Correlation between Growth Inhibitory Exhibition and Suspected Allelochemicals (Phenolic Compounds) in the Extract of Alfalfa (Medicago sativa L.)

Tran Dang Xuan*, Eiji Tsuzuki, Hiroyuki Terao, Mitsuhiro Matsuo and Tran Dang Khanh

(*United Graduate School of Agricultural Science, Kagoshima University, Kagoshima 900-0065, Japan; Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan)

Abstract: Acidic fractions of the extracts from the three alfalfa cultivars Batasu, Rasen, and Yuba exhibited a varietal difference in the inhibitory effect on hypocotyls and radicle growth of alfalfa (cv. Nasuwakaba) and rice (cv. Koshikihari) seedlings. The extract from Rasen possessed the strongest inhibitory activity, and that of Batasu was the lowest. In a TLC bioassay with lettuce seedlings, inhibitory zones of the extracts were located at an Rf value of 0.6-0.8, and maximum inhibitory zones at Rf of 0.1-0.2. Inhibitory zones were analyzed by HPLC. Eight phenolic compounds were identified in the extracts from Rasen and Batasu, and six compounds in that from Yuba. However, the content of these phenolic compounds varied with the cultivar. The content of each phenolic compound was the highest in Rasen, followed by Yuba and Batasu, although the content of p-hydroxybenzoic acid was equivalent in all cultivars. We suppose that the degree of inhibitory exhibition of allelopathy may be related to the presence and concentrations of allelochemicals (phenolic compounds), however, the allelopathic activity of the plant might be determined by interactions of all these compounds, not just a single chemical.

Key words: Bioassay, HPLC, Inhibition, Phenolic acids, TLC, Varietal difference.

The definition of allelopathy was coined by Molish (1937) as the biochemical interactions between all types of plants including both inhibitory and stimulatory reciprocal biochemical interactions. Recent evidence has shown that virtually all plant parts such as leaves, root, pollen, trichomes, bark, seeds, and fruits possess allelopathic potential (Kohli and Batish, 1998). Numerous plants exhibit allelopathic potential and many of them [alfalfa (Medicago sativa L.), barley (Hordeum vulgare L.), buckwheat (Fagopyrum esculentum Moench), asparagus (Asparagus officinalis L.), rice (Oryza sativa L.), corn (Zea mays L.), taro (Colocasia esculenta (L.)] have strong allelopathic properties. Alfalfa and buckwheat pellets applied to the paddy field were suggested to be useful as an alternative natural herbicide to reduce the herbicide dependence in weed control (Tsuzuki et al., 1999, Xuan et al., 2002). With the purpose of improving the efficacy of alfalfa in paddy weed control, allelopathic potentials of eight alfalfa varieties in Japan, Batasu, Hitawakaba, Kitawakaba, Makiwakaba, Natsuwakaba, Rasen, Tachiwakaba, and Yuba were examined. Rasen and Yuba were assessed to have the strongest inhibitory activity and Batasu was the lowest from the inhibitory effects of the extracts from fresh and dried plants and the leachates of germinating seeds on the growth of lettuce (Lactuca sativa L. cv. Great Lakes) (Xuan and Tsuzuki, 2002). Moreover, when the dried plants of Rasen and Yuba harvested at flowering stage were applied into paddy field at two days after transplanting at a concentration of 1 ton ha⁻¹, respectively, and they exhibited a strong inhibition against weed growth, approximately 90 and 95% weed reduction at 40 days after transplanting, respectively. Especially, application of dried Rasen recorded 80.6% increase of rice yield as compared with control (no herbicide or no weed management), while the plants treated with the herbicide had a 71% increase of yield. However, it was only increased 29% when Yuba was applied (Xuan et al., 2003).

The allelopathy has been widely studied but the mechanism has not been clarified yet (Kohli and Batish, 1998). A number of chemical compounds have been demonstrated to be involved in allelopathy and they are identified as secondary metabolites (Rice, 1984). They included a variety of phenolic acids, flavonoids, quinines, terpenoids, steroids, purines, long-chain fatty acids and acetylenes, organic acids, unsaturated lactones, and others. The phenolic compounds, derivatives of benzoic and cinnamic acids, have been the most commonly recognized as allelopathic compounds produced by higher plants (Rice, 1984). Recently, several active allelochemicals, which were speculated to cause intras and inter-specific injuries have been identified in alfalfa plants. Medercapin, 4-methoxymedicapin, sativan, 5-methoxysativan (Dornbos et al., 1990) and saponins (Guenzi et al., 1964) were identified as growth-inhibiting substances in alfalfa. On the other hand, ferulic acid and salicylic acid, which inhibited the growth of radish
(Raphanus sativus L.), have also been isolated from both aqueous extracts and decomposition residues of the plants in soil (Nakahisa et al., 1994).

In the present study, we fractionated the acidic fraction of extracts from three alfalfa cultivars, Batasu, Rasen and Yuba, by HPLC, and examined their effects on the growth of alfalfa (cv. Nasuwakaba) and rice seedlings. The phenolic compounds in the extract from each cultivar were identified, and their concentrations were compared with the growth inhibitory effect of each extract in relation to allelopathy.

Materials and Methods

1. Plant materials

Seeds of the three alfalfa varieties: Batasu, Rasen and Yuba were provided from the Yukijirushi Seed Company (Kumamoto City, Japan), and were sown on May 2, 2000 in concrete blocks (length: 60 cm; width: 60 cm; height: 100 cm) filled with soil taken from the experimental field where previously no alfalfa was cultivated. These plants were grown by conventional methods. At the vegetative and full-bloom stage on April 19, 2002, the plant tops were harvested at 10 cm height. The samples were dried at 80°C for 10 h before being crushed into a powder.

2. Preparation of acidic fraction from the extracts from alfalfa plants

Procedure for getting acidic fraction was carried out according to Fujii et al. (1991) with some modifications. One gram of dried powder of each alfalfa cultivars was mixed with 50 ml solution of MCW (methanol: 2, chloroform: 1, distilled water: 0.8) and was shaken in an ultrasonic bath for 30 s. The solution was then centrifuged for 15 min at 2000 rpm under room temperature, and the extraction was repeated three times. The supernatant was separated and the precipitate was added to 50 ml of 1 M NaOH and shaken at 40°C for 4 h. This suspension was centrifuged at 2000 rpm for 15 min and the supernatant was passed through the filter paper (Toyo Roshi company, Japan). This supernatant was adjusted to pH 7.0 with 2 M HCl, regulated to 50 ml by evaporation under reduced pressure, and then adjusted to pH 2.0 with 2 M HCl. It was centrifuged (2000 rpm, for 10 min) and filtrated (Toyo Roshi company, Japan) again.

The filtrates were then extracted 3 times with 50 ml of ethyl acetate (acidic fraction), which was dried with sodium sulfate (anhydrous). Subsequently, they were filtrated and evaporated to dryness on a rotary evaporator at 40°C. MeOH was added to the acidic fraction at a rate of 1 ml 1 mg (1000 ppm), and it was filtered through a 0.20 μm filter (Dismic-25cs, Toyo Roshi, Japan) for the analysis by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

3. Bioassays

Seeds of alfalfa (Medicago sativa L. cv. Nasuwakaba), and rice (Oryza sativa L. cv. Koshikihari) were used for the bioassay. Seeds of alfalfa and rice were surface-sterilized in sodium hypochlorite (5% available chlorine) for 20 min and immediately rinsed many times with distilled water. These seeds were subsequently placed into a 500 ml glass pot saturated with distilled water and incubated in the growth chamber (28°C, 67.2 μmol m⁻² s⁻¹) for 3 days. Seedlings with 2 mm shoot and three distinct seminal roots were randomly selected for bioassays.

The acidic fraction of the extracts from the three alfalfa cultivars was evaporated to dryness using a rotary evaporator at 40°C. The residues were dissolved in distilled water at a concentration 50, 100, 250, and 500 ppm. Five ml of each solution was added to filter paper in a Petri dish (dia : 6 cm), and 10 seedlings of alfalfa or rice were placed in each disk. Distilled water was used as a control. All Petri dishes were placed in the growth chamber (25°C, 67.2 μmol m⁻² s⁻¹), and additional water was not given during the treatment. After 7 days, hypocotyl and radicle length were determined.

4. TLC analysis

The acidic fraction was subjected to TLC by the ascending method with a plate coated with a 500 μm layer of silical gel G (Merck) and a solvent system of chloroform (CHCl₃) : MeOH : acetic acid (45 : 8 : 4). The chromatogram was divided into 10 zones, and each zone was eluted with 7 ml of distilled water. Lettuce is commonly used as tested plant for bioassay of allelochemicals because it is highly sensitive at a low concentration (Chung et al., 2001). In this experiment, 10 seeds of lettuce (Lactuca sativa L. cv. Great Lakes) were transferred into a 3 cm petri dish lined with filter paper, and 2 ml of the elute was added. They were placed in the growth chamber (25°C, 67.2 μmol m⁻² s⁻¹). Hypocotyl and radicle lengths were determined after 7 days of culture. The zones, which showed inhibitory activity were eluted in MeOH at a concentration of 1000 ppm, and then used for HPLC analysis.

Fifteen standard chemicals: Benzoic acid, caffeic acid, ferulic acid, gallic acid, protocatechuic acid, p-coumaric acid, p-chlorobenzoic acid, p-hydroxybenzoic acid, salicylic acid, sinapic acid, syringic acid, trans-cinnamic acid, vanillic acid, catechin and vanillin [Nacal-tesque (Japan), Sigma (Germany) and Wako (Japan)] were applied to the TLC simultaneously with the acidic fraction and the Rf values as well as colors in broad-band UV light were determined.

5. HPLC analysis

Benzoic acid, caffeic acid, ferulic acid, gallic acid, protocatechuic acid, p-coumaric acid, p-chlorobenzoic acid, p-hydroxybenzoic acid, salicylic acid, sinapic acid, syringic acid, trans-cinnamic acid, vanillic acid, catechin
Table 1. Effect of the acidic fractions from the 3 alfalfa cultivars on the elongation of hypocotyls and radicles of alfalfa and rice seedlings. Each value is a mean ± standard error (n = 8).

| Concentration (ppm) | Alfalfa (cv. Natsuwakaba) | Rice (cv. Koshihikari) |
|---------------------|---------------------------|------------------------|
|                     | Hypocotyl (mm) | Radicle (mm) | Hypocotyl (mm) | Radicle (mm) | Hypocotyl (mm) | Radicle (mm) |
| 0                   | 5.1±0.7b        | 12.5±2.1b       | 12.3±2.7b       | 16.1±3.1b   | 12.3±2.7b       | 12.3±2.7b   |
| 50                  | 11.5±2.3a       | 21.9±1.5a       | 22.3±4.1a       | 24.6±2.5a   | 19.1±1.8a       | 24.6±2.7a   |
| (+125.5)            | (+75.2)         | (+51.9)         | (+81.3)         | (+51.9)     | (+55.3)         | (+60.0)     |
| 100                 | 11.6±1.4a       | 18.8±2.8a       | 24.7±2.1a       | 20.2±3.5a   | 13.6±2.6b       | 25.3±2.8b   |
| (+127.5)            | (+49.2)         | (+106)          | (+100.8)        | (+25.5)     | (+10.6)         | (+25.5)     |
| 250                 | 6.5±1.6b        | 9.9±3.3c        | 18.8±2.6a       | 17.7±2.4ab  | 7.1±1.6c        | 8.5±2.3c   |
| (+27.5)             | (20.8)          | (+53.0)         | (+100.8)        | (+25.5)     | (1.9)           | (5.0)      |
| 500                 | 4.1±1.2c        | 8.6±2.1c        | 18.1±3.1b       | 14.3±2.7b   | 5.5±0.8e        | 4.3±1.6d   |
|                     | (19.6)          | (31.2)          | (12.1)          | (11.2)      | (55.5)          | (73.3)     |

LSD (0.05) among effects of acidic fractions from three alfalfa cultivars on the growth of alfalfa and rice, at each concentration:

- Alfalfa:
  - 50 ppm Hypocotyl: NS Radicle: 1.5
  - 250 ppm Hypocotyl: 2.9 Radicle: 3.0

- Rice:
  - 50 ppm Hypocotyl: NS Radicle: 2.8
  - 250 ppm Hypocotyl: 3.9 Radicle: 3.7

NS: not significantly different.

Means, within a column, followed by the same letter are not significantly different at P<0.05 according to Duncan’s multiple range test.

Means in the parentheses are inhibition percentage over control, and (+) shows promotion percentage over control.

and vanillin and samples were subjected to the each HPLC with an amount of 5 μl. Concentrations in the samples were calculated by comparing peaks areas of standards with those of the standards.

HPLC were performed using a TSK gel column ODS-80Tm (4.6 mm Φ×15 mm), temperature of 40°C. Two solvents, 2% of acetic acid (solution A) and 100% methanol (solution B), were established to be the gradient B in solvent A was: 0-28 min, 15-40%; 28-42 min, 40-62%; 42-63 min, 62-99%. The flow rate was 1.8 ml per min. Wave–length of ultraviolet absorption of detector (absorbance) of 254 nm. Retention times of the standard compounds and the major peaks in the extracts were recorded.

6. Statistical analysis

The HPLC and TLC experiments were repeated four times. The bioassays were repeated twice with four replications in a completely random design. Data were treated in a fashion of Duncan’s multiple range test.

Results and Discussion

1. Bioassay

The effects of the acidic fraction of the extracts from Batasu, Rasen and Yuba on hypocotyl and radicle elongation of alfalfa (cv. Natsuwakaba) and rice seedlings were examined. At the concentration of 50 and 100 ppm, the extract (acidic fraction) of the three alfalfa cultivars strongly promoted the growth of both alfalfa and rice seedlings. The maximum promotion of hypocotyl and radicle elongation was observed in alfalfa treated with Batasu extract at 100 ppm (127.5%) and 50 ppm (75.2%), respectively. The extract of Rasen at 100 ppm slightly inhibited the hypocotyls and radicle elongation of alfalfa (3.9 and 7.2%, respectively) and hypocotyl elongation of rice (3.0%). At 250 ppm, the extracts from Rasen and Yuba significantly inhibited the growth of both alfalfa and rice seedlings, as compared with control, except for hypocotyls elongation of alfalfa seedlings, which was slightly promoted (2.9%).
extract of Batasu significantly suppressed the radicle elongation of alfalfa seedlings but promoted hypocotyls elongation of rice seedlings (Table 1). At 500 ppm, the extract from all alfalfa cultivars suppressed the elongation of both hypocotyl and radicles of alfalfa and rice seedlings. The extract from Rasen exhibited the strongest inhibition and that of Batasu was the lowest. The results suggested that the radicle is much more sensitive than the hypocotyls to the acidic fractions.

The data in Table 1 indicated that the inhibitory effects of alfalfa extracts varied with the cultivars. At 50 ppm, the extract from the three cultivars did not significantly suppress the growth of the alfalfa seedlings than radicle elongation \((P < 0.05)\). At 100 and 250 ppm, a significant difference was found between the effects for the extracts from Rasen and Batasu in both alfalfa and rice seedlings, except for the radicle elongation of rice seedlings. A significant difference was not observed between the effects of the extracts from Yuba and that of Rasen or Batasu, with the exception of radicle elongation of alfalfa seedlings at 250 ppm (Table 1). At 500 ppm, the inhibitory effect of the extracts on either alfalfa or rice seedlings was significantly different among the three alfalfa cultivars used for extraction.

The results of this experiment verified that among the extracts (acidic fraction) of the three alfalfa cultivars, the extract from Rasen showed the strongest inhibitory effect followed by that of Yuba and Batasu in this order, on the elongation of hypocotyl and radicle of alfalfa and rice seedlings. This is consistent with the report by Xuan and Tsuzuki (2002), that the extracts of plant powder and leachate of germinating seeds of Rasen and Yuba showed the strongest inhibition for germination and growth of lettuce, and that of Batasu was the lowest. In the TLC bioassay, inhibitory zones were found at \(R_f 0.6-0.8\), and the maximum inhibition at \(R_f 0.7-0.8\) in all cultivars (Fig. 1). However, an additional inhibitory zone was observed at \(R_f 0.1-0.2\) in the extracts from Rasen and Yuba (Fig. 1). Inhibitory zones were scraped off, and eluted with methanol for HPLC analysis.

### 2. HPLC

The fraction at \(R_f 0.1-0.2\) of TLC exhibited peaks 4 and 6 in HPLC profiles of the extracts from Rasen and Yuba, respectively (Fig. 2). The fraction at \(R_f 0.6-0.7\) of the extract from Rasen showed peaks 1, 2, 3, and 4, and that from Batasu showed peaks 1, 2, 3, and 6 in the HPLC profiles. The fraction at \(R_f 0.7-0.8\) from Rasen showed peaks 3, 5, 7, 8, 9, 10, 11, 12, and 13, and that from Batasu showed peaks 4, 5, 7, 8, 9, 10, 11, 12, and 13 in HPLC profiles (Fig. 2).

The extract of Rasen had 12 peaks in HPLC profile, and the extracts from both Yuba and Batasu had 13 peaks (Fig. 2). However, only eight peaks in the extracts from Rasen and Batasu, and six peaks in the extract from Yuba were identified by comparison with the authentic chemicals (Fig. 2, Table 2). In addition, the identified peaks (phenolic acids) in the extract varied with the cultivar, except for \(p\)-hydroxybenzoic acid, vanillic acid and ferulic acid, which were observed in the extracts of all cultivars.

Table 2 shows the retention time and concentration of each phenolic acid identified in the extracts from the three alfalfa cultivars. The number and the kind of phenolic compounds identified in the extracts varied with the cultivar of alfalfa, and only three phenolic
Retention time (min)

Peaks 1: Gallic acid
2: p-coumaric acid
3: Protocatechuic acid
4: p-hydroxybenzoic acid
5: Unknown
6: Vanillic acid
7-8: Unknown
9: Syringic acid
10: Benzoic acid
11-12: Unknown
13: Ferulic acid

Peaks 1: Gallic acid
2: Protocatechuic acid
3: p-hydroxybenzoic acid
4: Catechin
5: Vanillic acid
6: Vanillin
7: Syringic acid
8-11: Unknown
12: Ferulic acid

Peaks 1: Unknown
2: p-coumaric acid
3-4: Unknown
5: p-hydroxybenzoic acid
6: Catechin
7: Vanillic acid
8: Unknown
9: Benzoic acid
10-12: Unknown
13: Ferulic acid

Fig. 2. HPLC profiles of the eluate of the inhibitory zones in TLC of the acidic fractions from three alfalfa cultivars; Absorbance (254 nm).

compounds, p-hydroxybenzoic acid, vanillic acid and ferulic acid were commonly detected in the extracts from the three cultivars. The concentration of p-hydroxybenzoic acid was nearly the same in all extracts from the three cultivars, but the concentration of vanillic acid and ferulic acid were the highest in the extract from Rasen and the lowest in that from Yuba (Table 2).

The fraction at Rf 0.1–0.2 of the extract from Rasen and Yuba showed an inhibitory effect, and had peaks 4 and 6, respectively; both of them were identified to be catechin (Fig. 2). However, this fraction from Batasu did not show any inhibitory effect (Fig. 1).

Ferulic acid was identified as a growth inhibitor in the extract from alfalfa (Nakahisa et al., 1994) and found in all three cultivars in this study. Many of the phenolic compounds identified in this experiment have been known as the growth inhibitors. Chung et al. (2001) isolated salicylic acid, p-coumaric acid, p-hydroxybenzoic acid, syringic acid, ferulic acid, and benzoic acid from four rice cultivars and proved that they are involved in allelopathic activity of the rice plant. Caffeic acid and ferulic acid isolated from buckwheat plants also had allelopathic activity (Tsuze and Yamamoto, 1983). Recently, Matsuo et al. (2002) identified four allelochemicals, caffeic acid, p-coumaric acid, ferulic acid and vanillin in four rice cultivars.

Probable major biosynthetic pathways leading to the production of autotoxic chemicals are the shikimic acid or acetate pathway (Rice, 1984). Autotoxic chemicals found in alfalfa are mainly cinamic acid and its derivatives such as ferulic acid, vanillic acid, hydroxybenzoic acid, p-coumaric acid, trans-cinamic acid, chlorogenic acid and caffeic acid (Newby et al., 1980; Hall and Henlerlong, 1989; Read and Jensen, 1989; Miller, 1996). Chon and Kim (2002) identified nine phenolic acids, caffeic acid, trans-cinamic acid, hydro-cinamic acid, coumarin, ferulic acid, m-coumaric acid, o-coumaric acid, p-coumaric acid, and salicylic acid in alfalfa
Table 2. Retention time and concentration of phenolic compounds identified by HPLC in the extract from three alfalfa cultivars.

| Chemicals          | Standard retention time (min) | Retention time in sample (min) | Concentration (mg g⁻¹) |
|--------------------|-------------------------------|-------------------------------|------------------------|
|                    | Batasu | Rasen | Yuba                | Batasu | Rasen | Yuba                | Batasu | Rasen | Yuba |
| Gallic acid        | 1.51   | 1.51  | 1.50                | 2.90±0.07 | 9.20±0.06 | -                  |
| p-Coumaric acid    | 1.87   | 1.88  | -                   | 7.20±0.01 | -        | 5.10±0.12          |
| Protocatechuic acid| 2.01   | 2.11  | 2.00                | 2.26±0.05 | 9.25±0.04 | -                  |
| p-Hydroxybenzoic acid| 2.76  | 2.80  | 2.77                | 2.25±0.02 | 2.05±0.05 | 2.11±0.15          |
| Catechin           | 3.00   | -     | 3.05                | -       | 10.6±1.3 | 6.72±0.15          |
| Vanillic acid      | 3.69   | 3.67  | 3.65                | 0.72±0.03 | 6.69±0.01 | 1.38±0.11          |
| Caffeic acid       | 4.00   | -     | -                   | -       | -        | -                  |
| Vanillin           | 4.41   | -     | 4.44                | -       | 6.32±0.05 | -                  |
| Syringic acid      | 4.98   | 5.01  | 4.94                | 0.90±0.07 | 1.94±0.13 | -                  |
| Salicylic acid     | 5.12   | -     | -                   | -       | -        | -                  |
| Benzoic acid       | 5.49   | 5.41  | -                   | 5.29±0.03 | -        | 8.44±0.21          |
| Ferulic acid       | 9.20   | 9.17  | 9.30                | 1.70±0.22 | 8.28±0.07 | 2.91±0.16          |
| Sinapic acid       | 12.16  | -     | -                   | -       | -        | -                  |
| trans-Cinnamic acid| 15.60  | -     | -                   | -       | -        | -                  |
| p-Chlorobenzoic acid| 17.41 | -     | -                   | -       | -        | -                  |

Values are means ± standard errors (n=4).

(-) Chemical not detected in the sample.

(Medicago sativa L. cv. Vernal). Mizutani (1999) separated the phenolic fractions in the extract from Sasa cernua by HPLC and identified five inhibitory compounds, p-coumaric acid, ferulic acid, vanillic acid, p-hydroxybenzoic acid and p-hydroxybenzaldehyde. HPLC analysis has also been applied to purify isothiocyanates isolated from the extract of Rorippa indica Hiern., and several phenolic compounds such as 1,4-dihydroxybenzoic acid, p-hydroxybenzoic acid, vanillic acid, and p-benzaldehyde found in the extract of barnyardgrass (Echinochloa crus-galli). Chung et al. (2001) and Chon and Kim (2002) also identified phenolic acids in alfalfa and rice plants by HPLC. The phenolic compounds shown in Table 2 are well-known allelochemicals, and many of them are observed in alfalfa plants and other allelopathic plants. Therefore, the presence of these suspected allelochemicals and their doses in alfalfa may be clarified by repeated TLC and HPLC purification.

The results of this study showed that Rasen had the strongest allelopathic potential, followed by Yuba and Batasu in this order. We suggest that varietal difference in the types and doses of allelochemical types may be reflected in that in their allelopathic inhibitory activities. On the other hand, a number of unknown peaks were observed in the HPLC profiles. They were peaks 8-11 in Rasen, peaks 3-4, 8, 10-12 in Yuba, and peaks 5, 7, 8, 11, 12 in Batasu. Further work is needed to identify these unknown compounds and to examine their inhibitory activities as well as the varietal difference in their contents.

Variatetal differences in allelopathic exhibition has been reported in several allelopathic plants including cucumber (Cucumis sativus L.) (Putnam and Duke, 1974), barley (Hordeum vulgare L.) (Didon, 2002), wheat grass (Agropyron glaucum) (Panchuk and Prutenkaya, 1973), and rice (Oryza sativa L.) (Dilday et al., 1994). Chung et al., (2001) surmised that the type of allelochemicals and their concentrations in rice plant varies with the cultivar, and proposed that the type and dose of allelochemical presence were strongly related to the allelopathic activity of rice plants.

In this study, a number of phenolic compounds were identified in the extract from the three alfalfa cultivars, and their concentrations examined. Further studies are needed to search for other allelochemicals in alfalfa plant and to examine allelochemical interactions, in relation to the allelopathic activity of the plant. Studies on allelochemicals of alfalfa in various sources such as root exudates, fallen leaves are also required in the future.

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