Study on the Allelopathy of Alfalfa (*Medicago sativa* L.)

I. Observation of allelopathy and survey for substances inducing growth inhibition*

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Received July 10, 1992

Abstract : Allelopathic effects of alfalfa (*Medicago sativa* L.) plants were investigated using soil previously grown with alfalfa (alfalfa soil). Alfalfa soil inhibited the emergence of alfalfa seeds and also the growth of alfalfa and radish. Methanol extracts from the alfalfa soil markedly inhibited the elongation of radish hypocotyl and alfalfa radicle, suggesting the presence of allelopathic substances. The incorporation of cut pieces of alfalfa shoots in the soil reduced seed emergence, hypocotyl elongation and dry weight in radish seedlings to 65, 73 and 81%, respectively, compared to the control. Aqueous extracts of alfalfa plants inhibited the elongation of radish hypocotyl and alfalfa radicle. Inhibition increased with increasing concentrations. Inhibition of radish hypocotyl and alfalfa radicle elongation was greater in root extracts than in shoot extracts. The presence of phenolic compounds as allelopathic substances in alfalfa soil, as well as in the alfalfa plant, was suggested using color reaction with diazotized sulfanilic acid.

Key words : Alfalfa, Allelopathy, Growth inhibitors, Growth injury by continuous cropping, *Medicago sativa*.

アルファルファのアレロパシーに関する研究 第1報 アルファルファのアレロパシーの観察とそれに関与する物質の検討 : 中久加菜・続 栄治・三溝孝司 (鹿児島大学連合大学院・宮崎大学農学部・長崎県庁)

要 旨 : アルファルファのアレロパシー現象を解明する目的のもとにアルファルファの植生土壌並びにアルファルファの水及びメタノール抽出液を用いて圃場及び室内実験を実施した。アルファルファの植生土壌はダイコンの出芽を抑え、アルファルファ及びダイコンの初期生育を抑制した。アルファルファの植生土壌のメタノール抽出液はアルファルファ及びダイコンの初期生育を著しく抑制した。アルファルファの地上部を土壌に混和し、アルファルファ及びダイコンの発芽ならびに初期生育を調査した結果、アルファルファの土壌混和処理はアルファルファ及びダイコンの発芽を抑制し、ダイコンの初期生育を抑制した。アルファルファの植生土壌及びその地上部のメタノール抽出液にはアレロパシー物質が存在し、これらの化学物質の中にはフェノール系化合物が含まれていることが示唆された。

キーワード : アルファルファ, アレロパシー, 生長抑制物質, 連作障害。

Alfalfa is widely cultivated in many regions of the world because it is easy to cultivate and useful as forage for domestic animals. Reduction of seed germination and seedling growth is known to take place in alfalfa when it is cultivated continuously in the one field^{3,5)}. Investigators have suggested several factors such as microbial activity, allelopathy and nutrient deficiency as possible causal factors in injury or continuous cropping^{2,3,6)}. Recently, Ramesh and Miller⁴⁾ suggested that growth reduction is caused by the leaching and exuding of toxic substances from alfalfa into the

soil by continuous cropping of alfalfa⁴⁾. Although this evidence strongly suggests the existence of toxic substances, their chemical features remain obscure. The present experiment was conducted to investigate the effects on seed germination and growth of alfalfa and radish of soil previously cropped with alfalfa and of the incorporation of alfalfa shoots into soil. The toxic substances in the aqueous and methanol extracts of alfalfa soil and in its shoots were also surveyed.

Materials and Methods

1. Observation of allelopathy

Experiments were conducted at the University Farm, Miyazaki University in 1988 and

* Part of this paper was presented at the 192nd meeting of the Crop Science Society of Japan in Nara in November 1991.

1989. Alfalfa (*Medicago sativa* L.) was grown in Wagner pots (1/2000 are) filled with field soil. Five months after planting, shoots were removed from the pots and 50 alfalfa and 25 radish seeds were sown in each pot. Ten days after sowing, 30 alfalfa and 10 radish seedlings were collected at random from each pot and their dry weight measured. The pots without alfalfa plants were used as controls. These soils had previously been grown with alfalfa (alfalfa soil), sweet potato, *Ipomoea batatas* Lam. (sweet potato soil) and soybean, *Glycine max* Merr. (soybean soil) was packed in unglazed pots (30 cm × 25 cm). Twenty-five radish seeds were sown in each pot, and emergence was recorded daily for 5 days.

One hundred grams of alfalfa shoots were cut into small pieces and then incorporated into Wagner pots (1/5000 are) containing field soil. Four days after the incorporation, seeds of alfalfa and radish were sown in each pot, and the dry weight was determined 10 days after sowing. These experiments were conducted with two or three replications.

2. Effect of methanol extracts of soils previously planted with alfalfa on the growth of radish

One kilogram of alfalfa soil was passed through a 2 mm — screen, extracted with 700 ml of 70% methanol for 24 hours, and then filtrated. The extracts were concentrated in a small volume under reduced pressure at 35°C. The concentrated solution was exposed to bioassay using radish seedlings.

3. Effect of aqueous extracts of alfalfa on early growth of alfalfa and radish

One hundred grams of shoots and roots of alfalfa were cut into small pieces and crushed by mixer (Hitachi VA-W 25), and then immediately extracted with 300 ml of distilled water for 24 hours and filtrated. Aqueous extracts were serially diluted to 1/2 to 1/5 of the original solution with distilled water. Each solution was exposed to bioassay.

4. Charcoal column and thin-layer chromatography

One hundred grams of alfalfa shoots were extracted with 70% methanol as described previously. The extracts were concentrated in a small volume under reduced pressure at 35°C. The concentrated solution was subjected to chromatography on a charcoal column (activated charcoal, Wako Pure Chemical

Industries. LTD., 1=25 mm, id=10 mm) with successive elution (acetone-water) to yield active fractions. Each fraction was evaporated to dryness and then used in a bioassay.

The fractions from the charcoal column which showed inhibiting activity for radishes were concentrated to a small volume and subjected to silicagel, (Merck) thin-layer chromatography using a mixture (ethanol: water, 3:2) as a developing solvent. The chromatogram was dried and fluorescence patterns were observed under Ultraviolet light (254 nm).

5. Bioassays

Radish (*Raphanus sativus* L., var. Hoshiriso) and alfalfa were used as test plants. Twenty seeds of alfalfa and radishes were planted on filter paper in a petri dish which contained 7 ml of test solution and distilled water. The germinated seeds were counted daily. Test plants were also planted on filter paper in a glass tube (55 mm × 35 mm) which contained 1 ml of test solution and distilled water. Petri dishes and glass tubes were then placed in a growth chamber set at 25°C under fluorescent light (about 5000 lux). Five days after incubation, the lengths of hypocotyl and root were measured.

Results and Discussion

1. Effect of soils previously cropped with alfalfa, sweet potato and soybean on early growth of alfalfa and radish

As shown in Fig. 1, compared with sweet potato and soybean soils, alfalfa soil reduced the rate of emergence of radish seeds. Alfalfa soil also significantly decreased the dry weight of alfalfa and radish seedlings in comparison with the control (Table 1).

Incorporation of alfalfa shoots into the soil reduced the rate of emergence to 65% of the control, hypocotyl length to 73%, and dry weight of radish to 81% (Table 2).

The incorporation also reduced the rate of emergence of alfalfa seeds (Fig. 2). Recently, Hall and Henderlong²⁾ reported that soil previously planted with alfalfa inhibited the emergence of alfalfa seeds²⁾. Their results suggested the presence of autotoxic substances in the soil.

The methanol extracts of alfalfa soil reduced the radicle length of alfalfa to 11% as

Fig. 1. Effect on the emergence of radish seeds of soil previously cropped with alfalfa, soybean and sweet potato.

Table 1. Effect on the dry weight of alfalfa and radish seedlings of soil previously planted with alfalfa.

Treatment	Alfalfa (g)	Radish (g)
Control	0.099 ± 0.008 (100)	0.285 ± 0.022 (100)
Alfalfa soil	0.071 ± 0.001 (72*)	0.182 ± 0.017 (64**)

*, **: Significant difference at 5 and 1% levels. () ; Percent compared to value of control.

compared with the control and completely inhibited the elongation of radish hypocotyl (Table 3). These results indicate that soil previously planted with or incorporated with alfalfa exhibits an allelopathic effect on the growth of the same or other plants.

2. Effects of aqueous extracts of alfalfa on seed germination and early growth of alfalfa and radish

Aqueous extracts of alfalfa shoots reduced the germination of alfalfa to 35% and radish seeds to 80% only when a high concentration was used (Table 4). In addition, the average length of time necessary for germination was prolonged when the aqueous extracts were

Fig. 2. Effect on the emergence of alfalfa seeds of alfalfa plants incorporated into soil.

Table 2. Effect on the growth of radish of the incorporation of alfalfa plants into soil (1988).

Treatment	Emergence (%)	Hypocotyl length (cm)	Dry weight (g)
Control	92 (100)	4.54 ± 0.46 (100)	0.323 ± 0.02 ¹⁾ (100)
Incorporated	60 (65)	3.34 ± 0.52 (73*)	0.263 ± 0.02 (81*)

1) ; Experiment in 1989

*; Significant difference at 5% level.

() ; Percent compared to value of control.

added, compared to the control. The differences were significant in high concentrations.

The effect of aqueous extracts from alfalfa shoots and roots on the growth of alfalfa is shown in Fig. 3. Inhibitory activity to radicle growth in extracts was much higher in alfalfa roots than in shoots. In particular, root extracts markedly inhibited the growth of alfalfa radicle even in a 1/3 dilution. Aqueous extracts of alfalfa also inhibited the elongation of radish hypocotyl (Fig. 3). Almost the same tendency for an inhibitory effect was observed in radish plants.

The results obtained here, combined with previously reported research that aqueous extracts of alfalfa inhibit the emergence of alfalfa seeds and elongation of seedlings of alfalfa and rice^{1,5)}, suggest that the alfalfa plant contains substances inducing allelopathy.

Table 3. Effect on the growth of alfalfa and radish of methanol extracts of alfalfa soil.

Treatment	Alfalfa radicle (cm)	Radish hypocotyl (cm)
Control	2.43 ± 0.53 (100)	3.67 ± 0.27 (100)
Alfalfa soil	0.31 ± 0.099 (11.3**)	— ¹⁾

1); Not measurable.

** ; Significant difference at 1% level.

() ; Percent compared to value of control.

3. Survey of allelopathic substances in alfalfa shoots by chromatography

The methanol extracts of alfalfa shoots were subjected to chromatography on a charcoal column with successive elution (acetone-

Fig. 3. Effect on the alfalfa radicle and radish hypocotyl of aqueous extracts of alfalfa.

Table 4. Effect on the germination of alfalfa and radish seeds of aqueous extracts of alfalfa shoots.

Treatment	Alfalfa			Radish	
	Dilution	Ratio of germination (%)	Average time required for germination (days)	Ratio of germination (%)	Average time required for germination (days)
Control		100	1.00	100	1.10
Dilution	1/5	100	1.80	95	1.32
	1/3	100	2.05	90	1.82
	1/2	100	2.90	85	1.83
	1	35	3.14	80	2.44

water) to yield active fractions (Fig. 4). The fractions, No. 1 to 3 from the charcoal column, reduced germination of radish seeds remarkably. Fraction No. 2, with the highest activity, was concentrated in a small volume and then analyzed by silicagel, thin-layer chromatography. The chromatogram was dried and examined under Ultraviolet light. Four spots were detected on the chromatograms. These were then eluted with distilled water from the chromatogram, and the eluates subjected to bioassay using radish plants. As shown in Figs. 5 and 6, the maximum inhibition was found at Rf 0.6-0.8 (B zone). The eluate obtained from the zone inhibited seed germination and elongation of hypocotyl of radish remarkably. The active zone turned yellow when sprayed with diazotized sulfanilic acid, suggesting the zone contained phenolic compounds.

It may be concluded that soils previously planted with alfalfa as well as the alfalfa plants themselves contain allelopathic substances. They are toxic to the growth of alfalfa and other plants. The chemical compounds may

Fig. 4. Radish germination bioassay of charcoal column chromatography fractions from methanol extracts of alfalfa.

* ; not observed.

Fig. 5. Radish germination bioassay of a thin-layer chromatography subfraction from Fraction 2 (acetone 10%) of charcoal column chromatography.

A ; Rf 0.0~0.6, B ; Rf 0.6~0.8, C ; Rf 0.8~1.0, D ; Upper solvent front.

Fig. 6. Radish hypocotyl bioassay of a thin-layer chromatography subfraction from Fraction 2 (acetone 10%) of charcoal column chromatography.

A ; Rf 0.0~0.6, B ; Rf 0.6~0.8, C ; Rf 0.8~1.0, D ; Upper solvent front.

* ; not observed.

play some part in growth inhibition due to the continuous cropping of alfalfa.

Acknowledgements

We thank Professor Keishi Shimokawa, Faculty of Agriculture, Miyazaki University, for his valuable advice. This study was supported by a Grant-in Aid (No. 61560017) from the Ministry of Education, Science and Culture, Japan.

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