Isolation of Allelopathic Substances in Rice Seedlings

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Abstract: In order to evaluate the allelopathic potential of the early developmental stage of rice plants (Oryza sativa L.), a search for allelopathic substances was undertaken in acetone extract of 10-d-old rice seedlings. Two growth-inhibiting substances were found in the neutral fraction of the extract after silica gel column chromatography, and one substance was further purified by thin layer chromatography, C18 Sep Pack cartridge and HPLC. The purified substance inhibited the growth of lettuce (Lactuca sativa L.) at concentrations greater than 0.3 μg mL⁻¹. These two substances may be released into the environment, either as exudates from living tissues or leachates from residues of the plant, and act as allelochemicals to other plants, which should be investigated further in the laboratory and the field.

Key words: Allelopathy, Growth inhibition, Lactuca sativa, Oryza sativa, Phytotoxicity.

As the use of man-made chemicals increases throughout the world, agricultural weed-control alternatives to the present commercial herbicide dominated programs are now being given wide consideration because of the concerns about natural environment as well as human health (Duke, 1986; Einhellig, 1996; Olofsdotter, 1998). Synthetic chemical herbicides may continue to be a key component in most integrated weed management systems, but controlling weeds through allelopathy is one strategy to reduce herbicide dependency (Duke, 1986; Putnam, 1988; Einhellig, 1996; Seigler, 1996).

Substances with allelopathic activity are present in many plants and in many organs, including leaves, flowers, fruits, and buds (Putnam and Tang, 1986; May and Ash, 1990; Mahall and Callaway, 1991; Inderjit, 1996). Chou and Lin (1976) reported that aqueous extracts of decomposing rice residues in waterlogged soil inhibited the root growth of lettuce seedlings. Common putative allelochemicals, such as p-hydroxybenzoic, vanillic, ferulic, p-coumaric, and p-hydroxyphenylacetic acids, were found in aqueous extracts of rice residues or straw (Kuwatsuka and Shindo, 1973; Chou and Lin, 1976; Chou et al., 1991).

Dekker and Megitt (1983) suggested that weed-control research should be concentrated on allelopathy during early developmental stages of crop plants, since most allelochemicals were released during germination and early growth of plants. However, the information about the allelopathic potential of germinating seeds of rice is limited. Thus, the objective of this research was to evaluate the allelopathic potential of neutral substances obtained from rice seedlings by using a Petri dish bioassay under laboratory conditions.

Materials and Methods

1. Plant material

Seeds of rice (Oryza sativa L. cv. Nipponbare) were surface sterilized in an aqueous solution of 25 mM sodium hypochlorite for 15 min, rinsed in distilled water for four times and allowed to germinate on two sheets of moist filter paper (No 1; Toyo Ltd., Tokyo) in the dark at 25°C. After three d, uniform germinating seeds were transferred onto two sheets of moist filter paper in trays at 25°C in a daily cycle of 12-h photoperiod. Light was provided from above with two white fluorescent lamps (3.2 W m⁻² at plant level; FL-20S, 20 W, National, Tokyo). After 7 d, the seedlings were harvested, rinsed with distilled water, immediately frozen in liquid nitrogen, and stored at -80°C until extraction.

2. Extraction

Frozen seedlings were homogenized in four volumes of 70% (v/v) cold aqueous acetone and the homogenate was filtered through filter paper (No. 1). The residue was homogenized again, by using the same solvent as in the first extraction, and filtered. The two filtrates were combined and evaporated in vacuo at 35°C to give an aqueous residue. The residue was adjusted to pH 7.5 with 1 M phosphate buffer and partitioned three times with an equal volume of ethyl acetate to obtain neutral substances contained in rice seedlings. After drying over anhydrous Na₂SO₄, the ethyl acetate phase was evaporated to dryness in vacuo at 35°C (neutral fraction).

3. Isolation and purification of inhibitor

The neutral fraction was chromatographed on a column (2 × 60 cm) of silica gel (100 g, Silicagel 60, 70–230 mesh; Merck, Darmstadt, Germany), and eluted stepwise with benzene containing increasing amounts of ethyl acetate (10% per step, v/v; 200 mL step⁻¹). After the eluting solvent reached ethyl acetate, the elution was completed with 200 mL methanol. The biological activity of the fractions was determined using a lettuce bioassay as described below. After evaporation, the
active residue was applied to a thin layer chromatography (TLC) plate (Silicagel 60 GF254; Merck), and developed with a mixture of chloroform and acetic acid (95:5, v/v) for 15 cm. Then, 10 equal segments of chromatogram were scraped off and eluted with a mixture of ethyl acetate and methanol (1:1, v/v). After evaporation, the active residue was dissolved in 20% aqueous methanol (2 mL, v/v) and loaded onto a reverse-phase C18 Sep-Pak cartridge (Waters, Tokyo). The cartridge was eluted with 20, 40, 60 and 80% aqueous methanol (20 mL each). After evaporation, the active component was finally purified by HPLC (0.8 cm id×30 cm, μBondapak C18, Waters; eluted at a flow rate of 2 mL min⁻¹ with 20% aqueous methanol, detected at 254 nm).

4. Bioassay

Seeds of lettuce (Lactuca sativa L. cv. Grand Rapids) were surface sterilized in an aqueous solution of 25 mM sodium hypochlorite for 15 min, rinsed in distilled water for four times and allowed to germinate on a shoot of filter paper (No. 2; Toyo Ltd.) in the dark at 25°C. After two d, seedlings having radicles of 6-8 mm in length were selected and used for the bioassay.

Each fraction from the chromatography described above was evaporated to dryness, dissolved in a small volume of a mixture of acetone and methanol, applied to a sheet of filter paper (No. 2) in a 3 cm Petri dish and dried. The filter paper in the Petri dish was moistened with 1 mL of a 0.05% (v/v) aqueous solution of polyoxyethylene sorbitan monolaurate (Tween 20; Sigma, St.
Louis, USA), and 10 lettuce seedlings were arranged on each Petri dish and grown in the dark at 25°C. Control seedlings were treated with plain solution without extracts. After 30 h, the lengths of the hypocotyls of the seedlings were measured.

Results and Discussion

The neutral fraction (1.5 g) of an acetone extract obtained from 10-d-old rice seedlings (500 g fresh weight) was subjected to chromatography on silica gel, and the biological activity of the eluted fractions was evaluated by the lettuce bioassay (Fig. 1). Inhibitory activity was detected in fractions 3 to 5 (elution with 20 - 40% ethyl acetate in benzene), and fraction 10 (elution with 90% ethyl acetate in benzene), respectively. However, the activity in fractions 3 to 5 was much greater than that in fraction 10.

Fractions 3 to 5 were combined, evaporated and the residue (97 mg) was further purified by TLC. Inhibitory activity was detected in the fractions of Rf. 0.3 and 0.4 in the lettuce bioassay (Fig. 2). The active residue (9 mg) was passed through a C_{18} Sep-Pack cartridge and the activity was detected in the fraction eluted with 40% aqueous methanol (data not shown). The active residue (2.4 mg) was finally purified by HPLC and the inhibitory activity was found in a peak fraction eluted between 35 to 36 min (Fig. 3), yielding an active component (0.2 mg). This purified substance inhibited the growth of lettuce hypocotyls at concentrations greater than 0.3 μg mL^{-1} (Fig. 4).

Dekker and Meggitt (1983) found that most of the allelochemicals in crop plants are released during germination and early developmental stage when the plants are most sensitive for competition with neighboring plants to secure resources such as light, nutrients and water. During this period, weeds also establish and create a basis for later major weed problems. Thus, the early developmental stage of crop plants might determine the possible crop yield at the end of the season (Putnam and Tang, 1986; Olofsdotter et al., 1995).

Many secondary plant metabolites are considered to be associated with the allelopathic effects of plants (Rice, 1984; Putnam and Tang, 1986; May and Ash, 1990). Under certain conditions, these compounds are released into the environment, either as exudates from living tissues or leachates from residues of the plants in sufficient quantities to affect the neighboring or successional plants (Bhowmik and Doll, 1982; Putnam, 1988; Inderjit and Dakshini, 1992).

In the present research, two growth-inhibiting substances were isolated by silica gel column chromatography from the neutral fraction of the acetone extract of 10-d-old rice seedlings (Fig. 1), and one substance was purified by TLC, Sep-Pack cartridge and HPLC, although the active component was not characterized. These results suggest that rice seedlings at the early developmental stage may produce growth-inhibiting substances that may be released into the environment, either as exudates from living tissues or leachates from residues after decomposition of the plant, and act as allelochemicals to other plants.

Understanding of the chemical basis of the allelopathic system in rice plants and also the experiment on the allelochemicals in the field are essential (Duke, 1986; Leather and Einhellig, 1986). Large-scale purification of the inhibiting substances is now underway.

References


