1976) Assay mixture was consisted of: 1.5 mL of 0.2 M phosphate buffer (pH 7.6), 0.4 mL pyrogallol 0.02 M and 0.1 mL enzyme extract. This mixture was incubated at 28°C for 3 min. The enzyme activity was determined by measuring the absorbance at 430 nm spectrophotometrically.

Statistical analysis: The experiment was carried out in completely randomized design with 4 replications The statistical significance of the difference between parameters was evaluated by means of Duncan-test on SPSS 11.5 and for each treatment and control 4 replications was selected. The results are given in the text as p, the probability values and $p \leq 0.01$ was adopted as criterion of significance.

RESULTS

The results of this study showed that by increasing amount of decompose *Eucalyptus* leaf in soil (3, 6%) length, fresh and dry weight of Phalaris root and shoot at $p \leq 0.01$ decrease (Table 1). Also, application of high concentrations of aqueous extract of *Eucalyptus* leaf (15, 30%) decreased fresh and dry weight of Phalaris root in comparison to control and 5% aqueous extract but they had a non significant effect on fresh and dry weight of Phalaris shoot at $p \leq 0.01$ (Table 2).

Present results also indicated that root length of Phalaris plants treated with 5, 15 and 30% concentrations of aqueous extract of *Eucalyptus* leaf were more than control while shoot length of Phalaris by increasing concentration of *Eucalyptus* leaf extract raised significantly ($p \leq 0.01$, Table 2).

Statistical analysis of the data showed that treatment with *Eucalyptus* leaf decompose decreased Phalaris leaf area more than spray of *Eucalyptus* leaf aqueous extract (Table 3).

Table 1: Effects of different amounts of decompose of *Eucalyptus* leaf (in each ratio 0: Control, 3, 6% with soil) on root and shoot length (cm), dry and fresh shoot and root (g) of Phalaris

	Phalaris					
	Root			Shoot		
	Length	F.W	D.W	Length	F.W	D.W
Control	29.488a	0.363a	0.075a	25.95a	2.583a	0.334a
Decompose (3%)	21.588b	0.220b	0.023a	14.850b	0.529 b	0.077b
Decompose (6%)	19.133b	0.137c	0.016b	6.228b	0.512 b	0.065b

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

Table 2: Effects of different amounts of aqueous extracts (0, 5,15 and 30%) of *Eucalyptus* leaf on root and shoot length (cm), dry and fresh shoot and root (g) of Phalaris

	Phalaris					
	Root			Shoot		
Extracts	Length	F.W	D.W	Length	F.W	D.W
Control	22.625b	0.413a	0.119a	25.975c	1.662a	0.264a
Aqueous (5%)	29.313a	0.488a	0.097b	24.663c	1.363a	0.227a
Aqueous (15%)	30.050a	0.221c	0.066c	28.308b	1.374a	0.227a
Aqueous (30%)	27.690a	0.322b	0.073c	30.613a	1.374a	0.225a

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

Table 3: Effects of different amounts of decompose of *Eucalyptus* leaf in ratio 0: Control, 3 and 6% with soil and aqueous extracts (0, 5, 15 and 30%) of *Eucalyptus* leaf on leaf area (cm^2) of Phalaris

	Eucalyptus leaf						
	Decompose (%)			Aqueous extract (%)			
Area	Control	3	6	Control	5	15	30
Leaf area	11.852a	4.809b	4.796b	12.092a	11.632a	10.119b	10.020b

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

Also, the analysis of data revealed that decompose of *Eucalyptus* leaf had more inhibition effect on fresh and dry weight of root and shoot of Phalaris than aqueous extract of *Eucalyptus* leaf (Table 1, 2).

These findings reveal that most of the growth parameters of Phalaris were decreased when exposed to different amounts of decompose and water extracts of *Eucalyptus* leaf. The reduction is more severe by *Eucalyptus* leaf decompose.

Table 4-7 show antioxidant enzymes activity in leaf and root of Phalaris.

Catalase activity in leaves and roots of Phalaris decreased by increasing the amount of *Eucalyptus* leaf decompose (Table 4, 5) while its activity in leaves and roots was increased at higher concentration of aqueous extract of *Eucalyptus* leaf (Table 6, 7).

At highest content of *Eucalyptus* leaf decompose (6%) peroxidase activity in Phalaris leaf decreased

Table 4: Effects of different amounts of decompose of *Eucalyptus* leaf (in ratio 0: Control 3, 6% with soil) on antioxidant enzyme activity on Phalaris root include: catalase, peroxidase, poly phenol oxidase and ascorbate peroxidase (OD min^{-1} g^{-1} Fw)

	Phalaris root			
Level of decomposition	Catalase	Peroidase	Ascorbate peroxidase	Polyphenoloxidase
Control	2.844a	0.065a	2.805b	0.192a
Decompose (3%)	2.826b	0.073a	2.823b	0.268a
Decompose (6%)	2.803c	0.075a	2.952a	0.212a

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

Table 5: Effects of different amounts of decompose of *Eucalyptus* leaf (in ratio 0: Control, 3, 6% with soil) on antioxidant enzyme activity on Phalaris leaf include: catalase, peroxidase, poly phenol oxidase and ascorbate peroxidase (OD min^{-1} g^{-1} Fw)

	Phalaris leaf			
Level of decomposition	Catalase	Peroidase	Ascorbate peroxidase	Polyphenoloxidase
Control	2.861a	0.636a	2.700b	0.176a
Decompose (3%)	2.833b	0.559ab	2.752b	0.159 a
Decompose (6%)	2.812c	0.378b	2.814a	0.160a

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

Table 6: Effects of different amounts of aqueous extracts (0, 5, 15 and 30%) of *Eucalyptus* leaf on antioxidant enzyme activity on Phalaris root include: catalase, peroxidase, poly phenol oxidase and ascorbate peroxidase (OD min^{-1} g^{-1} Fw)

	Phalaris root			
Extracts	Catalase	Peroidase	Ascorbate peroxidase	Polyphenoloxidase
Control	2.794b	0.067a	2.947d	0.225a
Aqueous (5%)	2.792b	0.076a	2.979c	0.205a
Aqueous (15%)	2.792b	0.066a	3.007b	0.194a
Aqueous (30%)	2.812a	0.078a	3.032a	0.187a

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

Table 7: Effects of different amounts of aqueous extracts (0, 5,15 and 30%) of *Eucalyptus* leaf on antioxidant enzyme activity on Phalaris leaf include: catalase, peroxidase, poly phenol oxidase and ascorbate peroxidase (OD min^{-1} g^{-1} Fw)

	Phalaris leaf			
Extracts	Catalase	Peroidase	Ascorbate peroxidase	Polyphenoloxidase
Control	2.838b	0.345a	2.891a	0.292a
Aqueous (%5)	2.836b	0.258a	2.874b	0.280a
Aqueous (%15)	2.851a	0.323a	2.869b	0.296a
Aqueous (%30)	2.851a	0.479a	2.873b	0.305a

Means±SD followed by different letter(s) of the same column indicates that the values are significant different at 0.01 determined by ANOVA and Duncan multiple range tests

(Table 6). Also, significant difference in peroxidase activity of Phalaris roots between control and several amounts of *Eucalyptus* leaf decompose (3, 6%) was not observed (Table 4).

Decompose and aqueous extract of *Eucalyptus* leaf was not significant effect on poly phenol oxidase activity in leaves and roots of Phalaris in compared to control (Table 4-7).

The data indicated that by increasing amount of *Eucalyptus* leaf decompose ascorbate peroxidase activity enhanced in Phalaris roots and leaves. The increase was significant at 6% concentration ($p \leq 0.01$, Table 4, 5).

The results also showed that increasing concentration of *Eucalyptus* leaf extracts significantly increased ascorbate peroxidase activity in Phalaris roots (Table 6). In Phalaris leaves, the higest ascorbate peroxidase activity was observed in control while a non significant difference was observed in the different concentrations of *Eucalyptus* leaf extracts (Table 7).

DISCUSSION

The results presented in Table 1 showed that increasing the amount of decompose *Eucalyptus* leaf in soil decreased length, fresh and dry weight of Phalaris of Phalaris root and shoot. Also, application of highest concentrations of aqueous extract of *Eucalyptus* leaf (15, 30%) decreased fresh and dry weight in Phalaris root in comparison to control and 5% concentration Shahid *et al.* (2006) reported that aqueous extract of *Eucalyptus camaldulensis* showed allelopathic effect on the growth of wheat and its weeds including weeds like *Avena fatua*, *Convolvulus arvensis*, *Rumex dentatus* and *Phalaris minor*.

Under laboratory conditions, the *Enterolobium contortisiliquum* plants showed tolerance to *Eucalyptus. grandis* oil, whereas *E. camaldulensis* oil caused loss of leaves, inhibition of height and diameter growth and a concomitant decrease in effective PSII quantum yield and the reduction of photosynthetic electron-transport chains (Duarte *et al.*, 2006).

The roots of plants exposed to allelochemicals became brownish, stunted and void of root hairs. This might be due to rapid inhibiting effect on respiration of root tips, which ultimately reduced elongation (Shahid *et al.*, 2006).

Present results also indicated that root and shoot lengths of Phalaris resulting from application of 5, 15 and 30% concentrations of aqueous extract of *Eucalyptus* leaf were significantly increased. Singh *et al.* (2006) reported that biological activities of receiver plants to allelochemicals are known to be concentration dependent with a response threshold. Responses are, characteristically, stimulation at low concentrations of

allelochemicals and inhibition as the concentration increases. Similar results were reported by Peng *et al.* (2004). It seems that concentration of *Eucalyptus* extract lower enough for root length of Phalaris to exhibit a stimulated response.

Morphological effects in growth may be the secondary manifestation of primary events, caused by variety of more specific effects acting at the cellular or molecular level in the receiver plants. Moreover, the inhibitory compounds might have reduced the uptake of nutrients which ultimately reduced shoot growth (Peng *et al*., 2004). Khan *et al.* (2005) also reported that water extracts of *Prosopis juliflora* and *Eucalyptus camaldulensis* and bark of *Acacia nilotica* significantly reduced germination percentage, seedling length and biomass yield of *Ipomoea* sp., *Asphodelus tenuifolius* establishment.

Present results revealed that most of the growth parameters of Phalaris were decreased when exposed to different amounts of decompose and water extracts of *Eucalyptus* leaf. The reduction is more severe by *Eucalyptus* leaves decompose.

Effects of decompose and aqueous extract of *Eucalyptus* leaf on antioxidant enzymes activity (catalase, peroxidase, ascorbate peroxidase and poly phenol oxidase) in root and leaf of Phalaris have been showed in Table 4-7.

Poly phenoloxidase and peroxidase activity in root and shoot of Phalaris were not significantly affected by decompose and aqueous extract of *Eucalyptus* leaf. However, catalase activity in root and leaf of Phalaris was reduced by leaf decompose of *Eucalyptus* and was increased by high amount of aqueous extract of *Eucalyptus* leaf. Also, present results indicated that ascorbate peroxidase in Phalaris root was significantly increased by aqueous extract of *Eucalyptus* leaf.

In general, various types of environmental stresses (including abiotic, xenobiotic and herbicidal) mediate their impact through oxidative stress caused by generation of Reactive Oxygen Species, ROS (Blokhina *et al*., 2003). ROS, such as singlet oxygen ($^1O_2$), hydroxyl radicals (HO•) and hydrogen peroxide (H_2O_2), are highly reactive and toxic molecules that can cause oxidative damage to membranes, DNA, proteins, photosynthetic pigments and lipids (Apel and Hirt, 2004). Recently, ROS generation and related oxidative stress has been proposed as one of the modes of action of plant growth inhibition by allelochemicals. However, very little is known about the action of allelochemicals/phytotoxins in inducing ROS-mediated oxidative damage. Bais *et al.* (2003) reported that catechin, a putative phytotoxin, inhibits plant growth due to a severe oxidative burst in root tips, resulting in cell death.

Recently, allelochemicals have been proposed to cause oxidative stress in target tissue and induce an antioxidant mechanism (Li and Hu, 2005; Singh *et al*., 2006). Activities of the antioxidant enzymes Super Oxide Dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (PX) were significantly elevated, thereby indicating the enhanced generation of Reactive Oxygen Species (ROS) by allelochemicals (Apel and Hirt, 2004; Niakan *et al*., 2008). Increased levels of scavenging enzymes indicates their induction as a secondary defense mechanism in response allelopathic compounds.

α-Pinene is one of the major components of volatiles released by a wide range of species throughout the world (May and Ash, 1990; Geron *et al*., 2001, 2002; Singh *et al*., 2006; Hartikainen *et al*., 2009). α-Pinene inhibits seed germination and primary root growth in maize and disrupts energy metabolism by acting as an uncoupler of oxidative phosphorylation and inhibiting the electron transport chain (Abrahim *et al*., 2003).

To avoid the cellular damage due to ROS generation, plants produce a number of antioxidant enzymes that are induced and provide secondary protection against oxidative stress (Blokhina *et al*., 2003; Apel and Hirt, 2004). Increased ROS generation due to α-pinene is also indicated by enhanced activities of scavenging enzymes such as SOD, CAT, APX. Activity of SOD in *Cassia occidentalis* roots increased significantly in response to α-pinene compared with controls (Singh *et al*., 2006). However, excessive ROS may cause a decrease in SOD and POD activities (Mishra *et al*., 1993).

Results of this study showed that antioxidant enzymes activity in root and shoot of Phalaris were affected of aqueous extracts and decompose of *Eucalyptus* leaf. Exposure to decompose and aqueous extract of *Eucalyptus* leaf induced oxidative stress through the enhanced generation of ROS which was accompanied by change in activation of antioxidant enzymes systems. Thus, we can used of decompose and aqueous extract of *Eucalyptus* leaf as natural herbicide.

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