Korean J. Crop Sci. 45(2): 134~138(2000)

Effect of Seed Leachates of 'Vernal' Alfalfa on Inhibition of Alfalfa Germination and Root Growth

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ABSTRACT: Most parts of alfalfa plant have been reported to contain autotoxic substances that inhibit seed germination and early seedling growth, however, the chemical(s) is not still studied much. Effect of seed leachates of 'Vernal' alfalfa (*Medicago sativa* L.) was evaluated for inhibition of alfalfa germination and root growth through bioassay. Alfalfa seeds were extracted in 1 L deionized water for 1 h after soaking and the leachates caused to reduce root length of alfalfa significantly as the soaking time increased. Crude seeds at 4 g L^{-1} exudated autotoxins that reduce significantly root length by 34% compared to the control, when the seeds soaked in deionized water for 24 h. However, the extracts did not affect final germination as well as speed of germination. Extracts from ground seeds significantly reduced speed of germination (GT 50) and root length. The results indicate that release of autotoxic substances fom seeds during seed imbibition was increased with increase of soaking time and seed amount, and that autotoxicity was more occurred in ground seeds than in crude seeds.

Keywords **:** alfalfa seed, autotoxicity, germination, root growth.

Autotoxicity, as a specialized form of allelopathy by which plants have detrimental effect on other plants of the same species (Putnam, 1985), has been verified especially in the effects of older alfalfa plants on new seedlings (Tesar, 1993). Afalfa (*Medicago sativa* L.), as a perennial legume forage crop, contains water-soluble compounds that are phytotoxic to same species (Chung and Miller, 1995; Hall and Henderlong, 1989; Hedge and Miller, 1992; Miller, 1983).

In alfalfa and many other species, the studies of autotoxic effects have been done on a germinating seed or seedling, but the full range of responses has not been determined. The effect of alfalfa autotoxicity on seeding immediately after alfalfa has been observed for a long time, and many general principles have been learned about the nature of the response and its management (Tesar, 1993; Miller, 1996). Although chemicals such as ferullic (Miller, 1996; Newby *et al.*, 1980) and saponin (Guenzi *et al.*, 1964; Miller, 1996), phenol-like compounds (Hall and Henderlong, 1989; Read and Jensen, 1989), medicarpin (Dornbos *et al.*, 1990), vanillic, hydroxybenzoic, *p*-coumaric acid (Newby *et al.*, 1980), and chlorogenic acid (Miller, 1996) have been implicated, there is no chemical that has been proven unequivocally to be the primary cause. This makes it very difficult to understand the problem and fully develop sound solutions.

During imbibition or bioassay, seeds could release diverse water-soluble compounds that influence other plant or itself. Initial water uptake by seeds is accompanied by the release of a large volume of gas and by a rapid leakage of substances, e.g. sugars, organic acids, and amino acids. Seeds absorbing some amounts of water could lose water-soluble materials by diffusion during imbibition. Such factors as the soaking duration, water temperature, aeration during soaking, and amount of water must be considered in evaluating the effect of seed leachates on germination and growth. Soaking injury could result from a lack of oxygen during imbibition, bacterial action, and harmful effect of pure water on imbibing tissues, leaching out of essential compounds or any combination of these (Woodstock *et al.*, 1981). Larson (1968) reported that water absorption by seeds with seed coat was slower than by seeds without seed coats. Removal of the seed coat allowed rapid imbibition resulting in seed injury presumably because of the loss of solute that included monosaccharides, disaccharides, amino acids, and other nitrogen containing compounds.

Water extracts of seeds, shoots, or roots of many plants have been reported to inhibit seed germination and early seedling growth (Evenari, 1949; Chung and Miller, 1995) and have been considered to be allelopathic. Inhibition of germination was not a good measure of phytotoxicity since seedling growth was often inhibited when germination was not (Cope, 1982). Chung and Miller (1995) and Chon *et al.* (2000) demonstrated that radicle or root length of alfalfa was more sensitive to extract than seed germination or hypocotyl length. McKee *et al.* (1971) found that water leachates of crown vetch (*Coronilla varia* L.) seeds caused abnormal seedling development but did not affect germination in most of 48 species.

Buta *et al.* (1987) reported that grass seed leachates were separated into organic and inorganic fractions using XAD-2

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polystyrene resin. The results show that organic fractions were inhibitory to lettuce seedling growth. Inorganic fractions were also inhibitory to lettuce seedling growth at high concentration but were stimulatory at low concentrations. However, germination of lettuce was not affected by leachate or their fractions. Elmore (1980) reported that the content of free amino acids known to inhibit germination of crop seeds were identified and quantified from velvetleaf (*Abutilon theophrasti*) seed and are responsible for the allelopathic effects. Grassel and Holm (1964) determined that substances diffusible from seeds of velevtleaf (*Abutilon theophrasti*ed Medic.) inhibited the germination of several group seeds including *Medicago sativa* L., *Raphanus sativa* L., *Brassica rapa* L. and *Lycopersicum esculentum* Mill. They further determined that the germination inhibitor(s) was associated with the free amino acid fraction, although they did not identify the individual components of this fraction. Aqueous extracts of the ground seeds of thirteen weed species delayed germination of the crop species due to free amino acids emanating from the seeds.

Chung and Miller (1995) ranked autotoxic effects of different plant parts of alfalfa as leaf (greatest), seed, root, flower, and stem (least). Emmert *et al.* (1998) reported that canavanine exuded from alfalfa seeds affect the growth and population biology of *Bacillus cereus* UW85, which is a biological control agent that suppresses disease of alfalfa (Handelsman *et al.*, 1990). The objectives of this study were a) to determine the effect of seed extracts on seed germination and seedling growth as affected by different soaking time and seed amount, and b) to make sure the difference in the autotoxic effects between extracts from crude and ground seeds through bioassay.

MATERIALS AND METHODS

Soaking Time of Seeds

To know autotoxic effects of exudates releasing during seed imbibition on root growth of alfalfa as affected by different soaking time, each 40 g seeds were separately soaked in 1 L deionized water for 1, 2, 3, 4, 5, 6, 12, 24, and 48 hours. The water containing the seed exudates after soaking was decanted, filtered with Whatman No.1 paper, and centrifuged at 3000 rpm for 2 hours. The supernatant was filtered through Whatman No. 42 paper. The extract was mixed in a 1:1 ratio with the autoclaved agar solution to give final concentrations of 20 g L^{-1}.

Amount of Soaking Seeds

To determine the degree of autotoxic effect under different seed amounts, seeds at 1, 2, 4, 8, 16 and 20 g were separately soaked in 1 L deionized water for 24 hours at 22°C in lighted room. The water containing exudates after soaking was collected and filtered with Whatman No. 1 paper. The extract was centrifuged at low speed (3000 rpm) for 2 hours. The supernatant was vacuum filtered through Whatman No. 42 paper, and mixed in a 1:1 ratio with the autoclaved agar solution to give final concentrations of 0.5, 1, 2, 4, 8, 10 g L^{-1}.

Extraction from Crude and Ground Seeds

Seed samples were divided into two groups. One group was extracted directly from dry-crude seeds by soaking in water for 24 hours at 24°C in a lighted room. The other was ground by homogenizing, then extracted. Extracts after soaking was collected and filtered with Whatman No. 1 paper. The extract was centrifuged at 3000 rpm for 2 hours. The supernatant was vacuum filtered through Whatman No. 42 paper, and mixed in a 1:1 ratio with agar solution to give final concentrations of 5, 10, and 20 g L^{-1}.

Bioassay and Data Analysis

Before mixing extracts with agar, Difco Bacto agar (16 g L^{-1}) was autoclaved for 30 min at 125°C and then equilibrated in a 50°C water bath along with a flask of stock extract. The stock extracts from seeds were mixed in a 1:1 ratio with the agar solution to give the desired final concentration. About 10 ml of extract-agar or water-agar (control) were poured into 9 cm-diameter plastic Petri dishes, covered, and allowed to solidify for 4 hours at room temperature. For all experiments viable seeds of Vernal alfalfa were surface sterilized for 15 min in sodium hypochlorite (0.525 g L^{-1}) rinsed, imbibed for 12 hours in deionized water at 22°C and carefully blotted using a folded paper towel. Twenty swelled seeds were distributed evenly on the extract-agar surface in each petri dish. The petri dishes were covered, sealed by wrapping in parafilm, and placed flat in a growth chamber programmed at 24°C during the 14-hour light period and 22°C during the dark period. Plates were illuminated at 400 mmol photons m^{-2} s^{-1} PAR provided by a mixture incandescent and fluorescent lamps. Number of germinated seeds (radicles 1-mm long) was determined at 12-hour intervals over a defined period. The data of germination were transformed to express GT 50, the time to reach 50% of the final germination by fitting the appropriate regression model using the regression procedure. Hypocotyl and root lengths were measured on all seedlings in each petri dish at 120 hours after placing seeds on the medium. When the F-test was significant ($p<0.05$) means were separated on the basis of least significant difference (LSD).

RESULTS AND DISCUSSION

Soaking Time of Seeds

Root growth was reduced by extracts and the reduction was dependent on soaking time. Hypocotyl length was not affected at all soaking time, whereas root length of alfalfa showed from 16 to 53% reduction at soaking time of 1 hour to 24 hours compared to the control. With increasing soaking time, extracts showed increased inhibitory effect on root growth. But there were no differences in phytotoxic effects on germination and hypocotyl growth by soaking time. After 1 hour, exudates from seeds were inhibitory to the alfalfa root growth (Fig. 1). Seeds absorbing some amounts of water lost water-soluble materials by diffusion during imbibition. The results showed that the soaking time of seeds influenced degree of autotoxicity, and that initial water uptake of seeds was occompanied by a rapid leakage of autotoxin(s).

Amount of Soaking Seeds

Root length was significantly declined as the concentration of seed extract increased from 0.5 to 10 g L^{-1}. At concentrations up to 2 g L^{-1} the root length (42 mm) was not affected, but seed extract at 4 g L^{-1} reduced significantly root length by 20% (34 mm) and at 8 g L^{-1} by 48% (22 mm), respectively, compared to the control (Fig. 2).

These results corroborate the report of Chung and Miller (1995) that seed extracts exhibited a pronounced reduction in radicle elongation of alfalfa. Hypocotyl, however, was less affected by all concentrations of extracts (Fig. 2). Hypocoty length was not very sensitive to the extract concentrations. We regularly observed the hypocotyl arching upward shortly after its growth initiated, thus escaping direct contact with the extract (Chon *et al.*, 2000). Chon *et al.* (1998), in their preliminary study on comparative effects of extracts from other plant parts, reported that extracts from seeds were less autotoxic than either those from leaves or stems. This study showed that leaf and stem extracts at 4 g L^{-1} reduced root length by 87 and 72%, respectively, while seed extracts at the same concentration reduced root length by 20%.

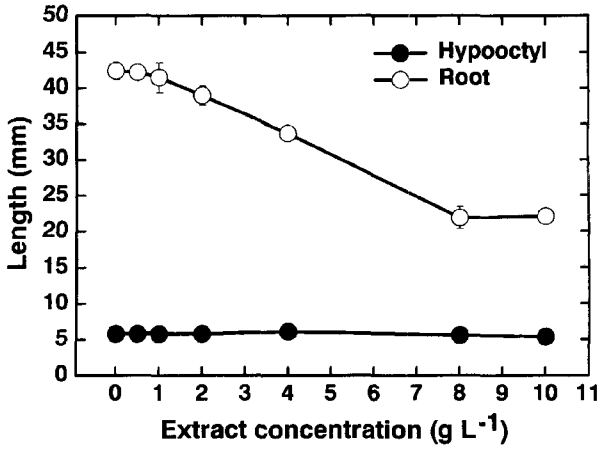

Fig. 2. Effect of alfalfa seed extracts on root and hypocotyl length of alfalfa at 5 days after placing imbibed seed on extract-agar as affected by different seed extract concentrations. Each bar represents standard error of the mean.

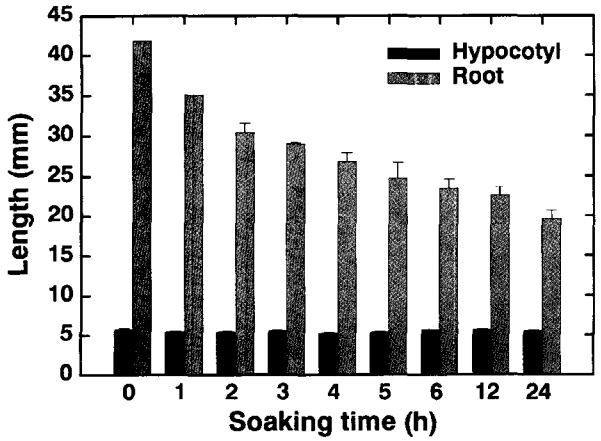

Fig. 1. Effect of alfalfa seed extracts on root and hypocotyl length of alfalfa at 5 days after placing imbibed seed on extract-agar as affected by different soaking time. Twenty grams of seeds were extracted in 1 L deionized water at 24°C in lighted room. Each bar represents standard error of the mean.

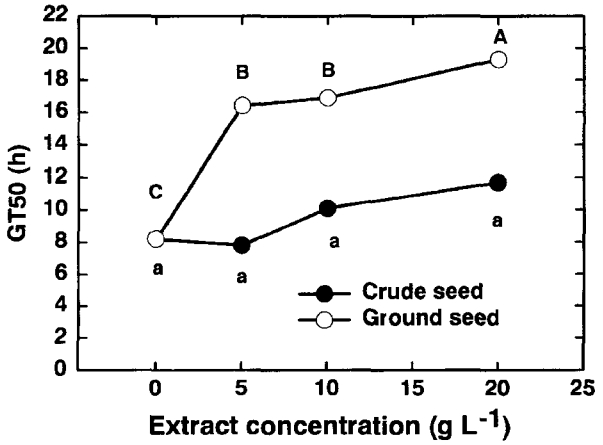

Fig. 3. Comparison in autotoxic effects between extracts from crude and ground alfalfa seeds on the time needed to reach 50% germination (GT 50) as the parameter of germination speed. The same letters are not significantly different at 0.05 probability level by Fisher's LSD test.

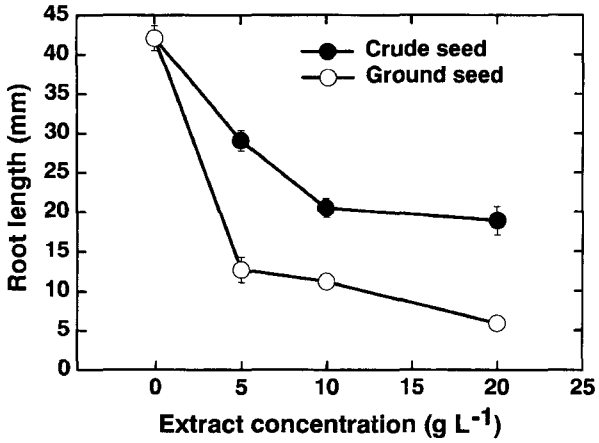

Fig. 4. Comparison in autotoxic effects between extracts from crude and ground alfalfa seeds on hypocotyl and root length of alfalfa at 5 days after seeding. Each bar represents standard error of the mean.

Extraction from Crude and Ground Seeds

Extracts from crude seeds did not affect seed germination and speed of germination. However, extracts from ground seeds reduced speed of germination (GT 50) significantly compared with the control (Fig. 4). Chung and Miller (1995) also reported that seed extracts reduced final germination to 39% at the 12 g L^{-1} extract concentration. Extracts from ground seeds reduced root length more than did that from crude seeds. Mean root length at extracts of 20 g L^{-1} from crude and ground seeds was 14 and 45% of control, respectively. Exudates from ground seeds contained more water soluble substance(s) which was inhibitory to root length than exudates from crude seeds. It is thought that ground seeds were more extratable than crude seeds. These results were supported by Larson (1967) who found that exudates from seeds without seed coats contain more solute than those from seeds with seed coats

CONCLUSION

Initial water uptake of seeds was at the same time accompanied by a rapid leakage of phytotoxic substance(s) during imbibition. We found that seed autotoxicity based on water-soluble extracts was increased with increase of soaking time and seed amount. Ground seeds exudated autotoxic substance(s) than crude seeds. Root growth was more sensitive to the autotoxic chemical(s) than was hypocotyl growth or seed germination. Results of this study indicate that growth effects in the bioassay are dependent on soaking time and concentrations of the leachate solutions, and that ground seeds were more extractable than crude seeds. Even though seeds imbibed for 12 hours put on medium during bioassay, some autotoxic chemical(s) could come out from the seed coats or the seeds themselves and affect the seed germination and seedling growth of test plant. Therefore, it should be considered that the effects of autotoxic or allelopathic chemical(s) could be released from seed itself on growth medium during assay.

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