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Maintenance of *Ephedra alata* Seeds Viability *via* Storage Containers

Abdulaziz A. Al-Qarawi, Elsayed F. Abd_Allah*

[1]Plant Production Department, College of Food & Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia.
Email: *eabdallah@ksu.edu.sa

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ABSTRACT

Sequential incubation of seed samples yielded 20 *fungal species belonging to* 13 *genera. The prevalent genera were Aspergillus* (*A. flavus and A. parasiticus*), *Fusarium* (*F. moniliforme and F. oxysporum*) *and Penicillium* (*Penicillium sp.*). *Such seedborne fungi differ in their colonization in different parts of seeds with most of them are colonized in seed coat, endosperm, and embryo of the seed. The usage of different storage containers for storing seeds indicated that the cotton cloth bags were the most favorable ones as they maintain seed moisture content* (*SMC*) *below the critical level resulting in minimum seed deterioration compared with other seed storage containers.*

Keywords*: *Seedborne Fungi, Ephedra alata, Storage Containers, Saudi Arabia

1. Introduction

Ephedra alata Decne, a gymnosperm belong to family Ephedraceae, is one of the oldest range and medicinal herb known in Saudi Arabia as well as in different rangelands in the world [5]. It was noted as it is accompanied with sand dunes formation in Saudi Arabia especially the mobile ones and therefore it is a very effective sand-binder and resistant to desertification [3]. The foliage of *E. alata* have acceptable aroma and used as foodstuff for animals especially camels, cattle and sheep. The deterioration of plant community had occurred in Saudi Desert due to abiotic factors such as soil salinity [7], edaphic factors of the soil [29] and soil drought [6]. On other hand, the biotic stresses such as seedborne fungi play an important and vital role in deterioration of seed quality [1,2]. Seedborne fungi use different mechanisms to deteriorate seeds such as production of both mycotoxins [2] and enzymes [26] which have attracted much attention of our investigations.

Application of prophylactic fungicides is not the preferred choice in range seeds especially in the sheltered areas. This raised the need to study safe alternative strategies to control seedborne fungi that attack range plants and might be transmitted to the aerial parts of the plants [4]. Seed storage containers play an important and considerable role in the production of healthy and vital seeds [21, 25; 27]. The successfulness of the storage containers restricted with surrounding factors such as seed nature, storage period, temperature, relative humidity, and seed moisture content. Consequently, the production of healthy and vital range seeds should managed through integrated approach.

The present study was designed to investigate the seedborne fungal flora of *E. alata* with special reference to their incidence in different seed parts. Furthermore, the effect of different storage containers on seed moisture content (SMC), seed vigor (SV), aflatoxins accumulation, and nutritional value of storage seeds (*E. alata*) was studied.

2. Materials and Methods

2.1. Seed Samples Collection

Seed samples of *Ephedra alata* Decne (approximately 100 g seeds per sample, each in replicates) were collected from King Khalid Center for Wildlife Research and Development at Thumama which belonging to Riyadh Region, Saudi Arabia during 2009. The samples were collected in sterile cellophane bags and held at 2℃ until analyzed according to the International Seed Testing Association [20].

2.2. Enumeration of Seedborne Fungi

From each seed sample, 400 seeds were surface-disinfected in Na-hypochlorite for two minutes followed washing with several changes of sterile saline water (8.5 gm NaCl in 1000 ml distilled H_2O). The disinfected

seeds were then incubated aseptically on potato dextrose agar (PDA, Difco Laboratories, Detriot MI). Rose Bengal (33 mg/ml, w/v) and Streptomycin (30 mg/ml, w/v) were added as bacteriostatic agents. Surface-disinfected seeds were spaced on Petri dishes (9 cm in diameter) and incubated at 28 ± 2℃ for 10 days. Similarly, surface-disinfected seeds were incubated on sterile moist filter paper with cellulose wadding as blotters and incubated as above. The fungal colonies developing around the seeds incubated on both agar plates and filter papers were examined and the fungi were identified microscopically [15] and the level of incidence were recorded. To enumerate seedborne fungi in different seed parts, surface disinfected seeds were soaked in sterile water for four hours and then dissected aseptically into different parts (coat, endosperm, and embryo). Each seed part aseptically used for investigation of seedborne fungi as described above.

2.3. Determination of Seed Moisture Content (SMC)

Each seed sample (100 gm) was grounded in a blender and known weight of the resultant powder was dried in an oven for 24 hours at 105℃, cooled in a desiccators and reweighed. The moisture content (MC) is expressed as percentage of the wet weight.

2.4. Storage Experiment

Seed samples (100 g each) were stored in four storage containers namely polyethylene bags, cotton cloth bags, tin cans and paper bags for six months at room temperature (25 ± 1℃) in the dark.

2.5. Seed Analysis

Vigor index (VI) of *E. alata* seeds were calculated Vigor index (VI) for each treatment was determined according to the following formula: VI = [mean of root length (cm) + mean of shoot length (cm)] X percent seed germination. Nutritional values (total lipids, total nitrogen, ash content, and fiber content) of stored seeds were determined according to AOAC [11]. Aflatoxins (B_1, B_2, and G_1) were extracted and cleaned up from storage seeds using chloroforme and cleaned using column chromatography according to AOAC [10]. Quantitative estimation of aflatoxins were carried spectrophotometrically [24] using standard aflatoxins (Sigma) as reference.

2.6. Statistical Analysis

For each experiment, the data were statistically analyzed using the analysis of variance procedure for completely randomized design. Treatment means were compared using the protected least significant difference (LSD) analysis according to Daniel [14].

3. Results and Discussions

In the present investigation, 31 seed samples of *E. alata* were analyzed to investigate their seedborne fungal flora by means of standard blotter and agar plate methods (**Table 1** and **Figure 1**). As suggested by Abd_Allah and Hashem [2] the comprehensive outcome recommended

Table 1. Incidence (%); case of isolations and occurrence remarks of seedborne fungal flora of *E. alata* following incubation on agar plate and blotter.

Fungal species	Incidence (%) of fungal species		Cases of isolation (No.)	Occurrence[Z]
	Blotter test	Agar plate		
Alternaria alternata	3.87	2.35	1	R
Alternaria sp.	4.36	2.21	3	R
Aspergillus flavus	14.65	16.37	42	H
Aspergillus nidulans	2.75	0	4	L
Aspergillus niger	8.36	12.75	13	M
Aspergillus parasitcus	18.52	21.18	37	H
Aspergillus sp.	16.32	12.16	7	L
Aspergillus terreus	7.15	8.13	6	L
Chaetomium globsum	0.95	1.15	1	R
Cladosporium sp.	1.78	0	2	R
Drechslera sp.	1.07	0	1	R
Epicoccum sp.	0.54	0.63	1	R
Fusarium moniliforme	3.27	3.85	7	L
Fusarium oxysporum	2.96	2.56	6	L
Penicillium sp.	7.36	8.81	7	L
Pythium sp.	0.62	0.87	1	R
Rhizoctonia solani	2.45	2.65	2	R
Rhizopus sp.	0.61	0.83	4	R
Sclerotium bataticola	1.54	1.35	2	R
Trichoderma sp.	1.27	1.75	3	R

Z: Out 31 *E. alata* seed samples. H = High occurrence (> 24 case); M = Moderate occurrence (from 12 to 24 cases); L = Low occurrence (from 6 to 11 cases) and R = Rare occurrence (< 6 cases).

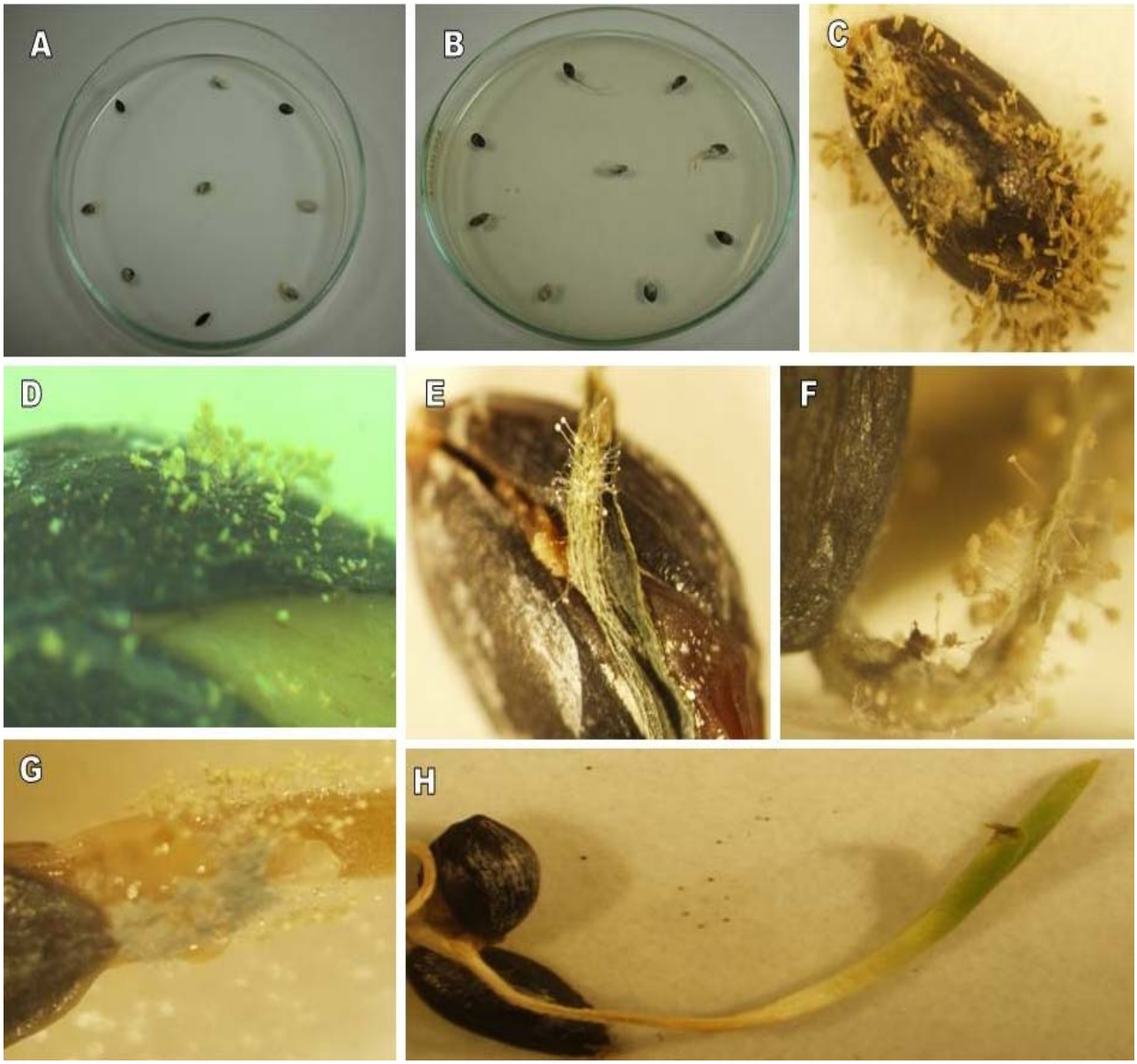

Figure 1. Detection of seedborne fungi associated with *E. alata*. A) Blotter and B) agar plate techniques used for enumeration of seedborne fungi. C-H) Stereo-microscopy of *E. alata* seeds acquaint the incidence of seedborne fungi on different seed parts.

that agar plate method was more conformable than moist paper (standard blotter) method in yielding more fungal flora (**Table 1**). The sequential incubation of seed samples yielded twenty fungal species belonging to thirteen genera, which are new to mycoflora of *E. alata* in Saudi Arabia (**Table 1**). The genes *Aspergillus* was the most predominant and represented by 6 species namely A. *flavus*, *A. nidulans*, *A. niger*, *A. parasiticus*, *A. terreus* and *Aspergillus* spp.. *Aspergillus* was followed by *Fusarium* (*F. moniliforme* and *F. oxysporum*) and *Penicillium* (*Penicillium* spp.), respectively. The other fungal genera (*Alternaria*, *Chaetomium*, *Cladosporium*, *Drechslera*, *Epicoccum*, *Pythium*, *Rhizoctonia*, *Rhizopus*, *Sclerotium*, *Trichoderma*) were rare in their occurrence (**Table 1**). Up to our knowledge, this is the first investigation for seedborne mycoflora of *E. alata* especially in Saudi Arabia although the contamination of Saudi herbs with toxigenic mycoflora was reported [8]. Nevertheless, similar mycological studies for many other seeds showed that *Aspergillus* and *Fusarium* were the most common genera as seedborne fungi [2; 16]. The component plating of *E. alata* seeds showed that most prevalent fungi colo-

nized seed coat (testa) followed by endosperm and embryo, respectively (**Table 2**). The highest colonization of storage aflatoxigenic molds such as *A. flavus* and *A. parasiticus* were in seed coat (21.97 and 11.53%, respectively) followed by endosperm (12.93 and 10.37%) and embryo (12.93 and 6.47%) (**Table 2**). Similar mycological investigations showed the colonization of *A. flavus* and *A. parasiticus* in different seed parts involving embryo [2; 28]. Therefore, the role of seedborne fungi (especially aflatoxigenic) as one of the major source of seed deterioration during storage should be studied throughout integrated management to minimize the chances of further storage losses and field infection [30]. It was reported the alteration in moisture content (MC) of stored seeds depends up on the hygroscopic nature of storage containers [9]. Similarly, in our study both polyethylene bags and tin cans caused no any significant alteration in SMC (compared with the initial SMC), however cloth and paper bags caused significant decrement in SMC (**Table 3**). The retention of superior SMC recorded here by both polyethylene bags and tin cans is probably attributed to impervious nature of previous storage containers compared with either cloth or paper bags [21].

There was a strong relationship between both storage periods and type of seed storage containers with seed health expressed as root depth, shoot height, percentage germination (**Tables 4(a-d)**). Prolonged storage periods were accompanied with decrease in vigor index ranged between slight and significant reduction depending upon the nature of storage containers. Such recorded decrease in vigor index (Seed germination, root depth and shoot height) strongly agrees with Basay *et al*., [12] and correlated with the alteration in SMC (**Table 3**) which act as a key factor influencing seed physical properties [27] and effectiveness of naturally seedborne fungal flora [2;9] which play vital role in diminution of viability and vigor of seeds [13]. The decrease in vigor index was significant with polyethylene storage container followed by tin cans, papers bags and cloth bags respectively (**Table 4(d)**). In the same connection, the polyethylene as impermeable storage container followed by tin cans, paper bags and cloth bags, respectively caused significant alteration in concentration of both O_2 and CO_2, which are the main cause of deterioration of agricultural products [18].

Table 2. Detection of seedborne fungi (Incidence [%] of fungal species) in different seeds parts of E. alata using standard blotter method.

Fungal species	Incidence (%) of fungal species			
	Surface disinfected seeds	Seed Coat	Endosperm	Embryo
Alternaria sp.	12.16	3.56	2.78	5.82
Aspergillus flavus	42.71	12.93	6.35	21.97
Aspergillus parasitcus	28.37	11.53	10.37	6.47
Fusarium moniliforme	3.45	1.86	0.93	0.66
Fusarium oxysporum	4.63	2.06	2.17	0.4
Sclerotium bataticola	4.36	1.89	1.75	0.72
Penicillium sp.	0.8	0.61	0.19	ND[Z]
Chaetomium sp.	1.62	0.86	0.76	ND
Drechslera sp.	0.86	0.51	0.24	0.11
Trichoderma sp.	0.12	0.09	0.03	ND
Pythium sp.	0.92	0.61	0.31	ND

[Z]ND: Not detected under the experimental conditions.

Table 3. Effect of various storage containers on seed moisture contentZ (SMC) [%] of E. alata stored for different storage periods (months).

Storage container	Seed moisture content (SMC) [%] of *E. alata* stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	9.58	9.54	9.53	9.51	9.44	9.41	0.2512
Cotton cloth bags	8.20	8.11	7.91	7.50	6.67	6.07	0.1947
Tin cans	9.63	9.57	9.50	9.45	9.43	9.39	0.1484
Paper bags	9.13	8.82	8.60	8.15	7.71	7.41	0.1955
L. S. D. at: 05	0.2337	o.1175	0.2067	0.1935	0.2378	0.2566	

Z: Initial seed moisture content was 9.72 (%).

Table 4-a. Effect of various storage containers on root depth (cm) of germinating seeds of E. alata stored for different storage periods (months).

Storage container	Root depth (cm) of germinating seeds of *E. alata* stored for different storage periods (months)						
	1	2	3	4	5	6	LSD. at: 05
Polypropylene bag	15.30	14.33	12.77	11.73	9.97	8.27	0.4398
Cotton cloth bags	18.60	18.43	17.97	17.50	15.67	15.27	0.7383
Tin cans	17.53	17.03	16.17	15.90	15.07	13.80	0.8323
Paper bags	18.27	17.60	17.03	16.53	16.00	15.07	0.6134
LSD at: 05	0.4280	0.4892	0.8117	0.4175	0.8044	1.0651	

Table 4-b. Effect of various storage containers on shoot height (cm) of germinating seeds of E. alata stored for different storage periods (months).

Storage container	Shoot height (cm) of germinating seeds of *E. alata* stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	9.63	8.97	8.40	7.30	6.37	4.83	0.5204
Cotton cloth bags	10.17	9.97	9.70	9.20	8.33	7.73	0.4836
Tin cans	8.97	8.67	8.07	7.50	6.40	4.90	0.4926
Paper bags	9.67	9.10	8.67	8.13	7.30	6.67	0.4438
L. S. D. at: 05	0.3689	0.4104	0.321	0.4644	0.6221	0.7590	

Table 4-c. Effect of various storage containers on percentage germination of E. alata stored for different storage periods (months).

Storage container	Percentage germination of *E. alata* stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	61.30	59.93	57.20	53.70	51.20	43.33	1.9266
Cotton cloth bags	70.17	69.00	65.03	63.00	59.00	55.20	1.2275
Tin cans	65.23	63.20	60.57	56.87	52.27	46.53	1.3697
Paper bags	69.30	65.27	61.70	57.13	53.73	51.07	1.6294
LSD at: 05	0.5040	0.9993	1.4174	2.1543	1.8938	2.2118	

Table 4-d. Effect of various storage containers on seed vigor indexZ of E. alata stored for different storage periods (months).

Storage container	Vigor index of *E. alata* stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	1528.41	1396.45	1210.73	1022.09	836.27	567.67	60.3480
Cotton cloth bags	2018.46	1959.60	1799.26	1682.10	1416.00	1269.60	83.1640
Tin cans	1728.68	1624.24	1467.73	1330.68	1121.99	870.17	73.3190
Paper bags	1935.78	1742.62	1585.69	1409.29	1251.99	1109.85	49.980
L. S. D. at: 05	32.05	60.769	65.249	72.736	92.857	91.266	

Z: Vigor index = Seed germination X [mean Root depth (cm) + mean Shoot length (cm)].

The effect of seed storage containers on aflatoxins production was investigated for seeds of many crops however, this approach does not provide a comprehensive view of the impact of range seeds. In our results, aflatoxins production were found to be inferior with employment of cloth bags followed by paper bags, tin cans and polyethylene bags respectively (**Tables 5(a,d)**). Such inhibition agrees with the findings of Paramawati *et al*.,

Table 5-a. Effect of different storage containers and storage periods (month) on the natural contamination of E. alata seeds with aflatoxin B1 (µg/Kg seed).

Storage container	Natural contamination with aflatoxin B_1 (µg/Kg seed) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	42.07	38.70	34.20	29.77	24.23	19.27	2.4825
Cotton cloth bags	14.53	12.00	6.20	2.70	0.00	0.00	5.8834
Tin cans	36.00	27.20	23.13	20.93	17.30	11.20	3.0896
Paper bags	27.70	24.93	22.93	14.50	9.17	0.00	3.2088
L. S. D. at: 05	4.3322	2.6583	5.9746	5.7786	2.1357	2.1357	

Table 5-b. Effect of different storage containers and storage periods (month) on the natural contamination of E. alata seeds with aflatoxin B2 (µg/Kg seed).

Storage container	Natural contamination with aflatoxin B_2 (µg/Kg seed) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	92.17	85.30	77.80	73.27	69.03	63.37	4.1764
Cotton cloth bags	45.07	34.93	28.63	18.50	11.17	9.50	3.5914
Tin cans	71.50	65.10	58.30	51.30	38.10	27.50	6.7281
Paper bags	77.17	66.27	55.67	46.87	37.43	24.27	4.9676
L. S. D. at: 05	6.8605	4.6379	3.6884	6.4785	5.8013	3.2707	

Table 5-c. Effect of different storage containers and storage periods (month) on the natural contamination of E. alata seeds with aflatoxin G1 (µg/Kg seed).

Storage container	Natural contamination with aflatoxin G_1 (µg/Kg seed) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	35.87	30.03	26.77	21.50	18.40	11.23	2.5326
Cotton cloth bags	ND	ND	ND	ND	ND	ND	0.00
Tin cans	23.10	18.10	12.10	10.70	ND	ND	4.3153
Paper bags	26.07	19.57	10.97	9.80	5.73	ND	2.6836
L. S. D. at: 05	5.0457	1.5258	1.3135	1.1019	4.6876	1.2105	

ND: Not detected under the experimental conditions.

Table 5-d. Effect of different storage containers and storage periods (month) on the natural contamination of E. alata seeds with total aflatoxinsZ (µg/Kg seed).

Storage container	Natural contamination with total aflatoxins^Z (µg/Kg seed) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	170.11	154.03	138.77	124.53	111.67	93.87	7.8536
Cotton cloth bags	59.60	46.93	34.83	21.20	11.17	9.50	7.0388
Tin cans	139.23	113.03	89.77	77.60	60.47	35.47	10.7660
Paper bags	105.17	79.00	62.40	47.80	29.93	11.13	5.5776
L. S. D. at: 05	12.8030	7.2977	9.0200	8.8850	6.5909	3.6033	

Z: Total aflatoxins (Sum. of B1 + B2 + G1).

[25]. It can explain in terms of the decrement alteration in SMC inferior requisite level for growth and aflatoxins production by seedborne fungi [2;9]. Data in our investigation (**Tables 6 (a-d)**) shows that prolongation of storage periods was accompanied with gradual deterioration in the biochemical aspects of seed such as lipids, ash, total nitrogen, and fiber contents. The employment of cloth bags followed by paper bags, tin cans and polyethylene bags respectively diminished such as sharpness in the deterioration of seed biochemical aspects (**Tables 6 (a-d)**).

Table 6(a). Effect of different storage containers and storage periods (month) on nitrogen content (mg/g dry weight) of E. alata seeds.

Storage container	Nitrogen content (mg/g dry weight) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	32.48	30.06	28.6	28.37	27.48	26.50	0.1773
Cotton cloth bags	48.65	45.54	43.97	43.67	42.08	40.83	0.1002
Tin cans	39.65	35.86	32.88	31.31	30.76	31.073	0.1273
Paper bags	44.21	42.55	41.30	39.89	38.62	36.68	0.1119
L. S. D. at: 05	0.1609	0.0981	0.1092	0.1084	0.0853	0.2270	

Table 6(b). Effect of different storage containers and storage periods (month) on fiber content (% of dry weight) of E. alata seeds.

Storage container	Fiber content (% of dry weight) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	5.03	4.49	4.25	5.02	3.84	3.48	1.1738
Cotton cloth bags	5.36	5.09	4.96	4.84	4.76	4.61	0.8601
Tin cans	5.20	4.39	4.07	3.77	3.38	3.13	0.1723
Paper bags	5.27	4.93	4.66	4.37	4.94	4.12	0.3679
LSD at: 05	0.2849	0.2246	0.2517	1.5158	1.1231	0.2852	

Table 6(c). Effect of different storage containers and storage periods (month) on lipids content (% of dry weight) of E. alata seeds.

Storage container	Lipids content (% of dry weight) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	6.03	5.12	4.44	3.51	3.02	2.40	0.4421
Cotton cloth bags	7.63	7.21	7.03	6.86	4.29	6.50	0.4052
Tin cans	6.35	5.97	5.41	4.97	4.28	3.81	0.5560
Paper bags	7.20	6.83	6.40	6.06	5.83	5.50	0.3554
LSD at: 05	0.5457	0.4259	0.4288	0.3882	0.3366	0.6253	

Table 6(d). Effect of different storage containers and storage periods (month) on ash content (% of dry weight) of E. alata seeds.

Storage container	Ash content (% of dry weight) of *E. alata* seeds stored for different storage periods (months)						
	1	2	3	4	5	6	LSD at: 05
Polypropylene bag	4.69	4.35	4.11	3.92	3.59	3.45	1.6900
Cotton cloth bags	6.52	6.40	6.25	6.20	6.09	5.89	3.5538
Tin cans	5.70	4.94	4.23	3.87	3.71	3.56	1.9654
Paper bags	6.22	5.921	5.67	5.40	5.18	4.91	0.7454
LSD at: 05	4.1092	3.1294	1.3800	1.5175	0.6931	1.4413	

Such results were in agreed with our data recorded previously concerning soybean seeds [17]. In this regard, the alteration in SMC due to the employment of different storage containers (**Table 3**) was the main cause of seed-borne fungal activities [2; 9] including production of hydrolytic enzymes such as proteinase [26], lipase [23] and lignocellulolytic enzymes [19] which were responsible for the biotic degradation of seed contents of protein, lipids and fiber [22]. Our results indicate that these biochemical events were correlated with maintenance of

high germination rate during storage. The success of storage container to preserve seed viability recorded in this investigation is still limited and more studies through integrated seed management program to maintain healthy range plants in the grassland is needed hence production vital seeds are necessary for desert ecological maintenance. These will be considered in the forthcoming investigation.

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