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Chemical Stress Induced by Heliotrope (*Heliotropium europaeum* L.) Allelochemicals and Increased Activity of Antioxidant Enzymes

[1]Kalantar Abdulghader, [2]Majid Nojavan and [1]Nabat Naghshbandi
[1]Department of Biology, Oshnaviyeh Payam Noor University,
Islamic Republic of Iran
[2]Department of Biology, IAU, (Urmia Unit), Islamic Republic of Iran

Abstract: The aims of this study were to evaluate the allelopathic potential of heliotrope on some biochemical processes of dodder. The preliminary experiments revealed that the effect of aqueous extract of leaves of heliotrope is higher than its seeds and roots. So, the aqueous extract of leaves was used in remaining experiments. Leaf extracts of 5 g powder per 100 mL H_2O inhibited the germination of dodder seeds up to 95% and that of radish up to 100%. While, the aqueous extract of vine leaves which is a non-allelopathic plant did not have any inhibitory effect on these seeds. Vine leaf was used as a control to show that the inhibitory effect of heliotrope is due to an inhibitory compound but not due to the concentration. The leaf extract of heliotrope at 0.0, 0.1, 1.0, 2, 3, 4 and 5 g powder per 100 mL H_2O reduced the radish seedling growth from 14 cm to about 0.5 cm and that of dodder from 7.5 cm to about 0.25 cm. The effects of heliotrope allelochemicals on some physiological and biochemical processes of radish was also Investigated. The activity of auxin oxidase increased in leaves and roots of radish. Suggesting that the reduced radish growth is due to the decreased active auxin levels in its leaves and roots. The activity of α-amylase was reduced, so reduction of starch degradation and lack of respiratory energy is the prime reason of germination inhibition in dodder and radish seeds. The level of soluble sugars increased. This is an indication of reduction of the activity of some respiratory enzymes and reduced consumption of these sugars. Proline levels were also increased, indicating that, the chemical stress is induced by leaf extract. Finally, the activities of GPX and CAT which are antioxidant enzymes were increased, along with increased extract concentration. These finding shows that the chemical stress induced by leaf extract produces super oxide ($O^{\bullet}_2$) and H_2O_2 which is neutralized to H_2O and O_2 by these enzymes.

Key words: Heliotrope, allelochemicals, Proline, GPX, CAT, auxine oxidase

INTRODUCTION

Allelopathy is the production of primary and secondary metabolites by allelopathic plants and releasing them into the environment for controlling the growth of neighboring plants (Putnam and Tang, 1986; Taiz and Zeiger, 2006). Allelochemicals are mostly toxic gases or organic acids (Duke, 2001), aromatic acids (Roth *et al.*, 2000; Ohno, 2001), Comarines (Blum, 1995), Quinones (Wink, 1983) and alkaloids (Corder, 1999). These compounds affect some physiological and biochemical processes of neighboring plants such as cell division (Lawrey, 1995), membrane permeability (Politycka, 1996), biosynthetic pathways of plant hormones (Einhellig, 1995), Photosynthesis (Leu *et al.*, 2002), chlorophyll biosynthesis (Zeng *et al.*, 2001) and enzyme action (Doblinski *et al.*, 2003).

The biological activity of only about 10% of 500000 plant species have been studied (Mendelson and Balick, 1995). The useful potential chemicals of remaining plants have not been worked out and brought to application stage (Mclauren, 1996).

Common heliotrope (*Heliotropium europaeum* L.) is a genus which belongs to Boraginaceae family (Diana *et al.*, 2002). This species has been known as a poisonous and medicinal plant (Mckenzie, 2001) and has some toxic effects on animals at higher dosages (Mifsud, 2004).

The allelopathic potential of heliotrope has not been studied. The aims of this study were to investigate its allelopathic effects on dodder and radish and find out the mode of action of it's allelochemicals on some physiological and biochemical processes.

Corresponding Author: Kalantar Abdulghader, Department of Biology, Oshnaviyeh Payam Noor University, Islamic Republic of Iran

MATERIALS AND METHODS

This study was conducted at fall 2003 in the plant physiology laboratory of Biology Department of Urmia University.

Seeds of radish and large seeded dodder were sterilized with 10% solution of sodium hypochlorite for 10 min and then washed with tap water and rinsed with double distilled water. Since the seeds of dodder had coat imposed dormancy, their dormancy was broken by concentrated sulfuric acid for 30 min.

The aqueous extracts of different organs of heliotrope were prepared by shaking 5 g of the powder of each organ in 100 mL of water for 24 h. Then, the solution filtered and centrifuged at 7000 g for 15 min. The supernatants were used as aqueous extracts for treatments.

In each Petri dish, 40 seeds of radish or dodder were laid on two sheets of Whatman No. 1 filter paper. Than 6 mL of each extract was added to each Petri dish. The petries were put in an oven at 25°C in the dark.

A completely randomized design was used in this experiment and each treatment was replicated six times. Distilled water was used as a control. Germination counts were done after 72 h and the length of seedlings were measured after 120 h.

Proline was measured using the Bates method (Bates *et al*., 1973). Proline standard curve was established using L-proline solutions of 0.0, 15.62, 31.25, 125, 250 and 500 μm concentrations. The absorption measurements were done with a LKB spectrophotometer at 520 nm.

Soluble sugars were measured by phenol sulfuric acid method. The standard curve was mode using glucose solutions at 0.0, 50, 100, 150, 200, 250 and 350 mg L^{-1} and the absorption measured at 485 nm.

Auxin oxidase activity was measured using salkofsky's reagent, as described by Pilet (1975). Absorption of the solutions measured at 600 nm wavelength.

α-amylase activity was measured using the method described by Robert and White house (1976). The standard curve was established by making starch solutions at 0.0, 0.5, 1.0, 1.5, 2.0, 2.0, 3.0 and 3.5 mg L^{-1} concentrations and the absorption of solutions were measured at 620 nm wavelength.

Catalase (CAT) activity was assayed by measuring the rate of disappearance of hydrogen peroxide using the method of Maehly and Chance (1959). The reaction mixture contained 2.4 mL of 50 mM phosphate buffer (pH 7.4), 0.1 mL of 1% H_2O_2 and 0.3 mL enzyme extract. The decrease in hydrogen peroxide was followed as a decline in absorbance at 240 nm.

Guaiacol Peroxidase Activity (GPX) was determined according to (Upadhyaya *et al*., 1985). The reaction mixture contained 2.5 mL of 50 mM phosphate buffer (pH 6.1), 1 mL of 1% hydrogen peroxide, 1 mL of 1% guaiacol and 20 μL enzyme extract. The increase in absorbance at 420 nm was followed for 1 min.

RESULTS AND DISCUSSION

Very little information is available on the mode of action of allelochemicals (Mendelson and Balick, 1995) and a refined understanding of the mechanism of action of these compounds is necessary for a better understanding of interactions among species in the agricultural ecosystems (Putnam and Tang, 1986).

Therefore, more must be learned about their compatibilities.

Seed germination: The effects of existing allelochemical in leaves and roots of heliotrope were examined on seed germination of dodder and radish. The effect of leaf extract was very meaningful and reduced the germination of both species close to zero percent (Fig. 1, 2). On the other hand, the effect of leaf extract was very stronger than root extract. The results indicate that, heliotrope is an allelopathic plant and the allelochemic compound is mostly found in leaves. Our findings correlate with the research results of many researchers by Einhellig (1995) and Duke (2001).

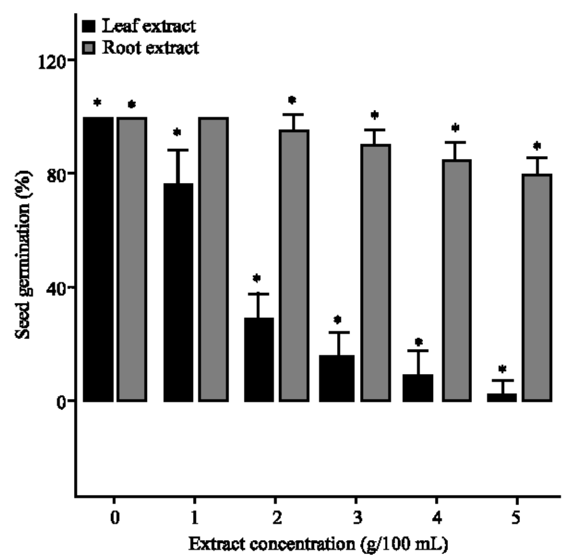

Fig. 1: The effects of leaf and root extracts of heliotrope on radish seed germination. The vertical bars are ±SE of the mean. *show significance at level of $p<0.05$

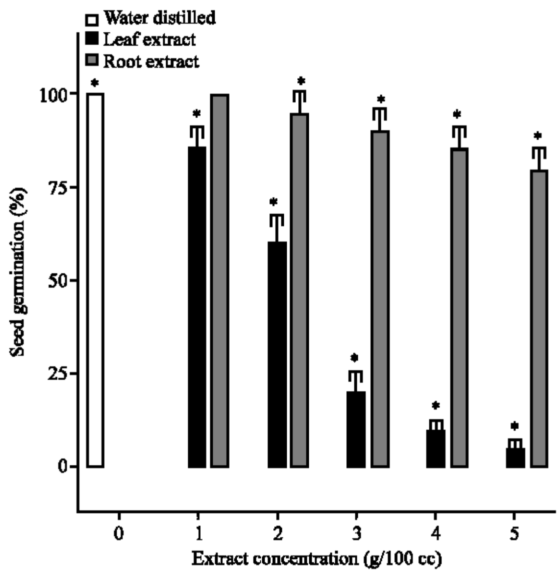

Fig. 2: The effects of leaf and root extracts of heliotrope on dodder seed germination. The vertical bars are ±SE of the mean. *show significance at level of $p<0.05$

Seedling growth: Since the effect of leaf extract was stronger, in remaining experiments we used it for treating radish and dodder. The effect of leaf extract at varying concentrations were significantly different, indicating that is reduces root and shoot growth (Table 1).

Reduction of longitudinal root and shoot growth indicates that auxin should be involved in this process (Putnam and Tang, 1986).

Proline synthesis: Normally under salt and drought stresses, the amount of proline increases dramatically. In this study, we examined the effect of allelochemical stress on proline synthesis at very low concentrations of leaf extract. With increasing extract concentration, proline increases hyperbolically (Table 2). In salt and drought stress proline regulates osmotic potential and facilitates water absorption. But its role in chemical stress is not known and needs more study.

Soluble sugars: In order to investigate the effect of leaf extract on energetic activities of the cell, the amount of soluble sugars in roots and leaves of radish were determined. The treatment concentrations were 0.0, 0.5, 1.0 and 1.5 g/100 mL H_2O. Both in roots and leaves of radish the soluble sugars increased linearly along with increased extract concentrations. This finding suggests that, some respiratory enzymes probably are blocked by allelochemicals existing in heliotrope leaves.

Table 1: Effect of leaf extracts on radish and dodder seedling growth

Leaf extract conc. (g/100 mL)	Seedling radish	Length (cm) dodder
0	11.0	7.5
1	7.5	3.5
2	2.6	1.0
3	2.1	0.8
4	0.5	0.3
5	0.0	0.2

Table 2: The effect of heliotrope leaf extract on proline synthesis in radish seed

Leaf extract concentration (g/100 mL)	0	0.5	1.0	1.5
Proline ($\mu g\ g^{-1}\ dw^{-1}$)	37.5±14.0	75.1±20.0	120.0±13.5	120.0±5.0

The data are the mean of treatments±SE of the mean ($\mu g\ g^{-1}\ dw^{-1}$)

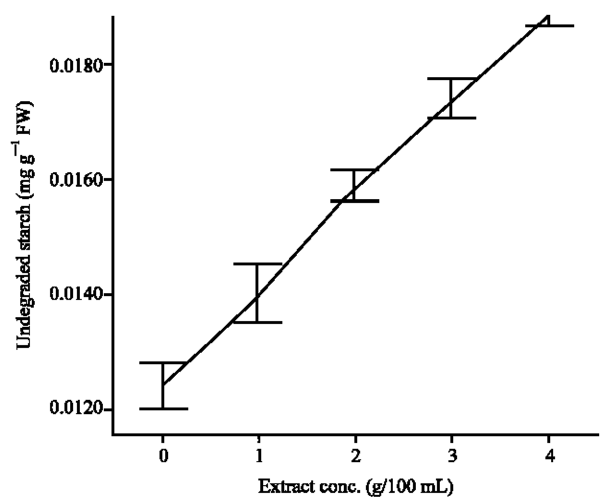

Fig. 3: Effect of leaf extract on α-amylase activity. The vertical bars are ±SE of the mean

Present finding of reduced root and shoot growth along with increased soluble sugar levels suggests for an imposed respiratory metabolism stress as an action mechanism by heliotrope allelochemicals.

α-amylase activity: The activity of α-amylase decreased linearly in response to increased extract concentration (Fig. 3). Decrease of the activity of this enzyme decreases starch degradation into glucose and hence the availability of metabolic energy to seed germination. This finding correlates with reduced germination of radish and dodder seeds. There are many reports that some allelochemics reduce growth by binding to GA or reduce Amylase synthesis or block its secretion from aleurone layer to the endosperm (Putnam and Tang, 1986).

Auxin oxidase activity: With respect to decreased longitudinal root and shoot growth by leaf extracts of

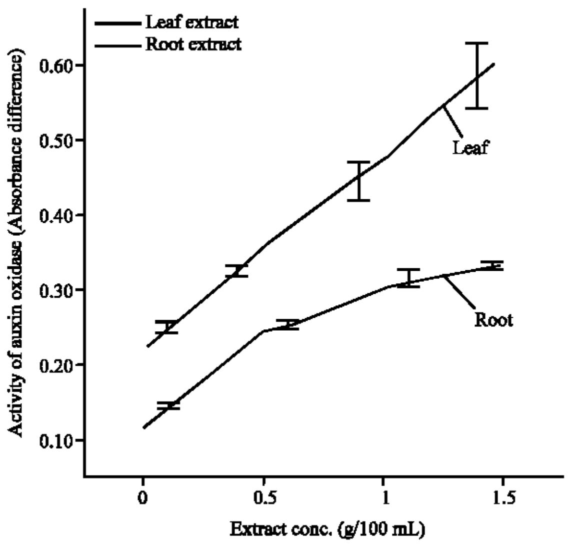

Fig. 4: The effect of heliotrope leaf extract on the auxin oxidase activity. The vertical bars are ±SE of the mean

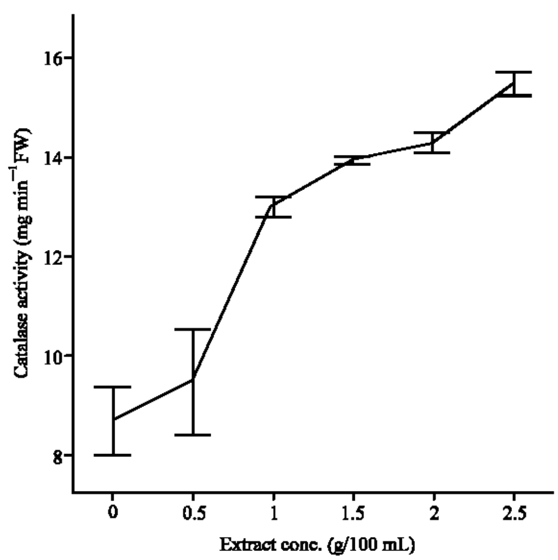

Fig. 5: The effect of leaf extract of heliptrope on catalase activity in radish seeding. The vertical bars are ±SE of the mean

heliotrope, measuring the auxin oxidase activity was a good criterion for understanding the mechanism of this process. The activity of auxin oxidase increased by increasing the concentration of heliotrope leaf extract from 0.0 to 1.5 g/100 mL H_2O (Fig. 4).

By increased activity of this enzyme, the suitable auxin level necessary for longitudinal root and shoot growth decreases. This finding justifies our data obtained in Table 1. Our data agrees with the findings of (Malik, 1997; Hoof-Wooter *et al.*, 1996).

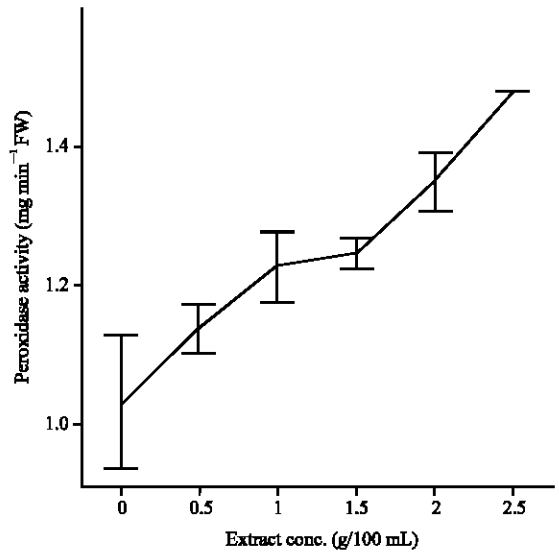

Fig. 6: The effect of the heliotrope leaf extract on guaicacol peroxidase activity in radish seedlings. The vertical bars are ±SE of the mean

Catalase (CAT) and guaicacol peroxidase (GPX) activities: Reactive Oxygen Species (ROS) such as $O^{\cdot}_2$, OH^- and H_2O_2 are produced under stress conditions in most plant species (Dierk and Claus, 2002). The ROS causes lipid peroxidation and DNA damage (Xunzhong and Richard, 2002). So, the toxic levels of ROS must be controlled in plants. There are antioxidant enzyme systems in plants that detoxify the ROS and block oxidative damage to cells (Dierk and Claus, 2002). To examine if heliotrope leaf allelochemical induces oxidative stress in radish or not, we measured the activities of CAT and GPX. The activities of both enzymes increased by increasing leaf extract concentrations from 0.0-2.5 g/100 mL H_2O (Fig. 5, 6). Increased activities of CAT and GPX are an indication of increased H_2O_2 production in radish seedlings under chemical stress induced by leaf allelochemics of heliotrope.

CONCLUSION

The results of our experiments show that, common heliotrope is not only a medicinal at higher dosages a toxic plant to mammals, but also an allelopathic plant. It is potentially an inhibitor of seed germination and seedling growth. We propose that, the active compound that has allelopathic potential and herbicidal activity to be isolated and characterized in the future.

The possible use of allelochemicals to defend crop plants against insects, nematodes, deseases and weeds are recently receiving considerable attention. In the near future allelochemics will be used as repellants, antifeedants and weed killer. On the other hand allelopathic plants will be cultivated in rotation with other crops for weed seed decay and stimulation or inhibition of weed seed germination.

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