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Full Length Research Paper

Potential allelopathic effects of sunflower (*Helianthus annuus* L.) on microorganisms

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Potential allelopathic effects of sunflower (*Helianthus annuus* L.) cultivar Hysun 38 on *Rhizobium*, *Azospirillum*, and phosphate-solubilizing bacteria (PSB) were studied by applying oxidase, QTS tests and Gram staining. Microorganisms for the control group were isolated from soil sampled before sowing sunflower. A commonly observed effect of allelochemicals was a decrease, compared to control, in the number of colonies of the three groups of microorganisms, isolated from the roots of sunflower in the case of *Azospirillum* and PSB and from the rhizosphere of sunflower in the case of *Rhizobium*. Isolates of *Azospirillum* and PSB from the rhizosphere of sunflower differed in their response: the number of colonies of *Azospirillum* remained the same as that in control but that of PSB was greater than that in control. QTS tests showed a range of responses, which varied with the group of microorganisms as well as with the habitat from which they were collected, namely roots of sunflower, rhizosphere of sunflower, and control. Allelopathy also influenced the results of Gram staining test but not those of the oxidase test.

Key words: Allelochemicals, sunflower, soil, *Azospirillum, Rhizobium,* phosphate-solubilizing bacteria.

INTRODUCTION

Allelopathy, as described by Hans Molisch, a German scientist who also coined the term in 1937, refers to all biochemical interactions (stimulatory and inhibitory) among plants, including microorganisms. The International Allelopathy Society defines allelopathy as any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of agriculture and biological systems (Anonymous, 1996).

The International Rice Research Institute (IRRI) and countries such as Japan, Egypt, and Korea have been actively studying the phenomenon. Putnam and Weston (1986) noted that substances with allelopathic potential are present in virtually all plant tissues including stems, leaves, roots, and seeds. These substances are released through such processes as volatilization, root exudation, leaching, and decomposition of plant residues. Allelochemicals are known to affect uptake of minerals by altering the function of cellular membrane in plant roots.

Sunflower, because of its high allelopathic potential, can actively influence the growth of surrounding plants, and more than 200 natural allelopathic compounds have been isolated so far from different cultivars of sunflower. Since Rice (1984) reported that pathogens also affect the amount of allelochemicals produced by plants, the objective of this study was to check the potential allelopathic effect of sunflower on microorganisms.

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MATERIAL AND METHODS

Collection of soil samples

Soil from the pots in which sunflower plants were to be grown was sampled twice: before sowing to serve as control samples and 35 days later, at the time of uprooting the plants, to serve as samples of the rhizosphere. Root samples were also collected from uprooted 35-day-old plants. Three replicates of soil samples were collected.

Isolating *Rhizobium*

To isolate *Rhizobium* from the rhizosphere soil, 1 g soil was suspended in 9 ml distilled water. The suspension was serially diluted, 20 µl each from three dilutions (10^{-1}, 10^{-5}, and 10^{-10}) transferred to tryptone medium, and incubated at 30°C. Pure cultures were obtained by streaking the initial culture on tryptone

agar tryptone medium was initially liquid but solidifies in Petri dishes after pouring and single colonies of *Rhizobium* were obtained by streaking the initial culture on tryptone agar and repeated subculturing as required repeated subculturing as required.

Isolating *Azospirillum*

Azospirillum was isolated from roots and soil samples collected before sowing, and from the rhizosphere soil. Serial dilutions were prepared as described above, 100 µl each from the three dilutions (10^{-1}, 10^{-5}, and 10^{-10}) were transferred to vials containing nitrogen-free medium (NFM), and the vials incubated at 30°C for 48 h. Vials that showed growth of *Azospirillum* were used for obtaining pure cultures on NFM plates. Single colonies on these plates were transferred to NFM as well as on NFM agar slants for further study. To isolate *Azospirillum* from roots, 1 g samples of roots were crushed in 9 ml distilled water. The resulting suspension was centrifuged at 3000 rpm for 15 min and 100 µl of the supernatant transferred to vials containing NFM. Pure cultures were obtained as described above.

Isolating phosphate-solubilizing bacteria

Phosphate-solubilizing bacteria (PSB) were isolated from each sample by serial dilution and plating. One gram soil was suspended in 10 ml sterile distilled water and thoroughly shaken, and serial dilutions were made from this suspension. For plating, 0.1 ml each from four serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) was removed with a micropipette and plated on different media specific to each microorganism to be isolated. After incubation, the total number of colonies formed was counted. Total count was determined as colony forming units (CFU):

CFU g^{-1} of moist soil = mean plate unit / (volume of sample plated) (dilution factor).

Phosphate-solubilizing bacteria mainly found in the rhizosphere were enumerated by using the specific media recommended for *Bacillus* and for *Pseudomonas* both PSB were isolated on Pikovskayas medium Phosphate- solubilizing bacteria mainly found in the rhizosphere were enumerated by using the specific media recommended for Bacillus and Pseudomonas: both PSB were isolated on Pikovskaya's medium Pikovskaya's agar (Pikovskaya 1948) containing insoluble tricalcium phosphate.

Purification of microorganisms

Colonies that had developed on each specific medium were picked with a sterilized inoculating wire loop and streaked on separate plates. All plates were then incubated at the specific combinations of temperature and duration specific for each group, as follow: *Rhizobium* was incubated at 37°C for overnight; *Pseudomonas* at 20°C for 24 h; and *Bacillus*, 37°C for 48 h.

Identification of bacterial isolates

Colony and cell morphology

The isolated cultures of bacteria were identified on the basis of the morphology of colonies and cells and biochemical tests. Bacterial isolates from cultures grown overnight in LB broth were spread on plates of the same medium. Morphology of the colonies (color and shape) was noted after 24 h. To study cell motility and shape, single colonies from the agar plates were transferred onto glass slides, mixed with a drop of sterile water, and observed under a light microscope (Nikon, Japan).

Gram staining

Slides of the isolated and purified bacterial cultures were prepared for Gram staining by the Vincent's method (1970). A drop of bacterial culture was taken and a thin smear prepared on a glass slide. The smear was air-dried, heat-fixed, stained with crystal violet for one min, lightly washed with distilled water, flooded with iodine solution for one min, and decolorized with 95% ethanol for one min. The smear was washed with distilled water, counterstained with safranin, washed with distilled water again, air-dried, and observed under a light microscope (Nikon, Japan) at ×100 magnification using an oil immersion objective.

Oxidase test

Oxidase test was performed to determine the presence of oxidase in the bacterial isolates (Steel, 1961). Kovac's reagent (1% N, N, N.N-tetramethyle-p-phenylene diamine; Kovac, 1956) was dissolved in warm water and stored in a dark bottle. Strips of filter paper were dipped in this reagent and air-dried. With the help of a sterile wire loop, 1-day-old colonies of bacterial cultures from agar plates were transferred on the strips of filter paper. Oxidase-positive colonies turned lavender, and then dark purple to black within five min. The oxidase test was performed on the strains of *Azospirillum* and *Rhizobium*.

Catalase test

Catalase test was performed to detect the presence of catalase in the colonies of both *Rhizobium* and *Azospirillum*. Bacterial colonies (24 h old) were taken on glass slides and one drop of H_2O_2 (30%) was added. Appearance of gas bubbles indicated the presence of catalase (MacFaddin, 1980).

Miniaturized identification System QTS 24

Physiological and biochemical tests were performed using the QTS 24 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan). Bacterial cultures (24 h old) grown on LB plates were suspended in saline (0.85% NaCl) and transferred to QTS kits. *Rhizobium* was grown on YMA.

RESULTS AND DISCUSSION

Isolation, identification, and characterization of microorganisms

The results of Gram staining of bacterial isolates from sunflower roots, rhizosphere soil, and control soil is shown in Table 1. For characterization of the isolates, 24-h-old bacterial cultures were tested using microbial identification kits (QTS 24) and the results recorded following overnight incubation at 30°C. The QTS results showed that PSB from each of the three habitats differed in their response to the following tests: VP, Gel, $GluNO_3$, Mald, Suc, Inos, Ado, Mel, and Rae. Isolates of PSB from the control and rhizosphere soils tested positive for VP whereas those from roots tested negative. In the case of

Table 1. Results of Gram staining of bacterial isolates from sunflower roots, rhizosphere soil, and control soil.

Treatment	*Rhizobium*	*Azospirillum*	Phosphate-solubilizing bacteria
Control soil	* *	* *	* *
Rhizosphere soil	* *	*	* *
Roots	*	*	*

Table 2. Morphological and biochemical characteristics of bacterial isolates from sunflower roots, rhizosphere soil, and control soil.

Test	Phosphate-solubilizing bacteria (C)	Phosphate-solubilizing bacteria (SF)	Phosphate-solubilizing bacteria	*Rhizobium* (C)	*Rhizobium* (SF)	Az. (C)	Az. (SF)	Az.
Source	Soil	Soil	Root	Soil	Soil	Soil	Soil	Root
Cells			Rods	Rods	Rods			
ONPG	+	+	+	+	+	+	+	+
CLT	-	-	-	-	-	-	-	-
MALO	+	+	+	+	+	+	+	+
LDC	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-
ODC	-	-	-	+	-	-	-	-
H_2S	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-
TDA	+	+	+	+	+	+	+	+
IND	-	-	-	-	-	-	-	-
VP	+	+	-	+	-	+	+	-
Gel	+	-	-	+	-	-	+	-
$GluNo_3$	+	-	-	+	-	+	-	+
Mald	+	-	-	-	-	-	-	-
Suc	-	-	-	-	-	-	+	-
Mann	+	+	+	+	-	+	-	+
Arab	-	-	-	-	-	-	+	-
Rham	+	+	+	+	+	+	+	-
Sorv	+	+	+	+	+	+	+	
Inos	+	-	-	+	-	-	-	-
Ado	+	-	-	-	-	-	-	-
Mel	+	-	-	-	-	-	-	-
Rae	+	-	-	-	-	-	-	-

Microbial identification kits (QTS 24, DESTO Laboratories, Karachi, Pakistan) were used for the biochemical tests. For the tests, 24-hour-old bacterial cultures were used and results recorded after 18 h of incubation at 30°C. ONPG = Ortho nitro phenyl β-D-galactopyranoside, CIT = sodium citrate, MALO = sodium malonate, LDC = lysine decarboxylase, ADH = arginine dihydrolase, ODC = ornithine decarboxylase, H_2S = H_2S production, URE = urea hydrolysis, TDA = tryptophan deaminase, IND = indole, VP = Voges Proskauer = Acetion, GEL = gelatine hydrolysis, GLU = acid from glucose, MAL = acid from maltose, SUC = acid from sucrose, MAN = acid from mannitol, ARA = acid from arabinose, RHA = acid from rhamnose, SOR = acid from sorbitol, INO = acid from inositol, ADO = acid from adonitol, MEL = acid from melibiose, RAE = acid from raffiose
C = Control soil, SF = Sunflower rhizosphere soil, and AZ = *Azospirillum*.

Rhizobium, QTS results showed marked differences between isolates from the control soil and those from the rhizosphere soil with respect to ODC, TDA, Ind, VP, Gel, Arab, Rha, Sor, $GluNO_3$, Mal, Suc, Inos, Ado, Mel, and Rae. In the case of *Azospirillum* also, isolates from different habitats differed in their responses: cultures from roots tested negative for VP, Gel, and Arab whereas those from soil (control and rhizosphere) tested positive. With $GluNo_3$, Mann, and Sor, the results were mixed: isolates from roots and rhizosphere soil tested negative whereas those from the control soil tested positive (Table 2).

Table 3. Results of oxidase test of bacterial isolates obtained from sunflower roots, rhizosphere soil, and control soil.

Treatment	*Rhizobium*	*Azospirillum*	Phosphate-solubilizing bacteria
Control soil	+	+	+
Rhizosphere soil	+	+	+
Roots	+	+	+

Table 4. Number of colonies of *Rhizobium, Azospirillum*, and phosphate-solubilizing bacteria from sunflower roots, rhizosphere soil, and control soil.

Treatment	*Rhizobium*	*Azospirillum*	Phosphate-solubilizing bacteria
Control soil	4.95×10^6	2×10^6	1.20×10^6
Rhizosphere soil	4.0×10^6	2×10^6	1.25×10^6
Roots	------	1×10^6	1.0×10^6

Oxidase rest

Azospirillum isolates from the control soil showed more intense oxidase reaction than that shown by isolates from the other two habitats, namely roots and rhizosphere. Within the two, the reaction was more intense in isolates from rhizosphere. *Rhizobium* isolates showed considerable variation in their response to the oxidase test (Table 3).

Number of colonies

The number of *Rhizobium* colonies was greater in soil samples from the control soil than in those from the rhizosphere soil (Table 4). These results are in agreement with the general observation that plants that produce allelochemicals reduce the population of *Rhizobium*.

The number of *Azospirillum* colonies was the highest in samples from the control soil, followed by those from the rhizosphere soil and from roots, in that order (Table 4).

The number of colonies of PSB showed the same pattern as that shown by Azospirillum colonies: the highest in the control soil, followed by that in rhizosphere soil and roots, in that order. These results agree with those obtained by Rice (1984), who maintains that allelopathy reduces the availability of phosphorus, and therefore the number of PSB (Table 4).

Conclusions

From the experiments it was concluded that sunflower roots lowered the number of colonies of the groups of microorganisms studied, namely *Azospirillum*, PSB, and *Rhizobium*. The effects of the three habitats were also apparent in Gram staining, QTS, and oxidase tests.

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