Variation in the Allelopathic Effect of Rice with Water Soluble Extracts

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ABSTRACT

The allelopathic effect of rice (*Oryza sativa* L.) on lettuce (*Lactuca sativa* L.) and ducksalad [*Heteranthera limosa* (Sw.) Willd.] was investigated with water soluble extracts. Ducksalad is a major weed in southern USA rice fields. 'PI312777' and 'Rexmont', which are suppressive and nonsuppressive to ducksalad in the field, were used to establish the bioassay of allelopathic activity in rice. Water soluble compounds, which were extracted from rice seedlings and adult plants, were applied to lettuce seeds. Leaf extract of PI312777 inhibited the root growth of lettuce more strongly than those of Rexmont. Extracts from the leaves of rice seedlings at the six-leaf stage inhibited the growth of ducksalad and lettuce, and a close relationship existed between the inhibitory effect and the two test plants. A wide range of variation in allelopathic activity among rice cultivars was assessed using water soluble extracts from their leaves and lettuce as a test plant.

Allelopathic plants release compounds into the environment through root exudation, leaching by dews and rains, and volatilization or decaying plant tissue (Rice, 1984). In most cases, the compounds inhibit the germination or growth of neighboring plants although sometimes the compounds stimulate their growth.

Allelopathy in crops may act as a biological weed control in the agroecosystem. The genetic improvement of the allelopathic effect in crops is a strategy for biological weed control in breeding programs. In the 1970s, germplasm assessment was extensively undertaken to detect allelopathic accessions of crops. Accessions with an allelopathic effect have been found in crops such as beet (*Beta vulgaris* L.), lupine (*Lupinus lutens* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), oat (*Avena sativa* L.), pea (*Pisum sativum* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and cucumber (*Cucumis sativus* L.) reviewed by Rice (1984). A total of 538 accessions of cultivated and wild cucumber were screened by the pot and field test, and several accessions inhibited the growth of weeds (Putnam and Duke, 1974). Out of more than 3000 assessions of oat, several were found with a fluorescent microscope to exude a large amount of an allelochemical, scopoletin (Fay and Duke, 1977).

Research on rice allelopathy has been undertaken to select allelopathic cultivars in the field or laboratory and to identify allelochemicals. Several accessions of rice germplasm in the field were found to decrease the growth of ducksalad (Dilday et al., 1994), which is a major weed in the southern USA and causes a 21% reduction in the yields of direct-seeded rice (Smith, 1988). In field experiments, some rice cultivars produced a weed-free radius of 10 to 15 cm around an individual plant while nonsuppressive cultivars were densely surrounded by ducksalad. Rice cultivars with an allelopathic effect to barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) were also screened by assessment in the field and laboratory (Olofsdotter and Navarez, 1996). Varietal differences in the allelopathic effect were detected using the plant box method, in which lettuce seeds were placed together with rice seedlings in agar media at the four- to seven-leaf stages (Fujii, 1992). Aqueous extracts of decomposing rice residues in soil inhibited the root growth of rice and lettuce seedlings (Chou and Lin, 1976). Six phenolic acids (*p*-salicyclic, *p*-coumaric, vannilic, syringic, ferulic, and mandelic acid) were isolated from decomposing rice straw and paddy soil (Chou, 1980). A total of 16 potential allelochemicals, including the above-mentioned compounds, have been found in rice (Olofsdotter et al., 1995). Allelochemicals, which are completely responsible for varietal differences in the allelopathic effect of rice observed in field or laboratory experiments, have not been identified. The utilization of allelopathy might be straight forward if allelopathic cultivars can be identified by the amount of allelochemicals, but this seems difficult. Genes for an allelopathic effect or production of allelochemicals have not been detected although quantitative trait loci (QTL) for an allelopathic effect against barnyardgrass were reported (Bach Jensen et al., 1999). A successful breeding strategy to incorporate allelopathic activity into advanced cultivars would include a large-scale evaluation of a broad array of germplasm, the identification of allelochemicals, and the mapping of genes on the chromosomes.

In genetic analysis for rice allelopathy to ducksalad, we first focused on bioassay improvement using a water soluble extract because it should be more simple and reproducible. This study aims to establish an improved bioassay using a water soluble extract to quantify genetic variation in the allelopathic effect among rice germplasm collections. The second objective is to select the parental lines for genetic analysis.

MATERIALS AND METHODS

Plant Materials

Two cultivars or lines, PI312777 and Rexmont, were selected to represent suppressive and nonsuppressive rice against ducksalad, based on earlier germplasm evaluation (Dilday et al., 1994). PI312777 is a progeny line derived from the cross combination 2* 'Taichung 65'/'Taichung Native 1'

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Abbreviations: QTL, quantitative trait loci; TN1, Taichung Native 1.

(TN 1), and Rexmont is a nonsuppressive commercial cultivar in the USA. These two cultivars were used to investigate which parts and stages of rice plants are the most suppressive against lettuce.

To determine more definitely the most suppressive parts of rice plants, 10 cultivars ('Dular', 'Koshihikari', 'Masrai', 'Muha', 'Musashikogane', 'Nepal no.1', 'Nepal no.18', 'North Rose', Taichung 65, and TN 1) were chosen according to previous studies (Dilday et al., 1994; Fujii, 1992; Olofsdotter and Navarez, 1996). The USDA-ARS Genetic Resources Information Network (GRIN) database indicates that PI312777, TN1, and Masrai were allelopathic to ducksalad in the field and that Rexmont, Taichung 65, Koshihikari, Dular, Muha, and North Rose were nonallelopathic. Nepal no.18 and Musashikogane were allelopathic to lettuce and Nepal no.1 was nonallelopathic with the plant box method described above (Fujii, 1992). Musashikogane and TN1 were allelopathic to barnyardgrass (Olofsdotter and Navarez, 1996).

PI312777, its parental cultivars (TN1 and Taichung 65), and Rexmont were also used to investigate the relationship between the allelopathic effect of a water soluble extract on lettuce and ducksalad.

Using the results from previous studies, a total of 100 cultivars from the germplasm collections in Japan were evaluated for their allelopathic effect. Some accessions that were already examined for allelopathic activity by other methods were included as references.

Extraction

Roots, stems (3 cm from the basal node), and leaves were sampled from rice plants (PI312777 and Rexmont) that were grown in the greenhouse at the four- and six-leaf stages. These parts were also sampled from adult plants of the two cultivars at the flowering stage and during the grain filling period. Extracts of other cultivars were prepared only from seedlings at the six-leaf stage, which was the most suppressive. All experiments had three replications.

Fresh samples were stored at −80°C and then freeze-dried. The samples were ground to a powder in a mortar and stirred in cold, sterilized distilled water. Ten milliliters of water per gram of fresh sample was added to the samples. The mixture was kept in a refrigerator for 2 h and then stirred on a rotary shaker for 1 h and centrifuged at 1500 rotations min.$^{-1}$ for 15 min. The supernatant was recovered and stored in a refrigerator until it was used as a crude water-soluble extract.

Bioassay with Lettuce

To evaluate the allelopathic effect of water soluble extracts, 50 seeds of the lettuce cultivar 'Great Lakes 366' were placed on filter paper in a petri dish. Three milliliters of the crude extract or sterilized distilled water, as a control, was applied to the lettuce seeds. The petri dishes were sealed and incubated at 25°C in the dark for 3 d. The number of germinating seeds was recorded, and the length of the roots and hypocotyls of 10 randomly selected germinating seeds was measured. Five replications were done for each extract. The data were processed using the GLM procedure of the SAS (SAS Inst., 1992).

Bioassay with Ducksalad

Two hundred fifty milliliters of 1% agar was pored into a 7-by-7-by-7-cm plastic box. Fifty seeds of ducksalad were seeded on the agar, and a mixture of 20 mL of crude extracts were applied with 20 mL of sterilized distilled water or 40 mL of sterilized distilled water as control. The surface of the agar medium was covered with water that was about 1 cm deep. The seeds were incubated at 30°C in the light for 7 d. The number of germinating seeds was recorded, and the lengths of the roots and shoots of 10 randomly selected germinating seeds were measured. Because the crude extracts were too toxic against ducksalad to detect the difference between PI312777 and Rexmont, they were diluted with the same volume of sterilized distilled water. Three replications were done for each accession.

RESULTS

Water soluble extracts from different tissues and growth stages of PI312777 and Rexmont were tested to find an appropriate extract showing the maximum varietal difference of allelopathic potential. The germination of lettuce seeds showed no difference among the extracts. The germination rate of lettuce was 87.1 ± 6.21% with the extract of PI312777 and 86.5 ± 6.04% with Rexmont, so no significant difference was observed between two rice cultivars. The effects on lettuce hypocotyl elongation was variable among replications.

All extracts showed an inhibitory effect on the root elongation of lettuce (Fig. 1). However, the extracts that were isolated from different tissues and growth stages

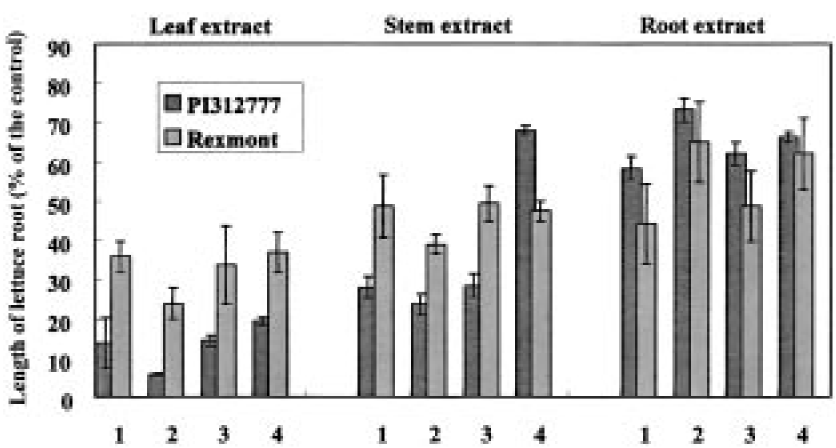

Fig. 1. Inhibitory effect of water soluble extracts from different tissues during the growth period of rice plants. 1, four-leaf stage; 2, six-leaf stage; 3, 1 wk before heading; 4, 2 wk after heading. Lettuce was grown in water as the control. Bars are the average of three replication of samples, and the lines indicate standard deviation.

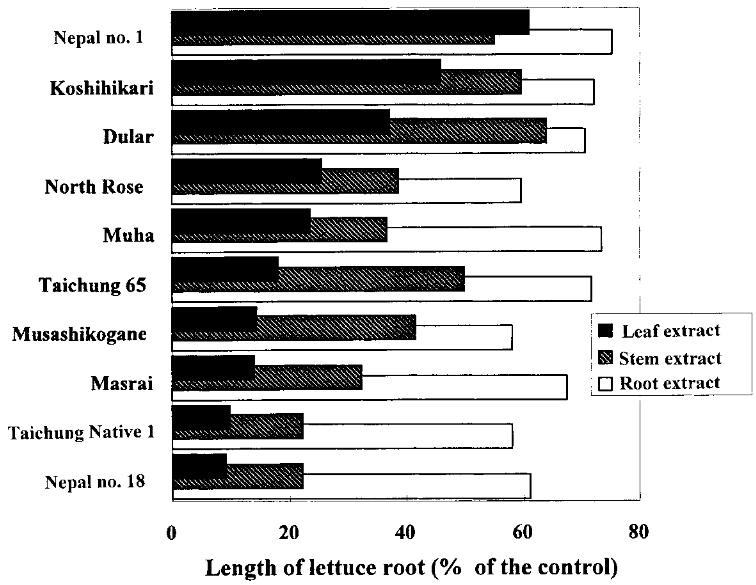

Fig. 2. Growth inhibitory effects of water soluble extracts from different tissues of rice cultivars on lettuce. Each bar is the average of three replications.

of PI312777 showed different degrees of an inhibitory effect on lettuce (Fig. 1). Leaf extract from rice plants at the six-leaf stage inhibited the lettuce root growth more than the extracts from other tissues and stages. The extracts caused necrosis in the tip of lettuce roots, and most of seeds with necrotic root tips could not develop a normal hypocotyl. The suppressive effects of the extracts from the plants during the grain filling period were low. The stem and root extracts were less effective compared with the leaf extracts. The extracts of Rexmont showed small age specificity and tissue specificity on lettuce growth compared with PI312777 (Fig. 1).

The suppressive effects of 10 rice cultivars were investigated using extracts from different tissues (Fig. 2). The results showed that the stem and root extracts were less inhibitory than the leaf extracts. The leaf extracts from cultivars previously reported as allelopathic had the largest inhibitory effect.

The relationship between the inhibitory effect of the extract to lettuce and ducksalad was investigated by applying leaf, stem, and root extracts from plants at the six-leaf stage. PI312777 and Rexmont were mainly used. The germination rate of ducksalad was 89.4 ± 3.47% with PI312777 extracts and 81.2 ± 7.42% with Rexmont extracts. The root length of ducksalad treated by the extract of PI312777 differed when treated by that of Rexmont, reflecting differences in the allelopathic effect between the two cultivars in the field. The extracts affected the shoot elongation of ducksalad, but this did not reflect the effect in the field. The suppressive effect of water soluble extracts from seedlings at the six-leaf stage on ducksalad root is shown in Fig. 3. The leaf and stem extracts inhibited the root length of ducksalad. The full-strength solution of the leaf extract from both cultivars was too toxic to detect varietal differences in the suppressive effect on ducksalad. When the extracts were diluted, the difference between PI312777 and Rexmont was clearly observed.

A correlation of the inhibitory effect of the leaf ex-

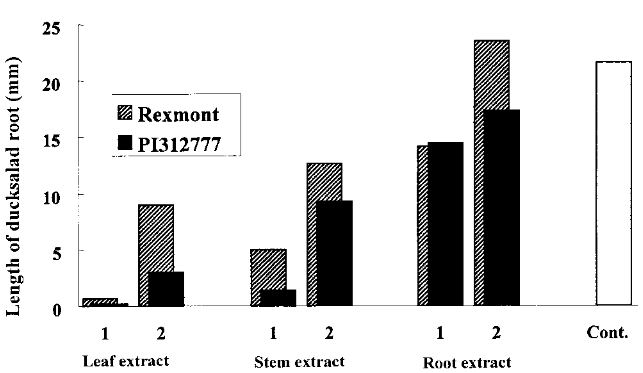

Fig. 3. Root growth inhibition of water soluble extracts from different parts of rice plants on ducksalad. 1, 40 mL of full-strength solution; 2, mixture of 20 mL solution and 20 mL water; Cont, 40 mL of water. Each bar represents the average ducksalad root length of three replications.

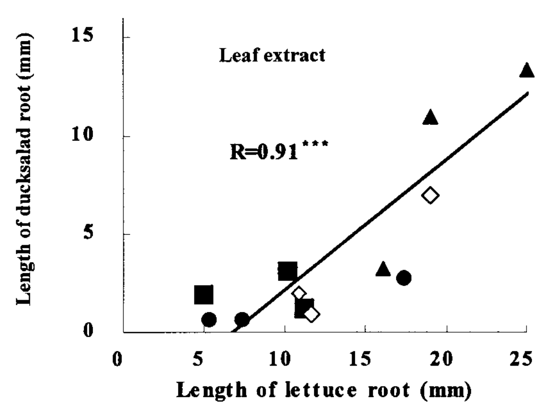

Fig. 4. Correlation between results from water soluble extracts from leaves of rice plants of four rice cultivars on lettuce and ducksalad root growth. ■, PI312777; ▲, Rexmont; ●, Taichung Native 1 (TN1); ◇, Taichung 65. Each point shows the average lettuce and ducksalad root length of three replications when applying the same extract. *, Significant at the 0.1% level.**

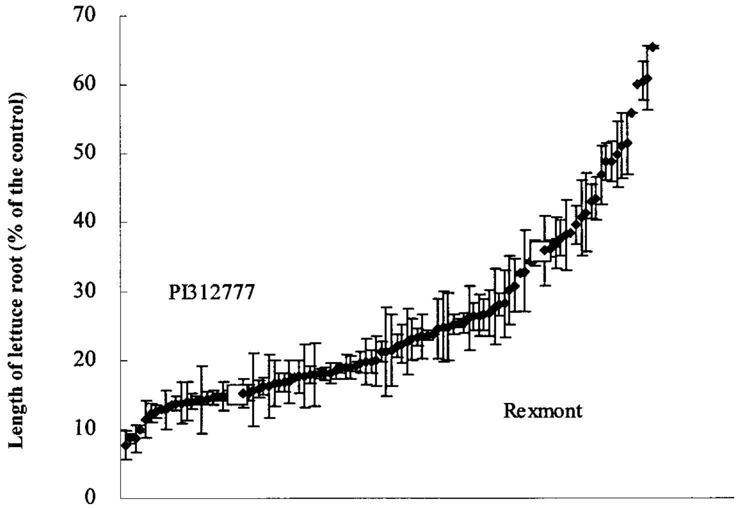

Fig. 5. Inhibitory effect of leaf extract from different cultivars on lettuce root length compared with the control. The bars represent deviation.

tract from four cultivars (PI31277, TN1, Taichung 65, and Rexmont) on the root growth of lettuce and ducksalad is shown in Fig. 4. The correlation coefficient between the inhibitory effects of water soluble extracts was 0.91 with the leaf extract, 0.58 with the stem extract, and 0.24 with the root extract, respectively.

To identify allelopathic cultivars, 100 cultivars from the rice germplasm collection were evaluated using leaf extracts from rice plants at the six-leaf stage and lettuce as a test plant. The results showed a wide range of variation in the inhibitory effect of rice (Fig. 5). The most inhibitory cultivar reduced the root elongation of lettuce by 96%, and the least inhibitory cultivar reduced it by 39% compared with the control. PI312777 and Rexmont showed an inhibitory effect of 90 and 69%, respectively.

DISCUSSION

This study focused on establishing an improved bioassay using water soluble extracts of rice and on choosing parental cultivars or lines for a QTL analysis.

Leaf extract from plants at the six-leaf stage made it possible to detect the difference between PI312777 and Rexmont in terms of their weed suppressive effect in the field. The varietal differences among the 10 rice cultivars that were observed confirmed that leaf extracts can be used to assess the growth inhibitory effect. This bioassay with water soluble extracts and lettuce as a test plant was easier than the field test for an allelopathic effect, which needs several years of replication.

A uniformity of seed quality, easy handling, and a high sensitivity to allelopathic compounds are required for a test plant to assess allelopathic activity. Lettuce has often been used as a test plant. A correlation between the allelopathic activity to ducksalad in the field and to lettuce was found in an experiment with water soluble extracts (Okuno et al., 1997). A correlation was also found between the inhibitory effect on the root lengths of lettuce and ducksalad when leaf extracts of the four cultivars were used in the present study (Fig. 2). Leaf extract from plants at the six-leaf stage was reconfirmed to be the most appropriate to assess the growth inhibitory activity of rice on ducksalad and lettuce.

The results of this study showed that the root extracts of rice were less effective than other extracts although some plants are known to release allelochemicals from their roots. In oat, the allelopathic effects against crunchweed [*Brassica kaber* (DC.) L.C. Wheeler var. *pinnatifida*] of several accessions appeared to be associated with the relative amount of scopoletin that was exuded from the roots (Fay and Duke, 1977). A weak association of the effect of the root extract with the allopathic effect in the field can be explained by an immediate release after translocation from the leaves. Another possibility is that rice allelochemicals are not released from roots but are leached directly from the leaves. Numerous allelopathic compounds have been identified in the rainfall leachate of several plant species. For example, an a-napthol derivative and scopoletin have been identified in the leachate of sunflower (*Helianthus annuus* L.) (Wilson and Rice, 1984).

Cultivars with a higher allelopathic potential than PI312777 were found from the 100 that were screened. These cultivars may be candidates for a genetic analysis of the allelopathic effect of rice and may be beneficial breeding lines in a practical utilization of allelopathy. There is no significant difference between local and improved cultivars. It seems that the allelopathic effect has not been under natural and artificial selection pressure, and this character does not conflict with agronomic features.

With the method refereed in this study, a QTL analysis of the allelopathic activity of rice will be undertaken. This information would be useful for a breeding program to improve the allelopathic activity of rice cutivars.

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Searching for Rice Allelochemicals: An Example of Bioassay-Guided Isolation

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ABSTRACT

A bioactivity-guided isolation method was developed with the objective of isolating the allelochemicals in rice (*Oryza sativa* L.). Roots of the allelopathic rice cultivar Taichung Native 1, grown hydroponically, were extracted and fractionated, with the activity of the fractions followed using a 24-well culture plate microbioassay. Some of the fractions obtained consisted of pure compounds, but none inhibited the growth of barnyardgrass [*Echinochloa crusgalli* (L.) Beauv.] at the lower concentration at which they were tested. Identified compounds were azelaic acid; ρ-coumaric acid; 1*H*-indole-3-carboxaldehyde; 1*H*-indole-3-carboxylic acid; 1*H*-indole-5-carboxylic acid; and 1,2-benzenedicarboxylic acid bis(2-ethylhexyl)ester. ρ-Coumaric acid, a known allelochemical, inhibited the germination of lettuce (*Lactuca sativa* L.) seedlings at 1 m*M*. However, ρ-coumaric acid was active against barnyardgrass only at concentrations higher than 3 m*M*. The two most active fractions obtained from the bioassay-guided isolation were still a mixture of compounds as analyzed by gas chromatography–mass spectrometry (GC-MS). Further fractionation is being done to isolate and identify the allelochemical(s) in these active fractions. This work has demonstrated the use of bioassay-guided isolation in identifying allelochemicals in rice and has correlated observed field activity with laboratory experiments.

Observation of apparent allelopathy in rice (*Oryza sativa* L.) has recently drawn great attention (Olofsdotter, 1998), and there is much interest in identification of the allelochemical(s). Identification of the phytotoxic compound(s) responsible for allelopathy will allow efficient generation of more allelopathic cultivars through traditional breeding or biotechnology-based genetic alterations. Such cultivars could become important tools in the development of advanced integrated weed management strategies for rice, which would be less dependent on synthetic herbicides.

The search for allelochemicals in rice necessitates evaluating the activity in a laboratory set-up to distinguish between competition and allelopathy, which cannot be distinctly separated in field studies (Olofsdotter et al., 1997). Depending on one's objectives, different methods could be followed in searching for active constituents from plants. These include bioassay-guided isolation, fractionation-driven bioassay, isolate and assay, and biochemical combinatorial chemistry approaches. The advantages and disadvantages of each of these methods are discussed in more detail by Duke et al. (2000a). We chose bioassay-guided isolation as the best way to proceed because the active component is not known.

Bioassay-guided isolation integrates the processes of separation of compounds in a mixture, using various analytical methods, with results obtained from biological testing. The process begins with testing an extract to confirm its activity, followed by crude separation of the compounds in the matrix and testing the crude fractions (Fig. 1). Further fractionation is carried out on the fractions that are determined to be active, at a certain concentration threshold, whereas the inactive fractions are set aside or discarded. The process of fractionation and biological testing is repeated until pure compound(s) are obtained. Structural identification of the pure compound then follows. This methodology precludes overlooking novel compounds that are often missed in studies that only identify those compounds with which the investigator is familiar. Moreover, the possibility of discovering an unknown molecular site of action is maximized (Duke et al., 2000b).

In carrying out bioassay-guided isolations of allelochemicals from rice, there are several important factors that must be considered. First, the rice cultivar to be

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Abbreviations: GLM, general linear model; GC-MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance.