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Full Length Research Paper

Efficiency of allelopathy of sunflower (*Helianthus annuus* L.) on physiology of wheat (*Triticum aestivum* L.)

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Pot-grown seedlings of wheat cvs. Margalla 99 and Chakwall 97 were treated with water mixed with extracts of sunflower leaves, stems and roots at 20 days after the seedlings emerged. Data on physiology of wheat seedlings including protein, proline, sugars, DNA, peroxidase, superoxide dismutase and chlorophyll contents were recorded; all increased with allelochemicals treatments when compared with controls.

Key words: Allelopathy, chlorophyll contents, deoxiribonucleic acid, peroxidase, protein, proline, sunflower, sugar, superoxide dismutase, wheat.

INTRODUCTION

There is increasing emphasis on sustainable agriculture and concerns about the adverse effects of extensive use of synthetic chemicals, such as contamination of the environment, greater plant resistance to herbicides and high costs. Consequently, research attention is now focused on reducing the dependence upon synthetic herbicides and finding alternative strategies for weed management. Allelopathy is one promising strategy, which can be put to good use in several ways in agro-ecosystems. Sunflower (*Helianthus annuus* L.) in general and cv. Hysun 38 in particular is increasingly recognized as an important crop in several areas of Islamabad, (Pakistan) given the suitability of the crop to local agroclimatic conditions, its importance as source of edible oil and protein, resistance to drought and its short duration, which makes it a suitable crop if sowing is delayed. However, yields of some crops following sunflower are lower than normal, possibly because of inadequate nutrition and chemical inhibition. Sunflower is often grown when rainfall is marginal and depletion of soil moisture by sunflower may be a factor, although this remains unproven. Both sunflower and the crops that follow it receive routinely specified amounts of fertilizers and there is no evidence that nutrient deficiency is the cause of lower yields. Sunflower is known to actively influence the growth of surrounding plants because of its high allelopathic potential and > 200 natural allelopathic compounds have been isolated from sunflower cultivars. Most of these known allelochemicals affect seed germination (Wardle et al., 1991). Wheat (*Triticum aestivum* L.) is the staple food of Pakistan and wheat straw an integral part of the daily ration of livestock. Various factors lower the productivity of wheat, such as delayed sowing, inadequate doses of fertilizers, water shortage, non-availability of improved seed, diseases and drought and now weed infestation has emerged as a serious problem. Weeds compete with the crop for nutrients, water, space, light and carbon dioxide and also interfere with its normal growth by secreting biomolecules into the rhizosphere. Thus, the objective of the present study was to assess the allelopathic effects of sunflower on contents of chlorophyll, protein, proline, deoxyribonucleic acid, sugar, superoxidase dismutase (SOD) and peroxidase (POD) in wheat.

MATERIALS AND METHODS

Sunflower plants (cv. Hysun 38) were grown in pots in the Department of plant sciences, Quaid-i-Azam University, Islamabad. 3 seeds were sown in each pot and were given a basal fertilizer dose of 2 g diammonium phosphate, 1 g urea, and 1 g potash. When the plants reached the vegetative stage (40 d after sowing), they were uprooted and separated into leaves, stems and roots. They were

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then washed thoroughly with distilled water, dried, pulverized in a mill and stored in a cool place along with anhydrous $CaCl_2$ to maintain dryness. Allelopathic extracts of sunflower at different concentrations were prepared as described by Bogatek et al. (2005). The solution was centrifuged at low speed (3000 rpm) for 15 min and the supernatant filtered through one layer of Whatman No. 42 filter paper. The extracts were stored at < 5°C until use. The extracts were determined to be free of fungal contamination. Seeds of 2 wheat varieties, cvs. Margalla 99 and Chakwall 97, were sown in pots (3 seeds per pot) and a basal fertilizer dose of 1 g urea and 1 g diammonium phosphate applied to soil in each pot at the time of sowing through either plain water (control) or water mixed with extracts of sunflower leaves, stems or roots (1 g of extract mixed with 9 ml of water). Contents of chlorophyll, protein, proline, sugar, SOD and POD were determined. The experiment had 3 replications.

Leaf protein contents

Protein contents of leaves was determined following the methods of Lowery et al. (1951) using BSA as the standard. Phosphate buffer (Stock solution), Monobasic sodium phosphate (27.6 g) was dissolved in 1000 ml of distilled water.

Chlorophyll content of leaves

The fresh leaves of wheat were collected at 50% flowering for extraction of chlorophyll. The chlorophyll estimation of leaves followed the method of Arnon (1949) and Kirch (1968). The crude preparation (1 ml) was mixed with 4 ml of 80% (v/v) acetone and allowed to stand in the dark at room temperature for 10 min. It was centrifuged at 2000 rpm for 5 min to clear the suspension. The supernatant, which contained soluble pigment, was used to determine chlorophyll. Absorbance of the solution was read at 645 nm for chlorophyll a and at 663 nm for chlorophyll b on a spectrophotometer against 80% (v/v) acetone blank. Total chlorophyll was determined with the equation of Arnon (1949). Total chlorophyll (mg/l) = (20.2 × a 645.A_{645}) + (8.02 × b 663.A_{663})

Sugar content of leaves

Sugar content of wheat leaves at flowering stage was estimated by the method of Dubo et al. (1956) as modified by Johnson et al. (1966). Fresh plant material (0.5 g) was homogenized with 10 ml of distilled water in a clean mortar. It was centrifuged at 3000 rpm for 5 min. Then to 0.1 ml of supernatant, 1 ml of 5% (v/v) phenol was added. After 1 h incubation at room temperature, 5 ml of concentrated H_2SO_4 was added. The absorbance of each sample was recorded at 420 nm. The concentration of unknown samples was calculated with reference to a standard curve made using glucose.

Proline content of leaves

Proline content of leaves was estimated at flowering stage by using the following method (Bates et al., 1973). Fresh plant material (0.1 g) was homogenized with 5 ml of 3.0% sulfosalicylic acid in a mortar. Samples were centrifuged at room temperature at 3000 rpm for 5 min. Supernatant was adjusted to 5 ml with distilled water, to which 5 ml of glacial acetic acid and 5 ml of ninhydrin (1.25 g of ninhydrin dissolved in a solvent prepared by mixing 30 ml of glacial acetic acid, 8 ml of orthophosphoric acid and 12 ml distilled water). The reaction mixture was shaken and the contents placed in tubes and heated in a boiling water bath for 1 h. Then tubes were cooled and the mixture extracted with 10 ml of toluene in a separating funnel. The absorbance of the toluene layer was recorded at 520 nm.

A standard curve was constructed from a dilution series of 10 - 100 µg of proline at increments of 10 µg. The concentration of the unknown sample was calculated with reference to the standard curve.

Peroxidase of leaves

To 3 ml of assay buffer (20 mM mixed phosphate buffer, Ph 7.0-7.5 at 30°C) was added 100 µl of 100 mM guaiacol and 300 µl of partially purified enzyme preparation. The mixture was incubated for 1 min at 30°C and the reaction initiated by addition of 1 µmol H_2O_2 (30% v/v). Activity was measured colorimetrically at 436 nm and calculated using an extinction coefficient of 6.39 mM/cm for the guaiacol dehydrogenation product (Putter, 1974).

SOD of leaves

SOD (EC 1. 15. 1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971) taking into account the notes by Beyer and Fridovich (1987). Leaf samples were homogenized in 4 volumes (w/v) of an ice-cold buffer containing 0.1 M Tris-HCL (pH 7.8), 0.1 mM EDTA and 0.05% Triton X-100. The homogenates were filtered through 4 layers of cheesecloth and centrifuged at 4°C for 30 min at 15,000 rpm. The crude extracts were dialyzed for 24 h against half-strength extraction buffer without Triton X-100, centrifuged at 4°C for 30 min at 15,000 rpm and the supernatants were used for SOD assay. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.053 mM NBT, 10 mM methionine, 0.0053 mM riboflavin and an appropriate aliquot of enzyme extract. The reaction was started by switching on the light and allowed to run for 7 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction as monitored at 560 nm.

DNA analysis

Total DNA was extracted according to Gumin and estimated using an UV spectrophotometer (Spectrophotometer 601) as adopted by Ogur and Rosen genomic DNA was extracted from 60 mg of frozen tissue (young leaves ground in liquid nitrogen) using Genomicprep cells and tissue DNA Isolation Kit (Amersham Pharmacia Biotech Inc.) according to the instruction manual. The DNA concentration in samples was measured by UV-absorption spectroscopy at 260 nm at which DNA gives a maximum absorption at a concentration of approximately 50 µg/g of double-stranded DNA. DNA was treated with 4 restriction enzymes (Bam HI, EcoRI, HinfI and SmaI at 5 U per reaction) according to manufacturer's instructions. The reaction mixture was incubated for 1 h at 37°C except SmaI (25°C). The restriction fragments were size-fractioned using 2% agarose gel electrophoresis. The agarose gels were stained with ethidium bromide and photographed under UV light. The results were documented by Image analyzer gel Doc 2000 (Bio Rad).

Statatistical analysis

The data were analyzed statistically using MStatC. A completely randomized design was followed.

RESULTS

Sugar contents of wheat

The maximum sugar contents for cv- Margalla 99 were in

Table 1. Effect of sunflower leaf, stem and root extracts on sugar contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	182.0 B	177.3 B
T2	192.3 A	185.0 A
T3	174.0 C	174.0 B
T4	185.7 B	176.7 B

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

Table 2. Effect of sunflower leaf, stem and root extracts on protein contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	1732 B	1613 C
T2	1781 A	1663 A
T3	1732 B	1630 BC
T4	1764 A	1651 AB

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

Table 3. Effect of sunflower leaf, stem and root extracts on proline contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	112.0 C	102.0 C
T2	126.0 A	112.0 A
T3	118.0 B	106.0 B
T4	124.3 A	109.3 A

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

T2 followed by T4, T1 and T3 (Table 1). For cv. Chakwall 97 the highest sugar contents were for T2 followed by T1, T4 and T3.

Protein contents of wheat

The maximum protein contents were for T2 followed by T4, T3 and T1 (Table 2) in 2 wheat varieties (Margalla 99 and Chakwall 97).

Table 4. Effect of sunflower leaf, stem and root extracts on deoxiribonucleic contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	497.3 C	490.0 B
T2	516.0 A	498.3 A
T3	504.0 BC	492.0 B
T4	512.0 AB	497.7 A

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

Table 5. Effect of sunflower leaf, stem and root extracts on chlorophyll contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	92.00 B	81.67 B
T2	95.33 A	83.00 A
T3	94.33 A	80.00 B
T4	94.67 A	82.33 A

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

Proline contents of wheat

The proline contents of wheat were affected by the sunflower extracts. Maximum proline contents were for T2 (which was similar to T4), followed by T3 and T1 (Table 3).

DNA in wheat

The maximum DNA contents were for T2 followed by T4, T3 and T1 (Table 4).

Chlorophyll contents

The maximum chlorophyll contents were for T2 followed by T4, T3 and T1 in cv- Margalla 99. For cv. Chakwall 97 the maximum chlorophyll contents were in T2 followed by T4, T3 and T1 (Table 5).

SOD contents of wheat

For cv. Margalla 99, the maximum SOD content was from

Table 6. Effect of sunflower leaf, stem and root extracts on peroxidase contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	11.67 B	10.00 C
T2	18.00 A	15.00 A
T3	14.00 B	12.67 B
T4	16.67 A	14.00 AB

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

Table 7. Effect of sunflower leaf, stem and root extracts on superoxidase dismutase contents of wheat seedlings 30 days after sowing (wheat varieties Margalla 99 and Chakwall 97).

Treatment	Margalla 99	Chakwall 97
T1	4.00 B	3.000 C
T2	11.00 A	8.670 A
T3	9.000 A	5.000 B
T4	10.00 A	7.670 A

Values followed by the same letter within a column are not significantly different.
T1, Distilled water; T2, leaf extract (1 g + 9 ml distilled water); T3 stem extract (1 g + 9 ml distilled water); T3 root extract (1 g + 9 ml distilled water).

T2 followed by T4, T3 and T1. For cv. Chakwall 97, the maximum SOD was for T2 followed by T4, T3 and T1 (Table 6).

POD contents of wheat

The maximum POD contents for cv. Margalla 99 were from T2 followed by T4, T3 and T1. For cv. Chakwall 97, the maximum POD was in T2 followed by T4, T3 and T1 (Table 7).

DISCUSSION

Studies of the intensity of carbon dioxide assimilation (an integral characteristic of photosynthesis) are very important. As a rule allelochemicals cause water deficiency and suppress photosynthetic assimilation of carbon dioxide, the extent of inhibition, however, differs with species and variety, being dependent on the amplitude of water balance decrease and plant age. The 2 wheat varieties used are stress tolerant varieties and so produced a different result, the decrease in the rate of photosynthesis caused by allelochemicals was not pronounced in these varieties, but weeds were affected by allelochemicals. Increased intensity chlorophyll contents in treatment treated with extract from leaves followed by that from roots and then stems was due to control of weeds in these treatments compared with controls, because weeds compete with the crop for light, water, shelter and nutrients. The chlorophyll contents were increased in the leaf-extract treatment followed by roots and stems. This was due to leaves of sunflower extracts having more allelochemicals followed by roots and stem, thus they have ability to control more weeds. This is possibly why chlorophyll contents increased in leaf-extract treatment followed by root extracts and stem extracts. Application of sunflower water extract suppressed weeds (Rice, 1974) and there are more allelochemicals in leaves, followed by roots and stems (Rice, 1984). So increased chlorophyll contents in leaf extracts were possibly due to control of weeds. The phenolics cause reduction in chlorophyll contents.

The effect of allelochemicals (e.g vanillic, ferulic, alkaloids and benzoic acid on DNA has been found by various workers. The affect of allelochemicals on weed control also caused increased protein contents. Activity levels of SOD and POD showed progressive significant increases with increased allelochemicals, compared to controls. This is likely related to stress, because allelopathy causes stress. Receiver-plants as well as donor-plants are active during the stress conditioned. The results agree with those of Fridovich (1986), who observed that stress increased the activities of leaf mitochondrial Mn-SOD and chloroplastic Cu/Zn-SOD. These are considered the primary scavengers in the detoxification of reactive oxygen species in plants and convert superoxide to H_2O_2 and O_2 and protect cells against superoxide-induced oxidative stress. The present results support the view that SOD and POD activity increased with increased allelochemicals. There were greater sugar contents in leaf-extract treatments, followed by root extract and the least effect was from stem extract. Additionally, cv. Margalla 99 was better than that of cv- Chakwall 97.The increased sugar contents in T2, T4 and T3 compared to T1 may be due to control of weeds. Varieties may differ in their sugar contents and be influenced by management and environmental conditions. Sugar is the major storage form of photoassimilate in leaves (Koch, 1996). Stimulation of enzyme synthesis for mobilization of seed reserves in germinating grains is thought to be caused by gibberellic acid, which is also known to stimulate the growth of intact plants (Salisbury and Ross, 1992; Arteca, 1995; Johri and Mitra, 2001). Allelochemicals increased proline accumulation in leaves and ABA is also known to enhance accumulation of proline in leaves. Thus the increased proline may be due to abscisic acid content, from the effect of allelochemicals.

Conclusions

Allelopathy of sunflower leaves increased contents of protein, proline, sugar, chlorophyll, peroxidase and

superoxidase dismutase in wheat. The leaf extracts were more effective than root extracts and the least effect was for stem extracts when compared with controls with no sunflower extracts.

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