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Oxalate and tannins assessment in *Atriplex halimus* L. and *A. nummularia* L.

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Abstract

The study was conducted at 3 locations in the arid region of Jordan to assess the seasonal changes of oxalate and tannins in *Atriplex halimus* L. and *A. nummularia* L. plants commonly used for revegetation of degraded rangelands. During spring and fall seasons, 20 shrubs of each species were selected randomly at each location, 20 similar twigs per shrub were clipped and analyzed for oxalate and tannins. *Atriplex halimus* contained higher levels of oxalate (7.00%) compared with *A. nummularia* plants (6.20%) (P < 0.001). Oxalate levels averaged 8.29 and 4.92% in spring and fall season, respectively. Plants of *A. halimus* accumulated more oxalate in spring than those of *A. nummularia*. Clipping had no effect on oxalate concentration. The seedlings of *A. nummularia* contained more oxalate than old plants whereas old shrubs of *A. halimus* contained more oxalate than the young seedlings. The browse of *A. halimus* contained more condensed and hydrolyzable tannins (1.05% and 0.67%, P < 0.0001) than *A. nummularia* (0.80% and 0.39%, P < 0.0001), respectively. Clipping had no effect on the levels of tannic phenols, condensed and hydrolyzable tannins. Young plants of the 2 species had higher levels of condensed tannins compared to older plants. However, seedlings of *A. nummularia* contained significantly higher levels of condensed tannins compared to *A. halimus* seedlings (1.57% and 1.47%, respectively). *Atriplex halimus* synthesized more oxalate, tannic phenols, condensed and hydrolyzable tannins than *A. nummularia*. These secondary metabolites may explain the low palatability of *Atriplex halimus* compared to *A. nummularia.*

Resumen

El estudio se condujo en t 3 localidades de la región árida de Jordania para evaluar los cambios estacionales del los oxalatos y taninos en *Atriplex halimus* L. y *A. nummularia* L., plantas comúnmente utilizadas para revegetar áreas de pastizal degradadas. Durante primavera y otoño en cada localidad se seleccionaron aleatoriamente 20 arbustos de cada especie y se cortaron 20 ramas similares por arbusto y fueron analizadas para determinar el contenido de oxalatos y taninos. *El Atriplex* halimus presentó más altos niveles de oxalatos (7.00%) que el A. nummularia (6.20%) (P < 0.001). Los niveles de oxalatos promediaron 8.29 y 4.92% en primavera y otoño respectivamente. En primavera, las plantas de *A. halimus* acumularon mas oxalatos que las de *A. nummularia*. El corte no tuvo efecto en la concentración de oxalatos. Las plántulas de *A. nummularia* contenían mas oxalatos que las plantas viejas mientras que los arbustos viejos de *A. halimus* presentaron a mas oxalatos que las plántulas. El forraje ramoneable de *A. halimus* contenía mas taninos condensados e hidrolizables (1.05 % y 0.67%, P < 0.0001) que *A. nummularia* (0.80% y 0.39%, P < 0.0001). El corte no tuvo efecto en los niveles de fenoles tánicos y taninos condensados e hidrolizables. Las plantas jóvenes de las dos especies tuvieron niveles mas altos de taninos condensados que las plantas viejas. Sin embargo, las plántulas de *A. nummularia* contenían niveles significativamente mas altos de taninos condensados que las plántulas de *A. halimus* (1.57% y 1.47%, respectivamente). *Atriplex halimus* sintetizó mas oxalatos, fenoles tanicos, taninos condensados e hidrolizables que *A. nummularia*. Estos metabolitos secundarios pueden explicar la baja gustocidad del *Atriplex halimus* comparado con el *A. nummularia*.

Key Words: ***Atriplex halimus, Atriplex nummularia*, tannins, oxalate, clipping, arid region**

Rangelands in Jordan cover 90% of the total surface area of the country. Serious declines in productivity have occurred over extensive areas of these lands. This decline takes the form of depletion of high value fodder species and their replacement by plants that are less productive, less palatable, and less nutritious than the original plants they replaced (Abu-Zanat 1995). Artificial revegetation may be a tool for rehabilitation of these degraded rangelands. Because of drought and scarcity of precipitation, fodder shrubs are preferred to herbaceous species for rangeland revegetation. In Jordan, the common fodder shrubs that are used for revegetation are *Atriplex halimus* L. and *A. nummularia* L. *Atriplex halimus* is native to Jordan and represents 60 to 70% of saltbush plantations in the Middle East region (LeHouerou 1994) whereas, *Atriplex nummularia* is an introduced species from Australia. However, they contain secondary chemical compounds, which may restrict grazing by herbivores. The type, level and activity of these chemical compounds depend on the age of plants as well as growing conditions (Cheeke 1995).

Atriplex species accumulate oxalates (James 1977, Libert and Franceschi 1987) up to 4–16% DM (Barry and Blaney 1987). Both soil salinity and moisture can alter the level of oxalate in the plant. Plants growing in saline soil need oxalates for osmo-regulation of Na, K and Cl ions (Cymbaluk et al. 1986). Under high

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soil moisture, *Atriplex halimus* plants were reported to accumulate higher levels of oxalate in leaves and twigs (Ellern et al. 1974). Plants containing 10% oxalate or more are considered toxic and should not be grazed (James 1977).

Tannins, on the other hand, are a chemically diverse group of water-soluble phenols, which bind proteins to form insoluble complexes (Hagerman et al. 1992). The level of tannins in the plant is not constant. Factors within the plant and the external environment influence the variability of tannin levels. Within a given species the proportion of tannins may change with plant maturity and/or climatic stress (Singleton 1981). Factors that may increase tannin level in plants are low soil fertility, drought and high temperature. Tannins inhibit cellulolytic and protolytic enzymes and decrease the production of volatile fatty acids, microbial DNA and RNA in the rumen. Feeding tannins to ruminant animals in large amounts have produced negative effects on the animal, inducing low feed palatability, inhibited digestion, and may cause systematic animal poisoning (Singleton 1981, Kumar and Singh 1984).

The objectives of this study were to: 1) assess the seasonal levels of oxalate and tannins in *Atriplex halimus* and *A. nummularia* in the arid region of Jordan, and 2) compare the effect of clipping versus no clipping on the level of tannins and oxalate in the browse of the 2 species.

Materials and Methods

Experimental Sites

The study was conducted at 3 locations: Khanasry (36° 03' 05" E, 32° 26' 4" N), Muwaqqar (36° 13' 30" E, 31° 46' 40" N) and Khalideyah (36° 17' 42" E, 32° 10' 36" N) research stations. These locations are classified in Jordan as arid lands with annual rainfall of 100–200 mm. Rainfall is basically irregular, sporadic, and poorly distributed. Maximum and minimum temperatures ranged between 11-35° C and 2-17° C, respectively with a relative humidity of 57%, which ranged between 86% in winter and 40% in summer. The soil is mainly silt-clay with a pH between 6.7–8.1.

Plant Material

In 1985, seeds of *Atriplex halimus* were collected from Jordan and seeds of *Atriplex nummularia* were introduced from Australia. All plant collections were propagated at the Khalideyah Nursery, Mafraq Governorate, which is the only source of all saltbush plantations existing at the experimental sites and range reserves in Jordan. In October 1996, seedlings of *A. halimus* and *A. nummularia* (9 months old) were transplanted in contour furrows at the 3 experimental sites. Old shrubs (5–10 years) of both species existed at all the sites.

Sampling

Browse from seedlings and old plants of *A. halimus* and *A. nummularia* was collected during May (spring season) and November (fall season) in 1997. At each location, 20 shrubs of each species and age class were selected randomly and tagged with plastic wires at the main stem. Twenty identical twigs (1 cm in diameter, 10–15 cm long) from each shrub were selected randomly from all sides of the shrub and clipped. The samples from each shrub were placed into plastic bags for weight determination and chemical analysis. In November 1997, regrowth of twigs previously clipped in May were re-clipped to determine the effect of clipping (a simulation of grazing) on the content of tannins and oxalate.

Chemical Analysis

Since the equipment for freeze drying is not available, the samples were dried using an air-circulating oven at 25° C for 48 hours according to Makker et al. (1988) and dry weights were recorded. The low drying temperature was selected to prevent the degradation of tannins by excessive heating (Makker et al. 1988). Waterman and Mole (1994) preferred that the drying temperature should be higher than 40° C to avoid oxidation by the still active enzymes and lower than 60° C to avoid heat damage and polymerization. The partially dried samples were ground to 0.2-mm mesh size and stored in plastic bags into a refrigerator at 3 ± 1° C for later chemical analysis. The dry matter of browse was determined by placing the residues of samples in an oven and dried at 100 ± 5° C for 48 hours.

Oxalate content was determined according to the procedure of the AOAC (1990). A sample weighing 1.5 g was treated with HCL (6 M), boiled for 30 minutes and the solution was filtered into a 100 ml volumetric flask. The solution was treated with 5 ml of tungstophosphoric acid reagent, after filtration the pH of the solution was adjusted to 4-4.5 using NH_4OH and acetate buffer. Test tubes were centrifuged at 1700 rpm for 15 minutes and the oxalate crystals were redissolved by 5 ml of H_2SO_4 (10 %) and titrated with $KMnO_4$ (0.01 M). All types of oxalates existing in the plant material were converted to oxalic acid, precipitated and titrated with $KMnO_4$.

Tannins were determined according to Makker and Goodchild (1996). Tannins were extracted from the sample (100 mg) using 10 ml of 70% ultra-pure acetone, immersed into an ice-cold sonicator and exposed to ultra-sonic for 15 minutes, transferred into a test tube and centrifuged at 3000 rpm for 20 minutes. The supernatant was separated and stored at 3° C. The dye Folin-Ciocalteau was added to 0.5 ml of the extract and absorbtion of the dye-phenolics complex was measured at 725 nm wavelength. The amount of total phenols as tannic acid equivalent was determined from a calibration curve (10-60 mg tannic acid/ liter). Tannins were precipitated using polyvinil pyrrolidones and 1.0 ml of the tannins extract was centrifuged at 3000 rpm for 15 minutes and the supernatant containing only simple non-tannic phenols was collected. The dye Folin-Ciocalteau was added to 0.5 ml of the supernatant and absorbance of the dye-phenolics complex was measured at 725 nm wavelength. Tannic phenols were calculated as the difference between total phenols and non-tannic phenols. Condensed tannins were determined by mixing the tannic extract with butanol-HCL reagent and ferric ammonium sulfate in a screw cap test tube. The test tube was heated in a water bath (97–100° C) for 60 minutes, cooled and absorbance was measured at 550 nm wavelength. Hydrolyzable tannins were calculated as the difference between tannic phenols and condensed tannins. Duplicates of each sample were analyzed for the confirmation of results. All results were expressed on a dry matter basis.

The least square analysis of variance was performed on data using the SAS General Linear Models (GLM) procedure for a randomized complete block design (SAS 1988). The first analysis compared experimental sites, shrub species, season and the appropriate interactions. The second analysis compared shrub species, age (seedlings versus old plants) and the appropriate interactions. The third analysis compared the effect of previous clipping on these components in secondary growth tissue and included experimental sites, shrub species, time of clipping and the appropriate interactions. Variables considered in the statistical analysis were oxalates, total phenols, non-tannic phenols, tannic phenols, condensed and hydrolyzable tannins. A protected LSD test was used to compare means.

Results and Discussion

Oxalate

Shrub Species

Oxalate content differed between *Atriplex halimus* and *A. nummularia* (Table 1). Regardless of location or season, *A. halimus* contained significantly (P < 0.001) higher levels of oxalate (7.01%) compared with *A. nummularia* (6.20%). The oxalate content in the 2 shrub species ranged between 4.17% and 10.67% and averaged 6.5%. The oxalate values reported in this study are within the range of values (4-1%) reported in various studies reviewed by Barry and Blaney (1987). A significant (P < 0.0001) location x shrub species interaction in oxalate content resulted from high levels of oxalates in *A. halimus* (7.84%) and low levels of oxalates in *A. nummularia* (5.97%) at the Khalideyah site. The soils of this site are characterized by high salt content.

Season

Seasonal variations greatly affected the level of oxalate in *A. halimus* and *A. nummularia.* The 2 saltbush species contained much higher levels of oxalate during spring (8.96% and 7.62%) compared to fall season (5.06% and 4.78%) for *A. halimus* and *A. nummularia*, respectively. Moisture stress during the fall season may have reduced oxalate levels, or mature tissue synthesized less oxalate (Ellern et al. 1974). During the fall season, there was no significant difference in oxalate content between the two-saltbush species, whereas, in the spring, plants of *A. halimus* accumulated more oxalate than *A. nummularia* (Table 1). There was a significant (P < 0.0001) location x season interaction in oxalates content due to variations in soil salinity among the experimental sites.

Table 2. Seasonal changes in levels[1] of total phenolic compounds, non-tannic phenols, tannic phenols, condensed tannins and hydrolyzable tannins in *Atriplex halimus* and *Atriplex nummularia* plants growing at 3 locations in the arid region of Jordan.

Location	Spring		Fall	
	A. halimus	*A. nummularia*	*A. halimus*	*A. nummularia*
	(%)			
Total phenols				
Khanasry	1.98ab[2]	1.22c	1.10c	1.09c
Muwaqqar	2.15a	1.69b	1.48bc	2.04a
Khalideyah	2.28a	1.43bc	2.28a	0.71d
Location mean[3]	2.14a	1.45b	1.62b	1.28bc
Season mean[3]	1.79a		1.45b	
Non-tannic phenols				
Khanasry	0.14c	0.28a	0.13c	0.05d
Muwaqqar	0.12c	0.21b	0.07cd	0.14c
Khalideyah	0.23b	0.25b	0.22b	0.09c
Location mean[3]	0.16b	0.25a	0.14c	0.09d
Season mean[3]	0.21a		0.12b	
Tannic phenols				
Khanasry	1.84a	0.95c	0.97c	1.03c
Muwaqqar	2.02a	1.48b	1.40b	1.91a
Khalideyah	2.03a	1.18bc	2.05a	0.61d
Location mean[3]	1.97a	1.20c	1.47b	1.18c
Season mean[3]	1.58a		1.33b	
Condensed tannins				
Khanasry	1.57a	0.74e	0.63e	0.68e
Muwaqqar	1.08c	1.15c	0.85d	0.72e
Khalideyah	1.25b	1.05c	0.89d	0.44f
Location mean[3]	1.30a	0.98b	0.79c	0.61d
Season mean[3]	1.14a		0.70b	
Hydrolyzable tannins				
Khanasry	0.27cd	0.21d	0.33cd	0.36cd
Muwaqqar	0.95ab	0.32cd	0.54c	1.18a
Khalideyah	0.78bc	0.13d	1.16a	0.17d
Location mean[3]	0.67a	0.22b	0.68a	0.57a
Season mean[3]	0.44b		0.62a	

[1]Least square means as % on dry matter basis. Standard errors of least square means are 0.11, 0.01, 0.11, 0.03 and 0.11 for total phenols, non-tannic phenols, tannic phenols, condensed tannins and hydrolyzable tannins, respectively.
[2]Means within columns and rows for each parameter followed by the same letter are not significantly different at the 0.05 level of probability.

Clipping

Clipping had no effect on the oxalate concentrations of saltbush plants, 4.99% in unclipped twigs versus 4.80% in regrowth. Clipping was confined to some twigs and not for the whole shrub. This lenient clipping was not severe enough to induce substantial differences in oxalate synthesis of saltbush plants.

Table 1. Seasonal changes in oxalate levels[1] in *Atriplex halimus* and *Atriplex nummularia* plants growing at 3 locations in the arid region of Jordan.

Location	Spring		Fall	
	A. halimus	*A. nummularia*	*A. halimus*	*A. nummularia*
	(%)			
Khanasry	8.53 ± 0.20b[2]	7.80 ± 0.18c	5.35 ± 0.18d	5.37 ± 0.18d
Muwaqqar	7.67 ± 0.20c	7.29 ± 0.18c	4.83 ± 0.18de	4.80 ± 0.16e
Khalideyah	10.67 ± 0.18a	7.77 ± 0.20c	5.00 ± 0.16de	4.17 ± 0.18f
Species mean[3]	8.96a	7.62b	5.06c	4.78c
Season mean[3]	8.29a		4.92b	

[1]Least square means as % on dry matter basis ± standar error.
[2]Means within columns and rows followed by the same letter are not significantly different at the 0.05 level of probability.
[3]Means within a row followed by the same letter are not significantly different at the 0.05 level of probability.

Shrub Age

A significant interaction (P < 0.001) existed between shrub species and age. Seedlings of A. nummularia contained more oxalate than old plants (9.65% and 7.63%, P > 0.0001) , whereas old shrubs of A. halimus (10.69%) contained more oxalate than the young seedlings (8.8%) (P < 0.0001). Young seedlings of *A. nummularia* contained more oxalate (9.65 %) than the seedlings of *A. halimus* (8.83%) (P < 0.005). During early stages of growth, there is a rapid rise in oxalate content followed by a decline in oxalate level as the plant matures (Davis 1981, Hodgkinson 1977).

Tannins

Shrub Species

Atriplex halimus contained more total phenols (1.88% vs 1.36%), tannic phenols (1.72% vs 1.19%), condensed tannins (1.0% vs 0.80%) and hydrolyzable tannins (0.6% vs 0.3%) than *A. nummularia* (P < 0.0001) (Table 2). Levels of tannic phenols in the 2 saltbush species varied between 0.61% to 2.05% and averaged 1.46%. These values are much higher than those reported by Davis (1981) for various saltbush species in different countries (0.32% to 0.9%). Variation in levels of tannins among saltbush plants and even within the same species could be attributed to differences in soil and climatic conditions of the sites (Barry and Blaney 1987). Difference in plant ability to synthesize tannins is well-documented (Mangan 1988, Davis 1981). These higher levels of tannins in the tissues of *A. halimus* may explain its low palatability compared to the browse of *A. nummularia.*

The type of tannins is more important than the quantity of tanninc phenols regarding the nutrition of animals. The hydrolyzable tannins have adverse effects on health and nutrition of animals compared to condensed tannins which mainly reduce the palatability of feeds. Plants of *A. halimus* contained more condensed and hydrolyzable tannins (1.05 and 0.67%) than those of *A. nummularia* (0.89 and 0.39%). The ratio of hydrolyzable to condensed tannins was 0.64 in *A. halimus* compared to 0.49 in *A. nummularia* plants. A significant ($P < 0.0001$) shrub species by location interaction in condensed tannins resulted from high content of condensed tannins in *A. halimus* (1.07%) and low levels in A. nummularia (0.75%) at the Khalideyah site. A similar pattern of shrub species by location interaction ($P < 0.0001$) was also noted for hydrolyzable tannins which peaked to 0.97% in *A. halimus* and dropped to the lowest levels (0.15%) in *A. nummularia* plants.

Season

In spring, saltbush plants contained higher levels of tannic phenols and condensed tannins (1.58% and 1.14%) compared to fall levels (1.33% and 0.70%, respectively) ($P < 0.001$). These results did not agree with the findings of Davis (1981), who observed no significant differences in the levels of tannin phenols in *A. halimus* plants compared to *A. nummularia* in spring and fall seasons.

The temporal variation in tannic phenols resulted from the variation of environmental factors prevailing in the 2 seasons. Saltbush plants synthesized more tannins in the spring season, apparently because of more favorable growth factors prevailing in spring compared to fall season, or due to the higher metabolic rate of the younger tissues. Twigs in the fall season had a low leaf: stem ratio compared to more leafiness in the spring. Since most of tannins exists in the leaves (Van Soest 1994, Barahona et al. 1997), it was expected that spring growth would contain higher levels of total and condensed tannins than the fall growth and regrowth.

Clipping

Clipping of saltbush plants had no effect on the level of total ($P > 0.3$) or condensed tannins ($P > 0.8$). This means that the severity of defoliation of saltbush shrubs was not enough to stimulate or hinder the ability of plants to synthesize tannic compounds. The lenient and confined clipping of selected twigs did not enhance the biosynthesis of total tannins as a defensive mechanism against herbivores.

Shrub Age

Significant difference ($P < 0.001$) in total tannins existed between young and old plants of *A. nummularia* (2.03% and 1.25%, respectively) but not in *A. halimus* (Table 3). However, condensed tannins were higher in various plants of both species. Young plants tended to produce more condensed tannins than old plants because they are more leafy. Young plants of *A. halimus* contained similar levels of total tannins as young seedlings of *A. nummularia*. However, seedlings of *A. nummularia* contained significantly ($P < 0.01$) higher levels of condensed tannins compared to *A. halimus* seedlings (1.57% and 1.47%, respectively).

Potential Toxicity Problems

Allison et al. (1977) reported that sheep ingesting between 39–51 g of calcium oxalate per day are likely to develop acute signs of toxicity. Theoretically, if sheep consume a diet solely of *Atriplex* plants grown in Jordan, they will ingest about 132 g of calcium oxalate (6.6% calcium oxalate =66 g per kg DM x 2 kg per day per sheep = 132 g) and are likely to develop acute signs of toxicity. Practically, the potential of toxicoses due to oxalate levels present in the browse of saltbush is low because of selectivity and adaptation of ruminants to high levels of oxalate. James and Butcher (1972) reported that sheep can consume a diet containing 6% oxalate without showing any toxicity signs. However, these animals developed diarrhea and refused to consume such a diet again. Oxalate poisoning occurs at the point where oxalate is absorbed faster than it can be degraded by ruminal microflora. Animals can gradually adapt increasing populations of oxalate degrading microbes in the rumen. Adapted animals can consume 30% more oxalate than unadapted ones (James 1977). In general, oxalate poisoning is a complex issue. Factors such as chemical form of oxalate, animal maturity, adaptation of animals to saltbush, the composition of the diet, and the availability of water for animals could influence the susceptibility of animals to oxalate poisoning.

Several studies dealt with the problem of toxicity due to high levels of tannins (> 6 %) in animals diet (Kumar and Singh 1984). However, animals refused to consume diets containing $\geq$ 2% tannins (Donnelly and Anthony 1969). Moderate levels of tannins (less than 4%) in forages can have beneficial responses in ruminants due to the increased amount of bypass proteins, resulting in higher growth rates (Nunez-Hernandez et al. 1991). The levels of tannins in the saltbushes grown in Jordan are not expected to have adverse effects on sheep grazing these plantations associated with abundance of herbaceous plants.

Conclusion

In summary, our study showed that the 2 saltbush species contained higher levels of

Table 3. Levels[1] of total phenolic compounds, non-tannic phenols, tannic phenols, condensed tannins and hydrolyzable tannins in *Atriplex halimus* and *Atriplex nummularia* seedlings compared to old plants growing in the arid region of Jordan.

	Seedlings		Old Plants	
	A. halimus	*A. nummularia*	*A. halimus*	*A. nummularia*
	(%)			
Total phenols	2.03b[2]	2.41a	2.25ab	1.50c
Non-tannic phenols	0.15c	0.37a	0.23b	0.25b
Tannic phenols	1.88a	2.03a	2.01a	1.25b
Condensed tannins	1.47b	1.57a	1.24c	1.08d
Hydrolyzable tannins	0.41b	0.47b	0.78a	0.17c

[1]Least square means as % on dry matter basis. Standar errors of least square means are 0.196, 0.089, 0.007, 0.087, 0.027 and 0.071 for total phenols, non-tannic phenols, tannic phenols, condensed tannins and hydrolyzable tannins, respectively.
[2]Means within a row followed by the same letter are not significantly different at the 0.05 level of probability.

oxalate, tannic phenols and condensed tannins during spring compared to fall season. *Atriplex halimus* is characterized by high levels of oxalate, tannic phenols, condensed and hydrolyzable tannins than *A. nummularia* which explanis its low palatability. Spring clipping of saltbush plants had no significant effect on oxalate or tannins concentration of subsequent clippings taken in fall. The levels of secondary metabolites found in our experiments indicate that if sheep were to consume a diet solely of *Atriplex*, they would be likely to develop acute toxicity symptoms. Future work should examine the different chemical forms of oxalates and tannins in the browse of saltbushes in response to different defoliation intensities and under different environmental conditions.

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