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Allelopathic Effects of Cool-season Turfgrass Mixture Clipping Extract on Four Weed Species and Detection of the Phenolic Compounds

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Abstract
In order to determine aqueous extract effects of cool-season turfgrass mixture clipping on germination and seedling growth of four weed species, laboratory and outdoor pot experiments were done at the experimental laboratory and greenhouse of the Doroud Parks and Landscape Organization, located in Doroud, Lorestan, Iran. The first factor was the concentration of sport turfgrass clipping extract (5, 10, 15, 20, 25% and 10, 25, 50, 75% (w/v) at laboratory and outdoor pot experiments, respectively) and the second factor was the type of weeds [dandelion (*Taraxacum officinale*), plantain (*Plantago major*), prostrate pigweed (*Amaranthus blitoides*) and common bermudagrass (*Cynodon dactylon*)]. *A. blitoides* was the most sensitive in both experiments. In both of the experiments, roots were more susceptible than shoots. For identifying phenolic acids, gas chromatography mass analysis displayed that this extract contained trans-cinnamic acid, 3,4,5-trimethoxybenzoic acid, syringic acid, p-coumaric acid, caffeic acid, gentisic acid, protocatechuic acid and ferulic acid. The results demonstrated that sport turfgrass clipping extracts have an inhibitory property on germination and initial seedling growth of noxious weed species and could be potentially used as preemergence bioherbicide.

Keywords: Allelochemicals, GC-Mass, germination, weed.

Introduction

Allelopathy has been defined as any direct or indirect harmful or useful effects by one plant on another through the production of chemical compounds that are released into the environment (Rice, 1984). The visible physiological effects from allelopathy interactions are frequently observed as inhibited or delayed seed germination or reduced seedling growth which is secondary expressions of primary effects on metabolic processes (Kruse *et al*., 2000). Chemical inhibition of growth by one plant upon another has been demonstrated among several species (Rice, 1995). Many grass species have been identified as allelopathic. The identification of allelopathic turfgrass cultivars could be a valuable tool which can be used to enhance integrated pest management as well as reduce synthetic herbicide applications (Lickfeldt, 2001).

Fales and Wakefield (1981) identified creeping red fescue (*Festuca rubra* L.) as allelopathic, and tall fescue has been studied extensively in allelopathy studies (Peters and Luu, 1985; Smith and Martin, 1994). Plant

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inhibitory compounds have also been detected in *Agrostis tenuis* Sibth. (Norrington-Davies and Buckeridge, 1994), *Buchloe dactyloides* (Nutt.) Engelm. (Wu LX and Harivandi, 1998), *Cynodon dactylon* L. [Pers.] (Waller *et al*., 1985), *Lolium perenne* L. (Mattner and Parbery, 2001; McCarty *et al*., 2010; Wardle *et al*., 1992; Wardle *et al*., 1996; Zuk and Fry, 2006), *Pennisetum clandestinum* Hochst. (Chou *et al*., 1987), *Poa annua* L. (Brede, 1982), and *Poa pratensis* L. (Chung and Miller, 1995). It has been shown how the grass *Agropyron desertorum* inhibits the growth of the shrub *Artemisia tridentata* (Caldwell *et al*., 1991) and the grass *Elymus lanceolatus* (Huber-Sannwald *et al*., 1998). Further, Mahall and Callaway (1991) have documented similar processes between *Ambrosia dumosa* and *Larrea tridentata*.

The most widely used biological assays for allelochemicals are seed germination and seedling growth studies (Aliloo *et al*., 2012). Numerous laboratory experiments have been conducted, but they probably do not reflect processes under field conditions (Inderjit and Callaway, 2003; Inderjit *et al*., 2001). In field experiments, allelochemical treatments are more realistic. In this situation, allelopathy cannot easily be separated from competition for resources (Nilsen 2002; Viard-Cretat *et al*., 2009).

Turfgrass seed can be sown in the form of monoculture or in seed mixture (Akbari *et al*., 2001). It is often sown in mixtures consisting of different species and cultivars. Mixture of turfgrass species ensures genetic diversity and higher adaptive potential (Beard, 1973). Turfgrass stands composed of multiple species may be better able to withstand environmental and pest stresses compared to monoclonal communities (Watschke and Schmidt, 1992). Weeds are the chief problem in turf zones. They compete with turfgrass for resources such as light, water, nutrient and physical space. They may belong to grasses, grass-like plants or broad leaf plants in the turf area. Esmaili and Salehi (2009) reported that *Taraxacum officinale* and *Cynodon dactylon* were important weeds in turf areas at one site in Iran. Christians (2004) reported important weeds in turf areas in his book. Although allelopathic effects of individual turfgrass on other plants have been reported (Wu *et al*., 2002), no study has yet been done on the allelopathic effects of mixture turfgrass on important weed species.

The aim of this investigation was to determine allelopathy effects of turfgrass mixture extracts on germination and seedling growth of four noxious weed species including dandelion (*Taraxacum officinale*), plantain (*Plantago major*), prostrate pigweed (*Amaranthus blitoides*) and common bermudagrass (*Cynodon dactylon*) and detection of the type of phenolic compounds in turfgrass clipping extracts.

Materials and Methods

Experimental location

The research was conducted at laboratory with Petri dishes and outdoor pots, using the field soil as the growth medium, at the experimental farm of the Doroud Parks and Landscape Organization, located in Doroud, Lorestan, Iran.

Preparation of turfgrass clipping extracts

A sport turfgrass (consisting of *Lolium perenne* L. 20% 'Bartwingo' and 35% 'Barrage'; *Poa pratensis* L. 35% 'Baron'; and *Festuca rubra* spp. *commutata* (Thuill.) Nyman. 10% 'Bargreen') clippings were collected in September 2012 using a rotary mower and mowing at 4 cm height. Collected clippings were dried in an electrical oven at 75ºC for 48 h and then milled. In order to produce the extract, 80 g of powdered material was plunged in 400 ml of deionized water for 24 hours at 40º C using a water bath. The mixture was then filtered through Whatman No. 42 paper and diluted to following concentrations: 0, 5, 10, 15, 20 and 25% (w/v) for laboratory study at Petri dishes and 0, 10, 25, 50 and 75% for outdoor pots.

Laboratory experiment

Laboratory experiment was done in Petri dishes in a lab germinator (Spencers, Model No: 3330-SSG-001 series). Five ml of turfgrass extract and a deionized H_2O (as control) were applied separately to 20 seeds including *Taraxacum officinale* L., *Plantain plantago*, *Amaranthus blitoides* and *Cynodon dactylon* L. [Pers.] in 9 cm Petri dishes lined with Whatman #1 filter paper. The dishes were sealed with parafilm and placed under artificial light for 16 h days at room temperature averaging 25ºC.

Outdoor pot experiment

Twenty healthy seeds of *Taraxacum officinale* L., *Plantain plantago* L., *Amaranthus blitoides* L. and *Cynodon dactylon* L. [Pers.] were planted in 20 cm-diameter×25cm-deep pots filled with field soil. One hundred ml of turfgrass extract and a deionized H_2O (as control) were applied. Germination percentage, root length, shoot length, root dry weight, shoot dry weight, seedling weight and seedling vigor index were measured on 14 days for both Petri dish and outdoor pot experiment. Seedling vigor index was calculated according to the following formula (1):

SVI = Germination (%) × [mean root length + mean shoot length]

Phenolic acid extraction

The method described by Wu *et al*. (1998) was used for the present study. For separation of phenolic acids from the crude clipping extract, the extract was acidified by adding 15 ml of concentrated sulfuric acid to 60 ml of the extract. After cooling down to room temperature, the solution was filtered through Whatman No. 1 paper and centrifuged at 3500×*g* for 5 min, and the clear extract was collected. Ten ml of ethyl acetate was added to the clear extract and shaken manually for 30 s. The organic (ethyl acetate) phase was carefully collected (using a 150-ml separating funnel), and the aqueous phase was repeatedly extracted three times using 4 ml ethyl acetate. The ethyl acetate extract was used for the following chemical analysis.

Esterification and gas chromatographic analysis

Before the analysis of phenolic acids using capillary gas chromatography (GC), esterification was conducted to increase the volatility of the compounds. The method described by Husek (1991) was used for the present study. Heptafluorobutyric-isobutanol amino acid (HFB-IBA) was used as an esterification reagent. The organic phase extract (about 70 ml in volume) was concentrated and the volume was reduced to about 200 μL by a stream of dry N_2 in an esterification tube. A reaction solution (1200 μL; dichloromethane and acetyl chloride 10:3, v/v) was added into the esterification vessel, capped, and heated at 135° C for 50 min. After heating, it was vaporized to dryness under a dry nitrogen stream. After cooling, 1200 μL of dichloromethane and 400 μL of heptafluorobutyric anhydride were added to the vessel, the cap was sealed and the vessel was heated at 130° C for at least 15 min. After the heating process, the vessel was removed and placed in an ice-bath for 2 min. The solvent was vaporized to dryness under a stream of dry nitrogen. The residue was redissolved with 500 μL of ethyl acetate for GC analysis.

An Agilent gas chromatograph was used for this study. The oven temperature ramps were at 75°C for 1 min, then increased at 25°C min^{-1} up to 280°C and held there for 1 min. The split injector was set at a rate of 46:1 and the temperature was 250°C. The FID detector temperature was set at 320°C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. Based on the retention time of each standard phenolic acid, the phenolic acids separated by GC chromatograms can be readily identified. For further confirmation, the method of direct standard addition was used. One μmol of each standard phenolic acid was individually added into the sample extract before esterification. The direct standard

phenolic acid addition response was detected by the GC chromatograms. A standard analytical curve method was used for quantitative analysis.

Statistical analysis

Analysis of variance was performed as factorial based on randomized complete block design (RCBD) with four replications in pot and laboratory experiments. Duncan's multiple range test was performed for mean comparison at 0.05 statistical level. Since interaction effects between studied factors in all traits were not significant, comparison of interaction effect means was not performed. The analysis of the allelopathic compounds by GC-Mass was repeated three times with three extracts. All collected data were analyzed using SAS software (41). The inhibition percentage for each trait was calculated from the equation below (Nikneshan *et al*., 2011):

Inhibition percentage = [(control- sample extract)/control]×100

Results

Laboratory experiment

Analysis of variance demonstrated that the main effect of concentration was significant and interaction effect was non-significant for all traits. The source of weed variation was only significant in root length, seedling weight and seedling vigor index traits. Treatment of seed with 25% clipping extract concentration completely inhibited all measured traits of all species (Table 1). *A. blitoides* and *C. dactylon* were more sensitive to the sport turfgrass clipping extract in all traits and, therefore, they were the most sensitive. Among the tested weed species, *T. oficinale* showed the least susceptibility to turfgrass clipping extracts. Root and shoot growth of all species reduced in all clipping extract concentrations, but it seems that roots were more sensitive than shoots (Table 1).

Outdoor pot experiment

Similar to the laboratory experiment, concentration and interaction between weed and concentration factors were significant and not significant in all traits, respectively. *A. blitoides* was the most sensitive. Treatment of seed with 75% clipping extract concentration severely inhibited all traits in

Table 1. Effects of species and concentration on inhibition percentage of measured traits at laboratory condition.

Treatment	Mean Traits						
Weed	Germination percentage inhibition (%)	Root Length inhibition (%)	Shoot Length inhibition (%)	Shoot Dry Weight inhibition (%)	Root Dry Weight inhibition (%)	Seedling Weight inhibition (%)	Seedling Vigor Index inhibition (%)
A. blitoides	70.50a†	76.26a	55.00a	70.66a	77.50a	84.31a	86.03a
C. dactylon	72.35a	78.76a	63.14a	70.50a	76.50a	81.95a	84.01a
P. plantago	68.61a	66.55b	55.17a	67.33a	72.00a	75.14a	82.66a
T. officinale	66.24a	73.72a	62.17a	65.33a	75.25a	74.80b	52.13b
Concentration							
5%	32.82d	31.48d	13.85e	25.20d	34.37d	50.69d	24.12c
10%	51.06c	58.22c	34.43d	50.41c	64.06c	67.65c	72.60b
15%	69.76b	83.69b	55.41c	71.45b	81.56b	82.55b	86.01ab
20%	93.48a	95.74a	90.67b	95.20a	96.56a	94.37a	98.32a
25%	100a	100a	100a	100a	100a	100a	100a

† In each column, means with the same letter are not significantly different from Duncan's multiple range test at %5 of statistical level.

all species but these reductions were not complete (Table 2). It was observed that the allelopathy effect was concentration dependent (Table 2).

Phenolic acids detection

Figure 1 presents the 8 phenolic acids in the clipping extract of a sport turfgrass consisting of 3 cool-season species. This extract contained trans-cinnamic acid, 3, 4, 5-trimethoxybenzoic acid, syringic acid, p-coumaric acid, caffeic acid, gentisic acid, protocatechuic acid, ferulic acids. These phenolic acids decrease in the order just described.

Table 2. Effect of species and concentration on inhibition percentage of measured traits at outdoor pot condition.

Treatment	Mean Traits						
Weed	Germination percentage inhibition (%)	Root Length inhibition (%)	Shoot Length inhibition (%)	Shoot Dry Weight inhibition (%)	Root Dry Weight inhibition (%)	Seedling Weight inhibition (%)	Seedling Vigor Index inhibition (%)
A. blitoides	37.49a†	27.88a	14.27a	43.35a	47.91a	44.31a	64.36a
C. dactylon	42.43a	25.81a	13.27a	27.82b	38.12ab	30.31b	51.78ab
P. plantago	32.82ab	22.74a	6.23a	26.19b	31.77b	27.44b	45.05b
T. officinale	23.71b	24.25a	10.18a	28.93b	48.43a	31.91b	45.20b
Concentration							
10%	8.3d	6.11c	2.68b	9.54d	14.27c	10.66d	15.98c
25%	22.45c	22.77b	4.33b	24.87c	31.87b	26.16c	43.21c
50%	46.59b	32.09a	15.30a	39.50b	56.66a	42.65b	66.52a
75%	58.11a	39.71a	21.64a	52.37a	63.43a	54.50a	80.68a

† In each column, means with the same letter are not significantly different from Duncan's multiple range test at %5 of statistical level.

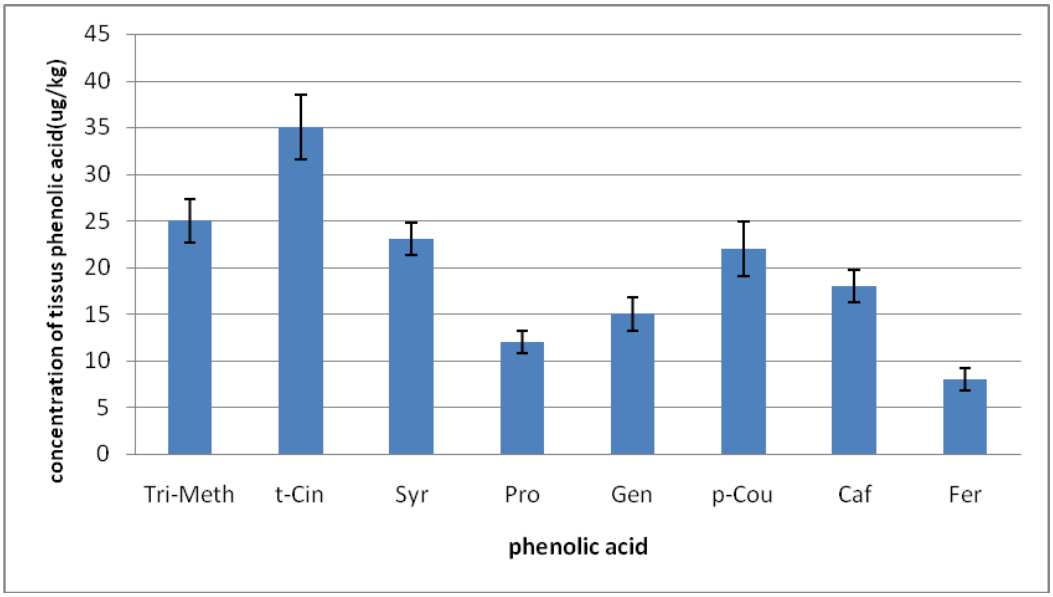

Fig. 1. Distribution of phenolic acids in clipping extracts made from a sport turfgrass. Abbreviations are as follows: Tri-Meth: 3, 4, 5-trimethoxybenzoic acid; t-Cin; trans-cinnamic acid; Syr: syringic acid; Pro: protocatechuic acid; Gen: gentisic acid; Cou: p-coumaric acid; Caf: caffeic acid; Fer: ferulic acid. Each column represents the mean of three replications and bars represent standard error of the means.

Discussion and Conclusion

The comparison of the two experiments showed that higher concentrations of clipping extract were required for a significant decrease in traits in pot than in Petri dish conditions. Treatment of seed with 25% clipping extract concentration could inhibit all traits in all species, but in pot experiment, 75% clipping extract could not completely inhibit any given trait. In pot experiment, it seems allelopathic compounds were probably adsorbed in the surface of soil and microbial activity caused degradation of allelopathic compounds. Moreover, the majority of soils are alkaline in arid and semi-arid regions such as Iran. Therefore, it might influence allelopathic compounds in pot conditions. Wu *et al*. (2002) noted that the allelopathy effect of allelechemical compounds is deeply affected by soil's physical and chemical conditions and soil's microbial activities. Sampietro *et al*. (2005) observed that phenolic compounds had less effect on target plants in unsterile soil. Inderjit *et al*. (2001) demonstrated that as allelochemicals move through the soil, they are altered by microorganisms, bound to organic matter, and distorted by polymerization. Zhao *et al*. (2010) stated that adsorption of phenolics to clay minerals may be one of their possible fates in soil.

In some studies both inhibitory and stimulatory effects of water extracts of plants have been reported (Gomaa and AbdElgawad, 2011; Ismail and Chong, 2002; Nikneshan *et al*., 2011). However, in both of our experiments, the inhibitory effect of extract was increased by increasing aqueous extract concentration and no stimulatory effect was found. Thus, the allelopathic property was dependent upon concentration. This result agrees with King (1996) who reported that the effect of perennial ryegrass extract on duckweed (*Lemna minor* L.) fronds was dependent on concentration. In rice, allelopathic property was found to be concentration dependent (Chung *et al*., 2001). Also, Bertin *et al*. (2003) observed that fine fescue (*Festuca rubra* L.) seedlings exuded large quantities of bioactive root matter into the agar medium, but older fine fescue plants had a more profound effect on test species.

Roots were more sensitive than shoots to turfgrass clipping extracts in all of the test weeds. Direct connection of roots to allelopathic compounds might be due to the fact that they are suppressed more than shoots. Similar results were observed by Jafariehyazdi and Javadifar (2011) and Gomaa and AbdElgawad (2011). Wu *et al*. (2002) observed that roots of cool and warm turfgrass were more affected than shoots by exerting phenolic compounds and stated that because seedlings with reduced root growth are unlikely to last in a well establish sward, this finding has practical importance.

So far, several phenolic acids have been identified to have allelopathic properties and have been measured in extracts from a variety of plant species. (Gomaa and AbdElgawad, (2011); Ismail and Chong, 2002; Wu *et al*., 1998). Phenolic compounds are major plant allelochemicals in ecosystem and they play a key role in allelopathy (Johan and Sarada, 2012). Rao and Buta, (1983) extracted 19 phenolic compounds from perennial ryegrass that negatively impacted lettuce seed germination. Wu *et al*. (2002) reported that gentisic acid was not detected in four cool season turfgrass but occurred in four warm season ones while both p-hydroxy-benzoic acid and vanillic acid were identified in cool and warm season turfgrass. In contrast, gentisic acid was observed in our extraction and p-hydroxy-benzoic acid and vanillic acid were not detected (Fig. 1). Salicylic acid was not detected in phenolic acid extraction. This is in agreement with Wu *et al*. (2002) finding: they did not detect salicylic acid in the four cool-season turfgrass, but they found it in four warm-season turfgrass species. It was suggested that phenolic acids could disrupt cell division, distort cellular structures and reduce respiration and photosynthetic rates

in plants exposed to them (Li *et al*., 2010; Yu *et al*., 2003). Other studies have mentioned change in plant enzymatic functions, inhibited protein synthesis, and inactivated plant hormones as inhibitory mechanisms from these allelochemicals (Batish *et al*., 2008; Li *et al*., 2010). Wu *et al*. (2002) noted that the amount and type of phenolic compounds released by plants as well as the allelopathic activity of these compounds may be greatly affected by seasonal changes, soil physical and chemical conditions, soil nutrients, and biotic factors such as plant density, growth conditions, stress of plants, and soil microbial activities. Therefore, clipping extract from turfgrass species in relation to management conditions may have different results.

In conclusion, the results of this study demonstrated that turfgrass clipping extract was a growth inhibitor in selected weed species. To guarantee the decrease of synthetic herbicide requirements in sustainable systems, the utilization of natural allelochemicals for weed suppression remains a viable option for reducing synthetic herbicide usage in the future.

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