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Effect of Alfalfa Extract, It's Concentration and Absorbents on Germination and Growth of Alfalfa

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알팔파 추출물, 추출농도 및 흡착제 처리가 알팔파 발아와 생육에 미치는 영향

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ABSTRACT : Crude aqueous extracts from dried leaves, stems, roots, and flowers from both field grown and greenhouse grown alfalfa plants inhibited alfalfa seed germination and seedling growth. The degree of inhibition was greater in the field grown plant extracts. Flowers extract of field grown plant most inhibited alfalfa germination and seedling growth. In the concentration study, the highest concentration of extract (9.0%, w/v) significantly inhibited total alfalfa seed germination by 50% as compared to control. In partitioning study using pot hydroponic culture of plant biomass into leaves, stems, root, LAR products of LWR and SLA exhibited significant variation among four species. This result support that the inhibitory effect of autotoxic substances presenting in alfalfa tissue may be possible interference with the patitioning of biomass into leaf component relative to the total biomass produced by the alfalfa plant. Toxicity of extract was not reduced by adding activated charcoal, Dowex-50W, amberlite to the extract. Toxic substances existing in most plant tissues but mainly above ground foliage are water soluble and stable and may persist in old alfalfa fields. Thus, it is recommended to remove as much as possible of the above growth parts, especially vegetative stage, before one tries to re-establish alfalfa in former field of alfalfa.

Key words : Alfalfa, Autotoxicity, Germination percentage, Seedling growth, LAR, LWR, SLA, Adsorbent

Alfalfa (*Medicago sativa* L.), a perennial legume forage crop, contains water soluble inhibitory substances which are autotoxic as well as inhibitory to other species. Autotoxicity is an intraspecific form or type of allelopathy that occurs when a plant species releases chemical substances that inhibit or delay germination and growth of the same plant species[17]. Many researchers have observed the phenomenon that fresh alfalfa shoots, roots and dry hay, old roots and soil residues release inhibitory compounds in the field, greenhouse, and laboratory [2, 3, 8, 10, 12, 15]. Miller [14, 15] has demonstrated the production and release of allelopathic chemicals which impact re-establishment of alfalfa stands. Al-

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so, Li [13] reported that alfalfa root aqueous extracts reduced alfalfa seed germination and root development when the extract was incubated at room temperature for one week. Pathogenic infections do not appear to be responsible for this inhibition [12]. When reseeding or interseeding alfalfa to thicken the stand, the incorporation of residues from old plants into the soil may produce water-soluble inhibitory substances that can effect new alfalfa seedlings [2].

In the study to solve of autotoxicity, fungicide treatment of seeds and herbicide spray treatments to rejuvenate an old alfalfa stand or to re-establish alfalfa into a pre-existing stand were usually not successful indicating an alleopathic reaction [5, 6].

The chemical mechanism of autotoxicity and allelopathy in alfalfa are poorly understood. Some researchers have identified inhibitory compound which are involved in alfalfa autotoxicity [3, 4, 7]. Hall and Henderlong [7] found alfalfa contains an autotoxic compound that characterizes a phenolic compound. Recently some chemical compounds have been isolated using the GC-MS as those that are related to alfalfa autotoxicity [4]. Medicarpin, Medicarpin 4-methoxymedicapin, sativan, 5-methoxysativan were isolated from alfalfa foliage. Among these compounds, medicarpin was implicated the allelochemical in alfalfa that causes autotoxicity. Medicarpin applied exogenously to alfalfa seeds reduced their germination by 58% after 6h when used in a filter paper bioassay [16]. More recently, chlorogenic, salicyclic acids were identified from fresh alfalfa leaves parts using HPLC method [3]. These compounds are found predominantly in the aqueous fraction of alfalfa tissue and seems not to be a direct result of microbial activity.

Interest in interplanting, double cropping, no-till planting and non-rotational cropping systems increase the need to understand allelopathy. Information on toxic compounds and the condition of growth where inhibition takes place is necessary to make a decision about repanting of alfalfa.

Thus, the present research was conducted (I) to determine which alfalfa plant extracts from both field grown plant and greenhouse grown plant contained more autotoxic substances; (II) to evaluate the effects of various concentrations of water extracts on seed germination and seedling growth and partition of biomass into leaves, stems, roots; (III) to determine whether or not the toxicity of the extract could be decreased with an adsorbent.

MATERIALS AND METHODS

1. Phytotoxicity comparison between field grown and greenhouse grown plant extracts

Mature fresh alfalfa plants, field and greenhouse grown, were separated into leaves, stems, roots and flowers, respectively. They were dried in a room temperature for 2 weeks and then were ground for Wiley mill with a 40-mesh screen and stored for a year in a plastic box. Ground each plant part was soaked in 100ml distilled water for 24 hour(h) at 24℃ in a lighted room to give concentration of 5% (w/v). These solutions were filtered through four layers of cheese cloth to remove the fiber debris and again filtered through filter paper (Whatman No. 42) and then centrifuged at a low speed (3,000rpm) for 4h. To prevent microorganism growth, the supernatant solutions were filtered using a 0.2 μm Nalgene filter ware

unit (Becton Dickinson Labware, Lincoln Park, NJ). These solutions were used for the phytotoxicity comparison between field and greenhouse grown plant extracts as the outlined below general bioassay procedures.

2. Concentration and biomass partition study with vegetative stage extracts

This experiment was conducted with water extracts of field grown plant harvested at the vegetative stage in petri dishes. Concentrations of 2.5, 5.0, 7.5 and 9.0% (w/v) were prepared by soaking dried ground tissue per 100ml of distilled water at room temperature for 24h in 250ml Erlenmeyer flasks. The next following procedures were the same outlined below general bioassay procedures. Pot hydroponic culture study shown in Fig. 1 was conducted to investigate the growth dynamics of alfalfa and to prevent root systems loss as pot sand culture occurred. 6 germinated seeds with 2.5cm in length per pot were placed in a growth pouch containing 5% (w/v) of dried tissue along with per 2,000ml of aerated Hoagland solution I [9]. Pot containing only Hoagland solution I was served as a control. This solution was added to the pots every 2 days to maintain the original volume of liquid. The alfalfa plants were grown at constant 26℃ and 16-h illumination /8-h darkness cycle for 18d. The photosynthetic photon flux density (PPFD) during illumination was 530 μmol photon $m^{-2}s^{-1}$. 18th day after seeding, the plants were harvested and separated into stem, leaf and root parts and measured for dry weight. Leaf area (LA) was measured by automatic leaf area meter (Type AAM5, Tokyo Hayashi Denco., Ltd., Japan.) Harvested plants were used to calculate LWR (leaf weight ratio), SWR (stem weight ratio), RWR (root weight ratio), SLA (specific leaf area) and LAR (leaf area ratio) with the some modifications procedures of Radford [18].

Fig. 1. The picture of pot hydroponic culture study.

3. Study of adsorbents treatment

Possibility of overcoming autotoxicity of the alfalfa was investigated by treating solid dried materials with activated charcoal, Amberlite and Dowex 50-W. The dried solid material procedue was applied with some modification of Chung [3] since MeOH extracts minus non-polar substances like lipids and chrolophyll was most inhibitory to alfalfa germination and seedling growth in hytotoxicity test. The fresh leaves harvested at vegetative stage(100g) were extracted by grinding with 80% MeOH in Waring blender for 10 min. The extract was filtered through four layers of cheese cloth, and then through filter paper (Whatman No. 1). The extracts were then concentrated under vaccum at 30℃ about 1/3 the original volume or until most of the MeOH had been removed. The resultant aqueous solution was washed with chloroform to remove nonpolar compounds such as lipids, glycerides, terpenes, and chlorophylls by shaking in a separatory funnel. This procedure was repeated three times and

the extracts combined. These chloroform washes are designated as aqueous extracts in the following discussion. The chloroform extracted aqueous layer was then concentrated by evaporation under vaccum on a rotary evaporator and freeze dried. Finally, solid dried materials (54g) were obtained from fresh leaves (1kg). Aqueous solution was made by dissolving solid dried material (1.5g) in 100ml distilled water. Amberlite (3g), and Dowex-50 (3g) were swollen in double distilled water (200ml) overnight. After swelling the adsorbents, distilled water was drained off and each adsorbent was treated with alfalfa aqueous solution filtered using a 0.2 μm Nalgene filter ware unit (Becton Dickinson Labware, Lincoln Park, NJ). Treatment with activated charcoal was a little different from that of other adsorbents. 5g of activated charcoal was directly applied with alfalfa aqueous extract. These treated extracts were used for the bioassay with an untreated aqueous extract and distilled water as a control. The next following procedures were the same below general bioassay procedures.

4. General bioassay procedures

The germination test was conducted as follows : one hundred alfalfa seeds (WL-320) were surface sterilized at the ratio of 10:1 (water: clorox) and rinsed with distilled water several times and were evenly placed on filter paper (Whatman No. 1) in sterilized 9cm petri dishes moistened with each extract. 8ml of each extract solution were added to a petri dish and distilled water was used as a control. All petri dishes were then placed in a lighted room at 24℃. Germination was determined by counting the number of germinated seeds at 24h intervals over 4 days period and expressed as total percent germinated. Radicle and hypocotocyl lengths were determined 5 days after seeding by measuring 24 representative seedling. After measuring the root and shoot lengths, the seedlings were separated and dried into leaves, stems and roots parts for measuring dry weight.

5. Statistical analysis of data

All of the above mentioned experiments were repeated twice under four replication with completely randomized design. Analysis of variance for all data was accomplished using the general linear model procedure of the statistical analysis system (SAS) program [20]. The pooled mean values were separated on the basis of least significant difference (LSD) at the 0.05 probability level.

RESULTS

1. Phytotoxicity comparison between field grown and greenhouse grown plant extracts

Extracts of both field and greenhouse grown plants inhibited seed germination and reduced seedling growth (Tables 1 and 2). Germination of the seeds treated with distilled water began after 1st day of seeding and exceeded 95% in 3th days (Table 1). However, germination of the seeds treated with the other extracts was significantly inhibited and did not reach 90% germination until 4th days after seeding. The total germination percentage among treatments except greenhouse grown stem extract were significantly different as compared to the control. An especially high degree of inhibition was produced by the field grown flower extracts. Flower extract of field grown plant was most

Table 1. Phytotoxicity comparison of leaves, stems, roots and flowers extracts of greenhouse and field grown alfalfa on alfalfa germination

Treatment		D[1]	D[2]	D[3]	D[4]	TOTGERM(%)
Control (Water) Extract		44.0a	39.5a	13.8	1.5c	98.8a*
Leaves	FGA[1]	8.3f	13.8d	20.0cd	17.0b	67.0de
	GHA[2]	11.5d	23.0c	34.8a	14.0b	83.3bc
Stems	FGA	15.3c	27.3c	24.0bc	18.8b	85.3bc
	GHA	23.5b	25.0c	26.0b	12.8b	87.3b
Roots	FGA	11.3de	14.3d	24.8bc	16.8b	67.0de
	FHA	15.3c	33.3b	34.5a	6.5c	89.5ab
Flowers	FGA	7.8f	13.5d	14.8e	15.0b	51.0ef
	GHA	8.8ef	10.5d	17.8de	38.5a	75.5cd
CV(%)		10.7	17.9	15.2	27.5	10.0

[1]FGA, Field Grown Alfalfa ; [2]GHA, Greenhouse Grown Alfalfa.

* Values within a column followed by the same letter are not significantly different at the 0.05 level as determined by least significant difference (LSD).

Table 2. Phytotoxicity comparison of leaves, stems, roots and flowers extracts of greenhouse and field grown alfalfa on alfalfa seedling development

Treatment		RL[1]	SL[1]	TOTL[1]	RW[1]	SW[1]	LW[1]	TOTW[1]
Control (Water) Extract		5.3a	3.3a	8.6a	0.60a	0.93a	1.70a	3.23a*
Leaves	FGA[2]	3.4d	3.1bc	6.5d	0.38cd	0.58d	1.15f	2.10f
	GHA[3]	3.7c	3.1bc	6.8c	0.40c	0.80b	1.50c	2.70c
Stems	FGA	3.2e	3.0c	6.2e	0.38cd	0.68c	1.28e	2.33e
	GHA	4.0b	3.2ab	7.2b	0.48b	0.90a	1.60b	2.98b
Roots	FGA	2.8f	2.8d	5.6f	0.35cd	0.75b	1.40d	2.50d
	GHA	3.7c	2.8d	6.5d	0.40c	0.60d	1.30e	2.30e
Flower	FGA	2.3g	2.7d	5.0g	0.33d	0.50e	1.00g	1.83f
	GHA	3.1e	3.0c	6.1e	0.35cd	0.55de	1.10f	2.00f
CV(%)		3.8	3.1	2.8	10.6	5.7	2.8	3.4

[1] RL, Root Length; SL, Shoot Length; TOTL, Total Length; RW, Root Weight; SW; Shoot Weight.

[2] FGA, Field Grown Alfalfa ; [3] GHA, Greenhouse Grown Alfalfa.

* Values within a column followed by the same letter are not significantly different at the 0.05 level as determined by least significant difference (LSD).

inhibitory to total germination percentage (51%). All of the extracts also inhibited either root or shoot growth (Table 2). The root growth of seedling treated with each extract was not well-developed. This extract caused most root brownish and black tips. The growth of root was more sensitive than that of shoot treated with each extract. A inhibitory effect on total seedling length was caused by root growth inhibition rather than shoot growth inhibition.

2. Concentration and growth study with vegetative stage extracts

The effect of various concentrations of plant extract on seed germination are shown in Table 3. As the extract concentrations increased from 2.5% to 9.0%, total alfalfa germination percentage was significantly decreased. In the highest concentration of extract (9%), seeds were germinated about 18% of the control number at 1st day, and by 4th day, they germinated only 50% as compared to the control. The lowest concentration (2.5%) of the extract significantly inhibited total seed germination by 15% of the control. In the biomass partitioning study by pot hydroponic culture, vernal cultivar partitioned more biomass roots and less into leaves and stems than other species, but DK-125 cultivar partitioned the most biomass into stems and roots (Table 4). The distribution of leaf biomass as leaf area was greatest in vernal cultivar, as indicated by their low specific leaf area (SLA), reflection its thicker leaves with low SLA. DK-125 cultivar had the lowest LAR, followed by WL-320 cultivar.

3. Study of adsorbents treatment

The extracts treated with adsorbents did not increase seed germination, or improve root or shoot growth as compared to the untreated extract. Activated charcoal, Dowex 50-W and Amberlite were not effective in

Table 3. Effect of various concentrations of vegetative stage extracts on alfalfa germination

Treatment(%)	D^1	D^2	D^3	D^4	TOTGERM(%)
Control (Water)	44.0a	39.5	13.8e	1.5c	98.8a*
2.5	31.0b	34.0bc	14.0a	3.8ab	83.8b
5.0	15.3c	43.0a	4.8b	4.3a	67.3c
7.5	8.8d	34.8bc	15.0a	2.0bc	60.5c
9.0	7.8d	32.5c	4.8b	4.8a	49.8d
CV(%)	3.1	6.5	2.3	1.8	6.9

* Values within a column followed by the same letter are not significantly different at the 0.05 level as determined by least significant difference (LSD).

Table 4. Effect on bimass partition of different cultivars by alfalfa extracts harvested at 18th after seeding

Species	LA^1	LWR^2	SWR^3	RWR^4	SLA^5	LAR^6
	cm^2	·····mg/mg·····			·····cm^2/mg·····	
Vernal	690a	0.59a	0.25c	0.16c	0.50c	0.29abc*
Arrow	634a	0.55b	0.27bc	0.17bc	0.58ab	0.32ab
WL-320	4.84b	0.52c	0.29ab	0.18abc	0.53bc	0.28bc
Dawn	5.02b	0.41c	0.30ab	0.19ab	0.64a	0.33a
DK-125	3.84c	0.49d	0.32a	0.20a	0.55bc	0.27c

1:Leaf Area(LA), 2:Leaf Weight Ratio(LWR), 3:Stem Weight Ratio(SWR), 4:Root Weight Ratio(RWR), 5:Specific Leaf Area(SLA), 6:Leaf Area Ratio(LAR)

* Values within a column followed by the same letter are not significantly different at the 0.05 level as determined by least significant difference (LSD).

Table 5. Effect of various adsorbents treatment on alfalfa germination and seedling growth

Treatment	Germination Percentage (%)	Total Length (cm)	Total Weight (mg)
Control	91.3	8.4	2.0
Untreated Extract	74.5	6.2	1.7
Charcoal Treated Extract	76.0	5.6	1.7
Dowex 50-w Treated Extract	75.0	6.3	1.6
Amberlite Treated Extract	72.0	6.5	1.6
LSD (0.05)	4.4	0.3	0.1

overcoming the inhibition of alfalfa aqueous extract (Table 5). On the contrary, treatment with these adsorbents enhanced the inhibition of the extract. Extract treated with amberlite significantly lowered total alfalfa germination percentage (72%), and extract treated with charcoal caused the lowest total seedling length (5.6cm). Dowex-50W and amberlite aqueous treatments inhibited total seedling weight by 20% as compared to control.

DISCUSSION

Strong evidence was presented to support the hypothesis that alfalfa is highly autotoxic and produces toxins that inhibit its re-establishment in old alfalfa sward fields [2, 3, 8, 10, 12]. Environmental factors affect the amount of toxic substances produced in alfalfa plant. In the phytotoxicity comparison between field and greenhouse grown plant extracts, aqueous extracts of field grown plant were more toxic to alfalfa germination and seedling growth than those of greenhouse grown plant (Tables 1 and 2). This result was similar to that of Rice[19] that plants grown in greenhouse do not produce as large quantities of inhibitors, chlorogenic acid, scopoletin, as the same kinds of plants growing in the field. This suggest that light quality plays an important role in the production of toxic substances. Greenhouse grown alfalfa plants may produce only limited quantities of toxic substances since ultraviolet (UV) light is absent in closed greenhouse. Plants vary their production of allelopathic chemicals depending upon the environment where they are grown and the stresses that they meet. All factors including soil acidity and microorganism population should be considered when growing alfalfa plants. Among separated plant parts, flower extracts were most inhibitory to seed germination. These results are also similar to those of Ballester et al. [1] who reported that the most inhibitory effect of Erica plants was released by flower extract. These results indicate that the alfalfa replant problem, autotoxicity, may be due to toxic substance released from alfalfa most tissues including flowers. This study suggests that a farmer must take a consideration whether or not it would be advisable to replant alfalfa based on the level of alfalfa plants still growing in the field. Also, to use natural compounds, this could be applied to enhance toxic substances production by exerting the proper stresses on the alfalfa plant. This is need to more investigate which environmental factors affect the quantity and quality of autotoxic substances produced in plants.

Result obtained in the concentration study of field grown alfalfa plant extracts at the

vegetative stage was similar to that of previous investigation [8]. As extracts concentration increased, germination inhibition percentage significantly increased (Table 3). Such a response may be due to the autotoxic principle increasing in the concentration in alfalfa plants with age of the tissue. This suggests that the autotoxic compounds may accumulate in sufficiently high quantity in the soil to cause autotoxicity. Toxic substances may be stable for a year or more since extracts from alfalfa tissues which had been harvested one year ago in this study still contained some toxic substance that caused inhibitory effects on seed germination and seedling development.

There was a significant difference in partitioning of plant biomass into leaves, stems, and roots among species in response to alfalfa residue extracts (Table 4). Among five species, significant variation in leaf area ratio (LAR) was produced by the differences in specific leaf area (SLA) combined with leaf weight ratio (LWR). The LAR is an important component of plant growth dynamics since it indicates the relative amount of photosynthetic assimilatory surface. A plant with a high LAR may have a higher relative growth rate than one with a low LAR in the same rates of photosynthesis per unit leaf area. Toxic substances of produced in alfalfa plant play significant functions in the influence of alfalfa residues on subsequent crop including alfalfa and weed growth by changing growth dynamics.

In the trial study to control alfalfa autotoxicity (Table 5), the result of adsorbents treatment was similar to that of Faix et al. [6] that adsorbents, during the treatment of the extracts with them, perhaps removed some of the compounds from the extracts that would otherwise counteract the growth inhibition, thereby did not reduce or destroy the toxic substances in the extract. It appears that alfalfa plant contains toxic substances that inhibit seed germination and seedling growth and inhibit plant functions. This autotoxic potentiality must be thought as part of the interference attributed to alfalfa in the field. This study provides the definitive evidence that when toxic substances produced by alfalfa plant are affected by environmental factors, they inhibit the germination and growth of alfalfa as an autotoxic plant inhibitor.

The change or quantification of toxic substances involved in alfalfa autotoxicity must be more investigated throughly in the future.

摘　　要

본 실험은 온실과 포장에서 재배한 알팔파를 잎, 줄기, 뿌리, 꽃 부분으로 나누어 수확, 건조, 하여 물 추출한 후 이 추출물들이 알팔파 발아와 생육에 미치는 영향을 조사 비교하였으며 포장상태에서 자란 알팔파 지상부 전체를 수확,건조, 추출하여 이를 농도별로 처리 알팔파 발아율을 검정하였고, pot실험을 통하여 알팔파 품종간 biomass 분배율 검정과, 알팔파 autotoxicity를 해결하기 위한 방법으로 추출물을 adsorbent로 처리하여 이들이 알팔파 독성성분을 변형시키는지를 검토하였다.

1. 온실과 포장에서 자란 알팔파의 추출물로 알팔파 발아 및 생육에 대한 영향을 비교한 결과 포장에서 자란 추출물질이 발아와 생육에 더 억제적으로 작용하였고, 특히 포장에서 생육한 꽃 추출물 처리가 가장 억제적으로 작용하였다.
2. 농도처리에 따르는 추출물 처리는 농도가 증가할수록 발아율이 억제되었고, biomass 분배율

에 대한 pot실험에서는 LWR 과 SLA의 산물인 LAR은 품종간 유의성을 보였다.

3. 알팔파 추출물에 대한 absorbents처리는 추출물의 독성을 변형시키지 못하였다.

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