Response of seeds and pollen of *Onobrychis viciifolia* and *Onobrychis oxyodonta* var. *armena* to NaCl stress

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Edited by: Edmilson José Ambrosano

ABSTRACT: Sainfoin (*Onobrychis viciifolia* Scop.) is an important forage legume crop with 52 species adapted to dry and poor soils in Turkey, but little is known about the effects of salinity on germination and seedling growth in arid and semiarid regions suffering from salinity problem. The seeds and pollen of two species of sainfoin *O. viciifolia* and *O. oxyodonta* var. *armena* (Syn: *O. armena*) were exposed to 0, 5, 10, 20 and 30 dS m^{-1} of NaCl under *in vivo* and *in vitro* conditions and evaluated for germination under salt stress by comparing germination percentage, mean germination time, root and shoot length, fresh and dry seedling weight and dry matter. Increased salinity levels generally resulted in decrease in all traits except time to germination, dry seedling weight and dry matter, which increased at high salinity levels. *O. viciifolia* seeds germinated and grew more rapidly compared to O. *armena* seeds under NaCl stress. No decrease in germination and seedling growth up to 10 dS m^{-1} was recorded. On the other hand, there was a clear difference for germination and seedling growth between *in vivo* and *in vitro* conditions. Lower values were obtained from *in vitro* experiments; suggesting that mineral salts, sucrose and agar may have resulted in higher osmotic potential inhibiting germination and seedling growth of species compared *in vivo* conditions. Decrease in pollen germination with increasing salinities was very sharp, indicating that pollen germination had higher sensitive to salinity. But, pollen grains of *O. armena* germinated rapidly compared to *O. viciifolia*. The results emphasize that *in vivo* experiments could be used for screening of NaCl tolerance in sainfoin cultivars without expensive chemicals and sophisticated equipments, but pollen germination is more appropriate for its wild relatives.
Key words: *in vivo*, *in vitro*, sainfoin, salinity, germination

Introduction

A total of 160 *Onobrychis* species exists throughout the world and are spread from Baltic States to Mediterranean region passing through all region lying in the Asia and Iran-Siberian element. In total 27 *Onobrychis* species are endemic to Turkey. Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume crop which is widely adapted to calcareous, well drained, poor and dry soils (Açiköz, 2001; Cavallarin et al., 2005). It has been grown extensively in Eastern Europe and West Asia (Sancak et al., 2003). It is highly tolerant to salinity and drought and improves soil fertility by fixing atmospheric nitrogen (Özaslan-Parlak and Parlak, 2008; Imanparast and Hassanpanah, 2009). It has erect stems and deep tap roots holding soil firmly to the deeper layer and prevents soil erosion. It is preferred to other forage plants on dry soils of Anatolia because it produces satisfactorily hay under adverse conditions (Cavallarin et al., 2005). *O. oxyodonta* var. *armena* (Syn: *O. armena* Boiss. and Huet) is a winter-resistant wild sainfoin species and is found extensively in meadows of Central Anatolia and Eastern Anatolia regions (Elçi, 2005).

Sainfoin is largely grown on low moisture soils of Anatolia where evaporation exceeds precipitation, resulting in salt accumulation on the soil surface (Kaya et al., 2003; Pessarakli, 1999). There are limited reports on the effects of salinity on seed germination and growth in *Onobrychis* species. Elçi (2005) indicated that it was suitable for salt infected soils and favorable yield could be obtained at moderate level of salinity. Greub et al. (1985) reported that sainfoin (*Onobrychis viciifolia* Scop.) was tolerant to NaCl, but it ranked lower than the grasses. Özaslan-Parlak and Parlak (2008) has indicated a decrease in plant height, dry hay yield and crude protein ratio due to increased salinity in irrigation water. However, it could not be determined clearly that differences between species against salt tolerance are mainly due to genetic or morphologic factors. Therefore, this study investigated the seed and pollen germination and the seedling growth of *O. viciifolia* and *O. oxyodonta* var. *armena* under various NaCl stresses using *in vivo* and *in vitro* techniques.

Materials and Methods

Seeds of *O. viciifolia* Scop. and *O. oxyodonta* var. *armena* (Syn: *O. armena* Boiss. and Huet) were collected from plants grown at same field, under similar cultural practices. Both species were tested against NaCl under *in vivo* and *in vitro* conditions. NaCl levels were adjusted as 5, 10, 20 and 30 dS m^{-1} (electrical conductivities of the solutions) of NaCl using a conductivity meter (Model WTW Cond. 314i, Germany). Distilled water served as a control (0 dS m^{-1}).

Four replicates of 50 seeds of each species were germinated between three rolled filter papers with 15 mL of respective test solutions. Seeds were treated with fungicide before planting; papers were replaced every two days to prevent salt accumulation. The rolled paper with seeds was put

into sealed transparent plastic bags to avoid moisture loss. Seeds were allowed to germinate at 20 ± 1°C in the dark for 14 days.

Surface sterilization of the seeds of both *Onobrychis* species was done using 50% commercial bleach for 15 min followed by rinsing with distilled sterile water. The surface-sterilized seeds were transferred to germination substrate containing MS medium (Murashige and Skoog, 1962), 30 g L^{-1} sucrose and 8 g L^{-1} agar supplemented with 2.72, 4.12, 11.30 and 17.22 g NaCl L^{-1} for obtaining EC values of 5, 10, 20 and 30 dS m^{-1}, respectively. The pH of medium was adjusted to 5.7 with 1 M NaOH or HCl before autoclaving at 121°C, 104 KPa for 20 min. Seeds were allowed to germinate at 20 ± 1°C under white fluorescent light with 16 h photoperiod.

A seed was considered germinated when the emerging radicle elongated to 2 mm. Germination percentage was recorded every 24 h for 14 days (ISTA, 2003). Mean germination time (MGT) was calculated following Ellis and Roberts (1980) to assess the rate of germination. $MGT = \sum Dn / \sum D$, where n is the number of the seeds newly germinated on day D, and D is the number of days from the beginning. Root, shoot length, fresh and dry seedling weights were measured on the 14th day. Dry weights were measured after drying samples at 70°C for 48 h in an oven.

Media reported to germinate pollen grains of sainfoin (*Onobrychis viciifolia* Scop.) described by Sancak et al. (2003) had a common medium containing of 100 g L^{-1} sucrose, 200 mg L^{-1} KNO_3, 150 mg L^{-1} $MgSO_4$, 150 mg L^{-1} $Ca(NO_3)_2$, 175 g L^{-1} PEG 4000, 150 mg L^{-1} H_3BO_3 and stigma extract at pH 6.5. Pollen was left to germinate for 1 hour at 24°C under cool white fluorescent light (35 μmol m^{-2} s^{-1}). Germination was observed under light microscope (Olympus BH-2). Pollen grains with pollen tube longer than the diameter of pollen were considered as germinated (Sancak et al., 2003).

The experimental design was two factors completely randomized design (CRD) with four replications. Data given in percentages were subjected to arcsin transformation before statistical analysis. For all investigated parameters, Analysis of Variance was performed using the MSTAT-C computer software (Michigan State University). Differences among the mean values were compared by Duncan's Multiple Range test ($p < 0.05$).

Results and Discussion

Seed germination percentage decreased by increasing NaCl in both species while the decrease was less in *O. viciifolia* compared to *O. armena* (Figure 1). The lowest germination (40.5%) was determined in *O. armena* at 30 dS m^{-1}. The lower doses of NaCl did not change the seed germination; however, seed germination drastically declined at doses higher than 20 dS m^{-1}.

Greater reduction in shoot length due to NaCl was very evident ($p < 0.01$) (Table 1). The longest shoots were obtained from *O. viciifolia* under all NaCl stresses. Shoot length was severely influenced by NaCl while the impact was more detrimental on *O. armena*. Increased NaCl diminished root length of the two species; however, this decrease was more prominent in *O. armena*.

Depending on the decrease in shoot and root length, fresh and dry seedling weights reduced gradually with the

Table 1 – Mean germination time (MGT), shoot length, root length, seedling fresh and dry weight and dry matter ratio of sainfoin species at various NaCl levels under *in vivo* conditions.

Variety	NaCl	MGT	Shoot length	Root length	Seedling fresh weight	Seedling dry weight	Dry matter
	dS m^{-1}	day	cm		mg per plant		%
O. viciifolia	0	1.94^{f}	5.69^{a}	4.18	152^{a}	9.3^{bc}	6.0^{f}
	5	1.95^{f}	4.91^{b}	5.13	128^{ab}	9.8^{bc}	7.6^{ef}
	10	2.07^{f}	3.25^{d}	3.13	94^{bcd}	9.8^{bc}	9.8^{cd}
	20	2.57^{de}	1.97^{e}	2.47	72^{cde}	10.8^{b}	14.8^{b}
	30	4.46^{b}	0.56^{f}	1.20	42^{ef}	12.3^{a}	28.8^{a}
Mean		2.60	3.27	3.22a	98	10.3	13.4
O. oxyodonta var. *armena*	0	2.33^{ef}	3.57^{cd}	4.52	109^{abc}	8.5^{c}	8.0^{de}
	5	2.85^{d}	3.90^{c}	4.21	106^{abc}	8.5^{c}	6.8^{ef}
	10	3.91^{c}	2.46^{e}	3.21	76^{cde}	8.3^{c}	10.5^{c}
	20	5.79^{a}	0.66^{f}	1.49	53^{de}	9.5^{bc}	15.8^{b}
	30	$-^{g}$	$-^{g}$	-	$-^{f}$	$-^{d}$	$-^{g}$
Mean		2.97	3.48	2.69^{b}	69	7.0	10.3
Summary of ANOVA							
Variety (A)		**	**	**	**	**	**
NaCl (B)		**	**	**	**	**	**
A × B		**	**	ns	*	**	**

*significant at $p < 0.05$, **$p < 0.01$ and ns: non-significant. Different letters at the same column show significant differences at 0.05 level.

increasing salinity stress (Table 1). No seedling growth was observed in *O. armena* subjected to 30 dS m^{-1}. Higher fresh seedling weights were recorded on *O. viciifolia* compared to *O. armena* at all NaCl levels. Dry Seedling weight showed a trend similar to that of fresh weight and showed decline in fresh seedling weight, dry weight enhanced with increasing NaCl levels. Increase in dry weight also resulted in increased dry matter of both *Onobrychis* species.

NaCl influenced seed germination of the species differently. Seed germination was not reduced at 5 dS m^{-1}, while 10 dS m^{-1} caused reduction in germination (Figure 2). Furthermore, higher NaCl concentration resulted in lower viability of the seeds. The detrimental effect of NaCl on seed germination appeared at 30 dS m^{-1}. However, mean germination time was delayed by increased NaCl. Apparent difference was recorded among the species. MGT increased with each increase in NaCl stress in the two species. Contrarily, NaCl level of 20 dS m^{-1} caused drastic delay in germination time of two species in general (Table 2).

For the species, root and shoot length decreased in response to increasing concentration of NaCl; however, this decrease was more prominent in *O. armena*. No root and shoot growth was recorded at NaCl levels of 30 dS m^{-1} (Table 2). The longest roots and shoots were obtained from *O. viciifolia* under all NaCl concentrations.

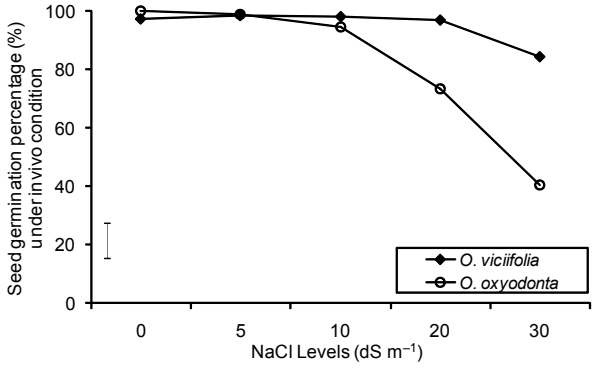

Figure 1 – Seed germination percentage of *O. viciifolia* (♦) and *O. oxyodonta* var. *armena* (○) under *in vivo* condition. The bar represents LSD value which is 12.5.

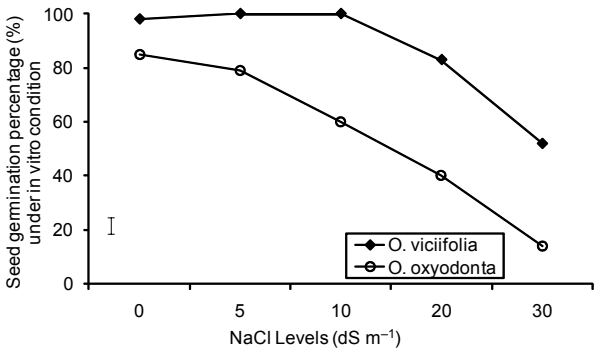

Figure 2 – Seed germination of *O. viciifolia* (♦) and O. *oxyodonta* var. *armena* (○) under *in vitro* condition. The bar represents LSD value which is 5.98.

Table 2 – Mean germination time (MGT), shoot length, root length, seedling fresh and dry weight and dry matter ratio of sainfoin species at various NaCl levels under *in vitro* conditions

Variety	NaCl	MGT	Shoot length	Root length	Seedling fresh weight	Seedling dry weight	Dry matter
	dS m^{-1}	day	cm		mg per plant		%
O. viciifolia	0	3.01^{d}	4.65^{a}	5.60^{b}	368^{b}	42.8^{bc}	11.3^{de}
	5	3.29^{d}	5.22^{a}	6.91^{a}	564^{a}	64.5^{a}	11.3^{de}
	10	4.34^{c}	4.59^{a}	4.95^{bc}	513^{a}	62.0^{ab}	11.0^{e}
	20	7.08^{a}	1.29^{e}	1.17^{e}	189^{c}	30.0^{cd}	15.3^{bc}
	30	-^{e}	-^{f}	-^{f}	-^{e}	-^{e}	-^{f}
Mean		3.54	3.15	3.72	327	39.9	9.8
O. oxyodonta var. *armena*	0	5.05^{c}	2.66^{c}	3.95^{cd}	334^{b}	44.0^{bc}	13.5^{cde}
	5	4.64^{c}	3.48^{b}	4.79^{bc}	193^{c}	28.0^{cd}	14.5^{bcd}
	10	6.07^{b}	1.96^{d}	3.61^{d}	118^{cd}	20.3^{d}	17.3^{ab}
	20	-^{e}	0.68^{e}	1.25^{e}	75d^{e}	16.3^{cd}	19.5^{a}
	30	-^{e}	-^{f}	-^{f}	-^{e}	-^{e}	-^{f}
Mean		3.15	1.76	2.72	144	21.7	13.0
Summary of ANOVA							
Variety (A)		ns	**	**	**	**	**
NaCl (B)		**	**	**	**	**	**
A × B		**	**	*	**	**	*

*significant at $p < 0.05$, **$p < 0.01$ and ns: non-significant. Different letters at the same column show significant differences at 0.05 level.

Compared to control, each increase in NaCl concentration resulted in remarkable decrease in fresh and dry seedling weights for the species. Although the species responded variable to different NaCl concentrations, the highest seedling fresh weight (564 mg per plant) and dry weight (64.4 mg per plant) were recorded at 5 dS m^{-1} for *O. viciifolia*. Depending on shoot and root length, seedling fresh weight fluctuated. In contrast, dry matter of the species was enhanced with increased NaCl except at 30 dS m^{-1} NaCl.

Pollen germination in control ranged from 83% for *O. viciifolia* to 96% for *O. armena* (Figure 3). Saline conditions induced a decrease in pollen germination. A very different response was detected between species at lower NaCl concentrations while none of the species were able to germinate at 30 dS m^{-1}. Especially, 20 dS m^{-1} caused a detrimental effect on pollen germination.

Germination and seedling growth of the investigated sainfoin species were influenced by salt concentrations under *in vivo* and *in vitro* conditions. However, the responses of these species to salt concentration were different. Reduction in seed germination is in conformity with Khajeh-Hosseini et al. (2003), Murillo-Amador et al. (2002), who found that decreased germination was due to increased salinity. The results of this study are also in agreement with Prakash et al. (1998), Gadallah and Ramadan (1997), Gadallah (1996), Prakash et al. (1995) and Francois and Bernstein (1964), who emphasized that different varieties showed variable response against various salinity levels. Similarly, Hampson and Simpson (1990), Murillo-Amador et al. (2002), Okçu et al. (2005), Kaya et al. (2006), Karlidag et al. (2009) reported that NaCl had only adverse effect on germination by creating osmotic potential in the medium and caused delayed seed germination. There was a clear difference for germination and seedling growth under *in vivo* and *in vitro* conditions. Lower values were obtained from *in vitro* experiments; suggesting that the sucrose, MS mineral salts and agar and in *in vitro* conditions might have caused higher osmotic potential inhibiting germination and seedling growth of species than under *in vivo* conditions. Germination and seedling growth were reduced due to increased salt concentration with varying responses for species while NaCl affected germination of seeds by creating an external osmotic potential preventing water uptake (Pessarakli et al., 1991; Kaya et al., 2006). These results agree with Murillo-Amador et al. (2002) in cowpea and Okçu et al. (2005) in pea but should not be approved as a general rule because opposite results were recorded by Atak et al. (2006) who found reduced seedling growth under NaCl salinity. Pollen germination was also adversely affected by increasing NaCl concentration. *O. armena* gave higher pollen germination at all NaCl levels; suggesting that there was no overlap in the response of seeds and pollen to NaCl. Martinez-Pelle et al. (1995) found different responses with respect to pollen germination under various salinity stresses in pistacia species.

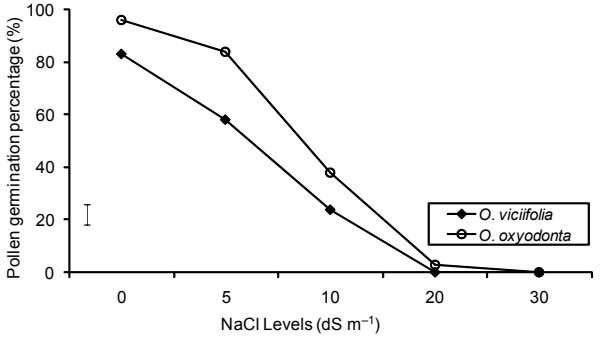

Figure 3 – Pollen germination of *O. viciifolia* (♦) and *O. oxyodonta* var. *armena* (○). The bar represents LSD value which is 7.02.

O. viciifolia gave higher seed germination and lower time to germinate under *in vivo* and *in vitro* conditions. However, the detrimental effect of NaCl on seedling growth was very evident in *O. armena* because no shoot and root growth was recorded at 30 dS m^{-1}. Furthermore, dry seedling weight and dry matter of the species enhanced with increased NaCl except at 30 dS m^{-1} NaCl for *O. armena*. Similar findings have been reported in *Bothriochloa persuta* L., *Dichanthium annulatum* and *Panicum antidotale* under salinity (Akhtar and Hussain, 2008). The results are in line with the findings of Rehman et al. (2008) in wheat, Steppuhn et al. (2001) in canola, field bean, dry bean and drum wheat and Li (2008) in *Limonium sinense*, *Sorghum sudanense* and soybean.

In conclusion, critical period of crop plant to salinity generally occur on germination and seedling growth stages. The relative time of seed germination and seedling emergence of plants influences the degree of tolerance. Thus, the species or cultivars which rapidly germinate or emerge gain advantages for tolerance to NaCl. NaCl caused an adverse effect on germination and seedling growth with delayed mean germination time in the species. Similar results were detected under *in vivo* and *in vitro* conditions while lower values were determined in *in vitro*. It seems that *O. armena* germinate and grow more slowly compared to *O. viciifolia*. Both sainfoin species could keep up with the salinity up to 10 dS m^{-1} during seed germination and early growth stages, and *O. viciifolia* was more tolerant to NaCl compared to *O. oxyodonta* var. *armena*.

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Received April 05, 2010
Accepted September 24, 2010