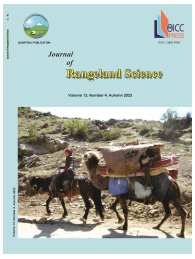

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Inter-specific variation for germination and seedling properties and evaluation of breaking seed dormancy in different species of Onobrychis Mill

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Abstract:
The genus Onobrychis Mill. comprises a few agronomically important forage legume species, with Sainfoin (Onobrychis viciifolia) being the most widespread. There are few studies on the dormancy pattern of Onobrychis wild species. This investigation was conducted to evaluate inter-specific variation for germination and seedling properties and evaluation of breaking seed dormancy in different species of Onobrychis during 2017. The treatments were control (seeds with pods were germinated in petri dishes immersed in distilled water), mechanical scarification, chilling, seedbed and chemical scarification. Seeds in the control treatment showed very low germination, ranging from 0% to 10% among species. In comparison, between physical treatments, seed pod removal significantly ($P < 0.01$) increased germination percentage and germination rate in all species. Germination percentages of seed pod removal treatment ranged from 50% (in O. vassilczenkoi and O. inermis) to 92.0% (in O. persica). The results indicate that the application of acid scarification treatment depends on species and duration of application of acid. Acid scarification for 7 minutes resulted in the increase in germination percentage and germination rate of O. caput-galli, O. inermis, O. vassilczenkoi and O. persica ($P < 0.01$). Whereas application of sulfuric acid for 15 minutes increased germination in O. montana, O. caput-galli, O. argyrea and O. arenaria much more than other treatments ($P < 0.01$). Chilling treatments had no significant effects on germination. Overall, results indicated that there was high variation among the different species of Onobrychis which is important for the improvement of seed germination in future programs for seed germination approaches. Based on the results, it can be suggested that sowing in peat and seed pod removal treatments can be the most effective methods in the germination of sainfoin species.

Keywords: Diversity; Scarification; Sainfoin; Wild relatives

1. Introduction

Sainfoin (Onobrychis Miller) includes annual and perennial species distributed from the Mediterranean region to Central Asia, especially Iran and Anatolia [1, 2]. The genus comprises about 170 species in two subgenera with 9 sections [Onobrychis subgenus (sections: Dendrobrychis, Lophobrychis, Onobrychis and Laxiflorae) and Sisyrosema subgenus (section: Anthyllium, Afghanicae, Heliobrychis, Hymenobrychis and Insignes)] [3, 4]. These species are relatively drought tolerant with nitrogen fixation activity similar to other forage legumes [5–7]. Sainfoin contains condensed tannins which prevent bloat in ruminants and improve protein digestion by grazing animals [2, 8].

Most species of Fabaceae have some testa-imposed dormancy preventing them from imbibing water even under suitable environmental conditions [9]. Seed dormancy is ecologically significant in the nature and causes delays in germination until suitable conditions for the establishment of seedlings can be created; also, it allows for the maintenance of an annual seed bank [10]. The germination of dormant seeds is slow and not uniform, limiting their applications. As the germinating seedling emerges from the pod, the radicle can be injured and infected by pathogens; for example, Alternaria and Fusarium spp. [11].

Several methods have been used to soften or break hard seeds artificially, such as mechanical and acid scarification [12], chilling [13], short time heating [14, 15] and

Table 1. Mean percentage of hard seeds, swollen seeds and viability of Onobrychis species.

Species	Hard seeds (%)	Swollen seeds (%)	Viability (%)
O. biebersteinii	6.67	9.33	87
O. chorassanica	17.33	4.00	76
O. vaginalis	36.00	13.33	65
O. viciifolia	0	9.33	92
O. vassilczenkoi	16.00	12.00	76
O. arenaria	4.00	38.67	80
O. inermis	1.33	41.33	88
O. persica	1.33	5.33	95
O. argyrea	2.67	12.00	90
O. montana	0	8.00	94
O. caput-galli	2.67	13.33	90

even priming [16]. Uzun and Aydin have reported that the most effective treatment in breaking hard seed dormancy of legume seeds is mechanic disruption [17]. Majidi and Barati have noted the beneficial effects of acid scarification on overcoming dormancy in two Onobrychis species [18]. Kimura and Islam have reported that the effectiveness of scarification depends on the species and the type of treatment [19]. In most legume species, physical dormancy is broken by different treatments, but it is not clear how this mechanism works [20]. On the other hand, the effect of each method on germination rate depends on the plant species and varieties due to different seed coat structures [21]. Despite previous studies on the effects of different methods on breaking hard seed dormancy in legumes, little information is available regarding seed dormancy break in sainfoin species. Carleton et al. studied the effect of seed pod and temperature on some cultivated species, O. viciifolia, and showed that the presence of seed pods at all studied temperatures reduced the speed of germination and seedling elongation [22]. Furthermore, there are few studies on the dormancy pattern of Onobrychis wild species. The objectives of this study were to evaluate seed dormancy in different species of genus Onobrychis and to determine the effects of mechanical, chemical and chilling methods on breaking the dormancy in O. viciifolia, and 10 related wild species.

2. Materials and methods

2.1 Plant matrials

The seeds of 11 species of Onobrychis (O. viciifolia Scop., O. argyrea Boiss, O. caput-galli, O. vaginalis C. A. Mey, O. montana DC, O. chorassanica Bunge ex Boiss, O. inermis Steven, O. vassilczenkoi Grossh, O. biebersteinii Sirj, O. arenaria (Kit.) DC and O. persica Sirj) were used in this study. The germplasm was obtained from The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), United States Department of Agriculture (USDA). All species were sown in the field after removing seed pods manually to reproduce seeds during 2017. Seed material was determined by a tetrazolium test with four replications of 100 seeds to test the viability of seeds [23]. To prevent microbial contamination, seeds were surface sterilized for 5 minutes in sodium hypochlorite, rinsed twice with tap water and then distilled sterile water. A range of dormancy breaking treatments were applied. Seeds were allowed to germinate at 20 ± 1°C in darkness and 70% of relative humidity. At the end of the germination test, hard and swollen seeds were determined for each species. Swollen seeds imbibed, but did not germinate, and hard seeds failed to absorb water. Percentage of hard seeds (viable seeds without germination) was calculated in each plot (Petridish). Germination was defined as seeds with approximately 2 mm primary root emerged from the seed coat. Germination percentage was recorded every 24 h for 10 days. After 10 days, the percentage of germinated seeds and germination rate were calculated according to ISTA [24]. Germination rate was calculated using the following equation:

$$GR = \sum_{1}^{i} \frac{n_i}{D_i}$$

where GR is germination rate, n_i is the number of germinated seeds on day i and D_i is the number of days after beginning the experiment [25].

The experiment was carried out as a completely randomized design (CRD) with four replications for each species in separate experiments described below. Mean length and dry matter of shoot and root were measured at the end of the period. The shoots and roots were weighed and then dried in an oven at 70°C for 24 hours before re-weighing.

2.2 Dormancy breaking treatments

- Mechanical scarification treatment implemented by removing seed pods manually un-pod (UP) through scratching the seed pod with a scalpel to guarantee water and gas transport scarification (SC).
- Chemical scarification: The seed pods were immersed in concentrated sulfuric acid (97%) for 5 (SA5), 7 (SA7) and 15 (SA15) minutes and thoroughly washed with tap and distilled water.
- Chilling treatments: Chilling treatments on germination were evaluated by exposing the seeds wet condition at 1°C during a week (CL). Then, seeds were subjected to germination test in petri dishes.
- Seedbed treatments: Consisted of three treatments; Scarification (P+SC): abrading pod with scalpel and sowing it in peat, Removal of seed pod (P+UP): removing seed pod carefully (by hand) and sowing in the peat, and seed with pod (P+P): the seed pods were sown in peat. Peat was filled in multi-celled trays having 75 cells with the depth of 3 cm and the diameter of 3 cm. Each seed was sown in one cell and washed with distilled water.

Control treatment (C): seed pods germinated in petri dishes immersed in distilled water.

2.3 Statistical analyses

The data were analyzed according to a completely randomized block (CRD) design. Significant differences among means were performed by the least significant difference

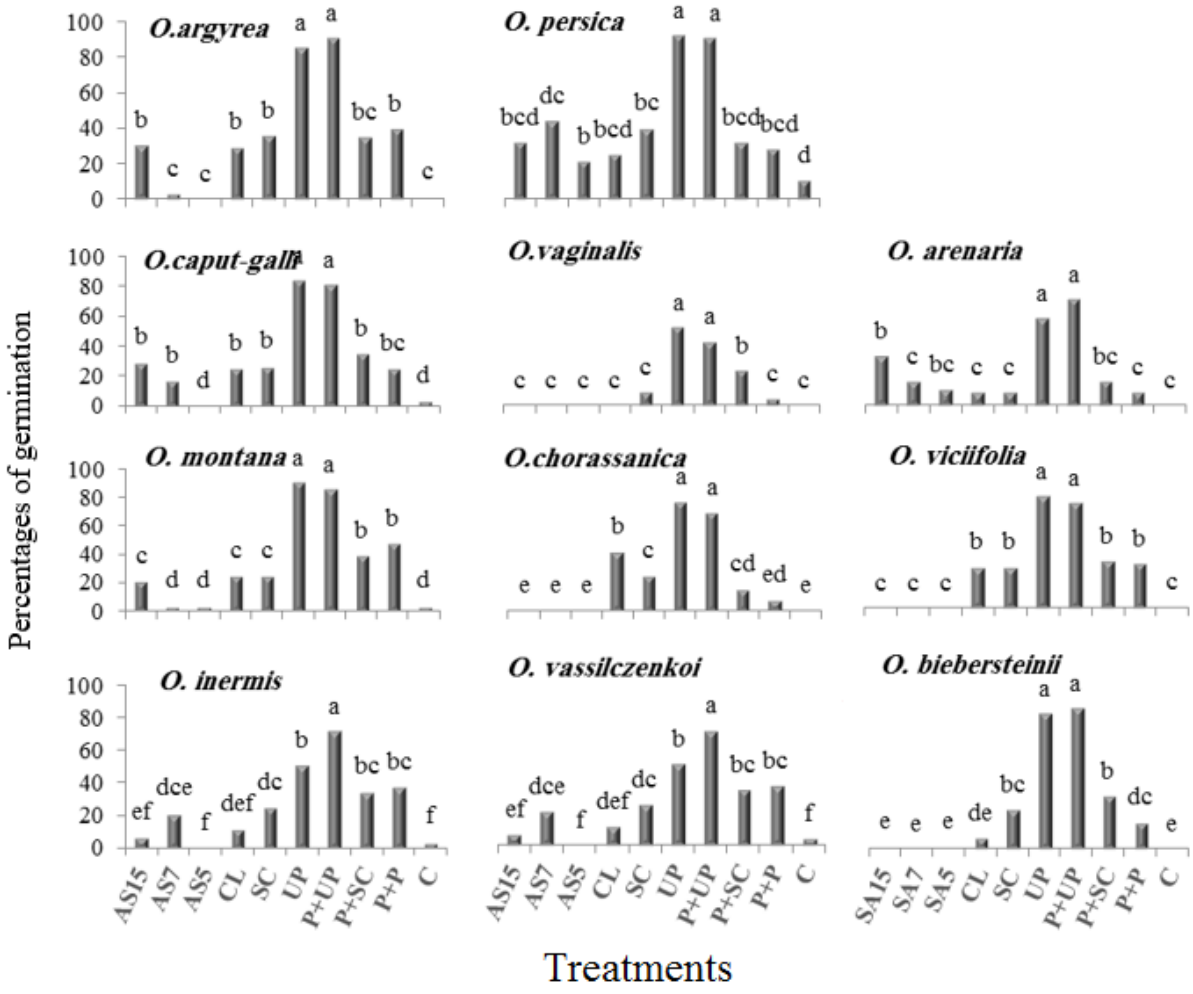

Figure 1. The effects of different treatments on the germination of Onobrychis species: acid scarification for 15 minutes (AS15), acid scarification for 7 minutes (AS7), acid scarification for 5 minutes (AS5), chilling (CL), scarification (SC), removing the seed pod (UP), the seeds without pods sown in peat (P+UP), scarification+sowing in peat (P+SC), seeds with pods sowing in peat (P+P), control (C).

(LSD) test ($P < 0.01$). Analysis of variance (ANOVA) was performed in SAS software version 9.1 and Graphs and Figs. were drawn by EXCEL software.

3. Results

3.1 Seed viability

Mean percentage of hard seeds, swollen seeds and viability for the 11 species are shown in Table 1. The viability

Table 2. Results of the analysis of variance for traits in different species of Onobrychis.
(** Significant at the $P < 0.01$ probability level, *** Significant at the $P < 0.001$. SOV: Sources of variation. DF: degree of freedom, GR%: percentage of germination, Rate: Rate of germination, RL: Root length, SL: Shoot length, RDW: Root dry weight, and SDW: Shoot dry weight. CV: Coefficient of variation.)

SOV	DF	GR%	Rate	RL	SL	RDW	SDW
Species	10	1658.76***	46.28***	7.85***	7.50***	18.25***	104.23**
Treatment	9	24121.14***	190.62***	118.27***	41.35***	24.46***	164.56***
S*T	90	309.78***	8.07***	2.56***	1.12***	1.20	1.65**
Error	220	80.49	1.12	0.69	0.17	5.65	45.30
CV(%)	-	15.25	11.36	12.89	9.68	12.27	18.23

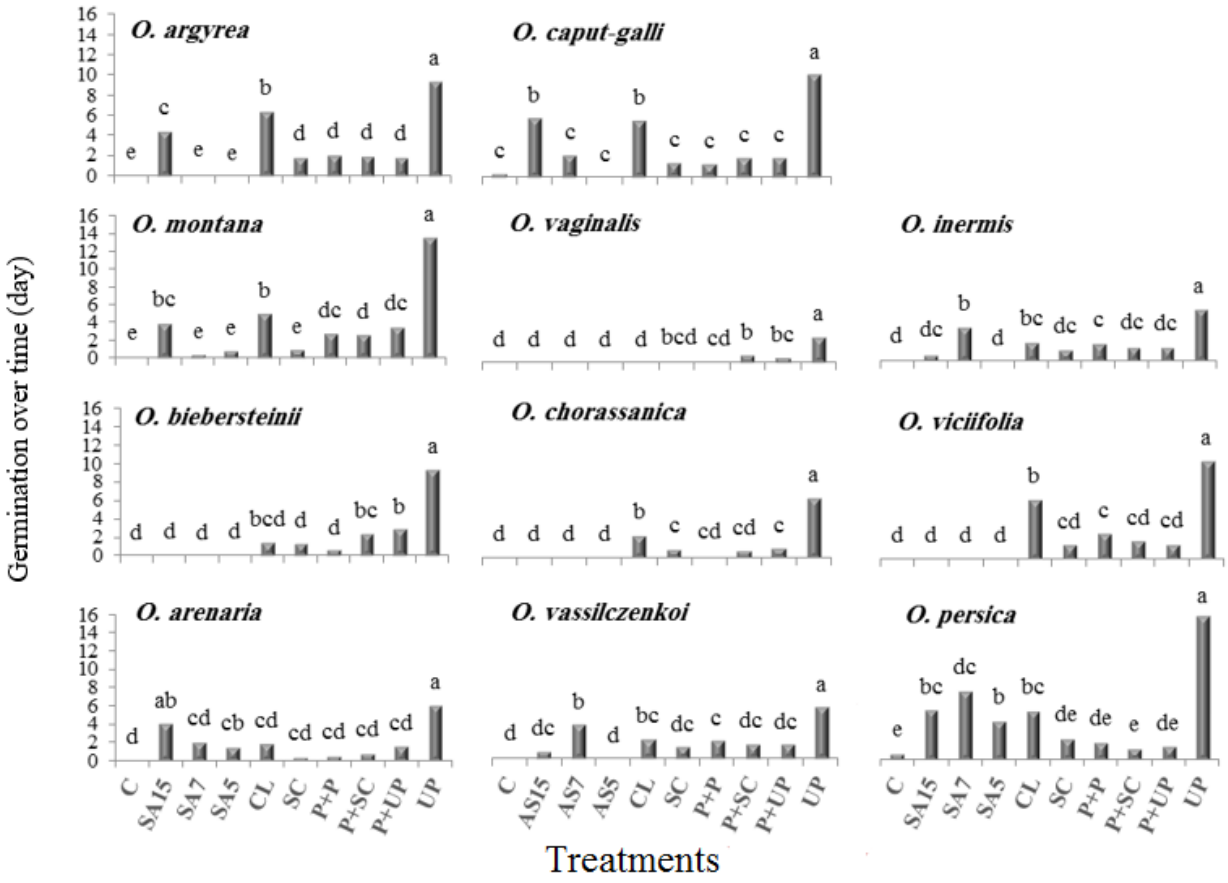

Figure 2. Germination rate of Onobrychis species under different treatments: control (C), acid scarification for 15 minutes (AS15), acid scarification for 7 minutes (AS7), acid scarification for 5 minutes (AS5), chilling (CL), scarification (SC), seeds with pods sowing in peat (P+P), scarification+sowing in peat (P+SC), the seeds without pods sown in peat (P+UP), removing the seed pod (UP).

percentage ranged from 65% in the O.vaginalis to 95% for the O. persica. The percentage of the hard seeds varied from 0% to 36%, and the lowest values were determined in O. montana and O. viciifolia in the section Onobrychis while the maximum hard seed percentages (17, 36 and 16%) were detected in O. chorassanica, O.vaginalis and O. vassilczenkoi in the section Hymenobrychis, respectively.

Results of the analysis of variance (Table 2) showed that the effects of species and treatment were significant for all traits ($P < 0.05$). The interaction of species and treatment was significant ($P < 0.05$) for all measured traits, except root dry weight (RDW). Germination percentage and rate were affected by the treatments in all of the studied species (Figs. 1, 2). Control treatments showed a very low germination ranging from 0 to 10% among species. Germination percentage and rate in all species were compared with those in control (C).

3.2 Mechanical scarification

Mechanical scarification significantly increased the germination of all species (Figs. 1 and 2). Among mechanical treatments, manual removing of the seed pod (UP) significantly increased both the germination percentage and rate in all species as compared to the control. The species of O. persica (92%), O. viciifolia (90%) and O. montana (90%) reached up to high germination with mechanical scarification. Mechanical scarification with scalpel (SC) also increased germination percentage and rate varied depending on the species. Also, mechanical scarification had a significant effect on shoot length, root length and root and shoot dry weight (Tables 3, 4).

3.3 Chilling scarification

The effects of chilling (CL) on germination was affected by the species (Figs. 1, 2). The CL did not affect seed germination percentage in O. vaginalis, O. biebersteinii, O. vassilczenkoi, O. inermis, O. arenaria and O. persica as compared to the control, but the rate of germination was significantly increased in all species except O. biebersteinii and O. vaginalis (Fig. 2). The CL also significantly affected root length, shoot length, and root and shoot dry weight (Table 3, 4).

3.4 Acid scarification and Seedbed treatment

Application of acid scarification for five minutes did not affect germination percentage and rate in the studied species (Figs. 1 and 2). However, acid scarification for seven and fifteen minutes significantly increased seed germination percentage and rate. Treatment with acid for 7 minutes (SA7) increased germination percentage and the rate of O.

Table 3. Mean comparisons of seedling shoot length (cm) (SL) and root length (cm) (RL) of 11 Onobrychis Species under different treatments.
(Treatments: C: control, UP: un-pod, SC: scarification, CL: chilling, SA5: sulfric acid for 5 minutes, SA7: sulfric acid for 7 minutes, SA15: sulfuric acid for 15 minutes, UN-P: un-pod+peat, P-P: seed pod+peat, S-P: scarification+peat, LSD: Least significant different at $P < 0.05$. In each row mean differences smaller than LSD value are significant at $P < 0.05$.)

Species	Traits	C	UP	SC	CL	SA5	SA7	SA15	UN-P	P-P	S-P	LSD
O. argyrea	RL	-	6.04	2.76	1.88	-	0.2	2.44	4.49	4.60	5.07	1.23
	SL	-	3.38	2.01	2.32	-	0.75	1.66	3.62	3.90	3.45	0.61
O. caput-galli	RL	0.93	3.88	2.44	2.62	-	1.39	1.71	4.59	4.92	4.10	1.43
	SL	1	1.97	1.77	2.05	-	1.56	2.2	2.54	3.78	3.11	0.77
O. vaginalis	RL	-	5.55	1.55	-	-	-	-	3.66	2.50	2.75	0.91
	SL	-	2.32	0.54	-	-	-	-	2.38	2.61	2.53	0.37
O. montana	RL	0.55	7.20	2	2.17	1.16	2.16	1.71	5.91	4.92	5.24	1.97
	SL	0.80	3.57	1.33	1.69	1.1	0.96	1.02	4.79	3.67	4.27	1.08
O. chorassanica	RL	-	6.56	3.2	2.75	-	-	-	3.86	4.41	3.44	0.85
	SL	-	1.81	3.81	1.53	-	-	-	2.20	2.36	2.31	0.22
O. inermis	RL	3.75	2.93	2.38	2.8	-	1.7	0.48	3.60	3.74	3.46	1.26
	SL	1.75	1.70	1.86	1.68	-	1.95	1.22	3.87	2.91	3.64	0.67
O. vassilczenkoi	RL	0.5	5.89	3.12	2.51	-	-	-	3.30	3.38	3.32	0.87
	SL	0.66	1.88	1.75	1.56	-	-	-	2.03	2.40	1.34	0.86
O. biebersteinii	RL	-	5.91	2.77	1.5	-	-	-	5.05	3.99	4.43	0.70
	SL	-	3.51	1.77	1.44	-	-	-	4	2.53	3	0.35
O. viciifolia	RL	0.16	632	3.09	2.16	-	-	-	4.67	5.09	4.85	1.5
	SL	0.50	3.21	2.18	2.22	-	-	-	3.55	3.60	2.63	0.78
O. arenaria	RL	-	3.58	0.66	0.77	2.19	1.05	1.18	6.47	3.93	4.52	1.67
	SL	-	1.51	0.38	1.5	1.36	1.76	1.66	3.08	2.85	2.33	0.61
O. persica Sirj	RL	3	7.91	2.98	2.56	2.60	1.50	1.45	4.10	4.88	4.03	2.24
	SL	1.63	3.84	1.63	2.41	2.27	2.10	1.70	3.70	4.35	2.56	0.99

caput-galli, O. inermis, O. vassilczenkoi and O. persica as compared to the control. Effect of longer duration of acid scarification varied. Acid scarification for 15 minutes (SA15) significantly increased the germination of O. montana, O. caput-galli, O. argyrea and O. arenaria. However, Acid scarification had no significant effect on shoot and root length and dry weight (Tables 3 and 4). The application of seedbed treatment had a significant effect on germination percentage in the sainfoin species. The seeds without pods sown in peat (P+UP) showed the maximum germination percentage for all species. For seeds with pods sowing in peat (P+P), it increased the germination of O. viciifolia, O. vassilczenkoi, O. argyrea, O. inermis, O. montana, and O. caput-galli as compared to the control. Mechanical scarification combined with sowing in peat (P+SC) also significantly increased seed germination in all species except O. arenaria, O. persica and chorassanica. The seedbed treatment had a significant effect on shoot length, root length, root dry weight and shoot dry weight.

4. Discussion and Conclusion

One of the major problems with forage legume is the seed dormancy caused by the hard seed coat. The first consequence of hard seed coat is inhibition of water uptake, which is crucial for seed germination [26]. In this study, we evaluated interspecific variation for seed dormancy and determined the effects of mechanical, chemical and chilling methods on breaking the dormancy in cultivated and 10 Onobrychis related wild species. Our results indicated that seed dormancy in this genus generally results from a hard impermeable seed coat and that mechanical scarification is effective in eliminating seed dormancy which was in agreement with the results in other legumes [27–29]. The results of the present study also showed that removing seed pod increased germination in all species, reaching to the high percentage of germination. Although seed pod removal could increase the percentage of germination up to 100% in a species, it had a small effect in O. vaginalis (52%), O. vassilczenkoi (50) and O. inermis (50), probably due to another type of seed dormancy in addition to physical dormancy. Majidi and Barati found that among physical treatments, only seed pod removal significantly increased seed germination percentage and rate in O. viciifolia and O. melanotricha [18]. Seed pods of the cultivated sainfoin (O. viciifolia) contain water soluble germination inhibitors and mechanical restriction appeared to be a minor factor in the total effect of the pod on germination rate.

Sowing in peat facilitated leaching of chemical inhibitors present in the seed pod. Peat enhanced microbial activities and included high organic matter, humic acid, fulvic asid and nitrate, which all stimulated seed germination [30, 31]. The combination of mechanical scarification and sowing

Table 4. Mean comparisons of seedling Shoot Dry Weight (SDW) and Root Dry Weight (RDW) (mg) of 11 Onobrychis Species under different treatments.
(Treatments: C: control, UP: un-pod, SC: scarification, CL: chilling, SA5: sulfric acid for 5 minutes, SA7: sulfric acid for 7 minutes, SA15: sulfuric acid for 15 minutes, UN-P: un-pod+peat, P-P: seed pod+peat, S-P: scarification+peat, LSD: Least significant different at $P < 0.05$. In each row mean differences smaller than LSD value are significant at $P < 0.05$.)

Species	Traits	C	UP	SC	CL	SA5	SA7	SA15	UN-P	P-P	S-P	LSD
O. argyrea	RDW	-	1.70	1.20	1.38	-	0.45	0.96	2.20	1.90	2.33	0.70
	SDW	-	9.66	8.03	8.32	-	9.50	10.20	8.14	8.76	9.06	3.67
O. caput-galli	RDW	0.66	0.77	1.87	1.27	-	0.79	3.50	2.28	1.94	1.99	2.50
	SDW	0.50	5.19	9.42	6.80	-	10.61	10.39	7.52	7.66	10.33	4.39
O. vaginalis	RDW	-	1.08	0.30	-	-	-	-	1.12	1.78	1.92	0.63
	SDW	-	19.24	0.19	-	-	-	-	3.11	6.40	26.12	23.61
O. montana	RDW	0.50	1.56	2.65	1.56	2.23	3.30	0.94	3.06	4.08	11.30	8.24
	SDW	2.16	7.90	13.48	8.68	10.10	1.5	12.70	8.33	9.98	8.29	3.09
O. chorassanica	RDW	-	2.10	1.13	1.46	-	-	-	1.53	1.66	6.49	4.63
	SDW	-	4.71	6.11	3.25	-	-	-	6.49	19.26	5.05	13.51
O. inermis	RDW	2.60	0.63	1.20	1.49	-	1.07	4.97	1.49	3.13	0.90	2.53
	SDW	7.50	3.65	7.20	6.22	-	7.61	26.88	7.80	7.84	6.51	14.76
O. vassilczenkoi	RDW	0.33	1.98	0.49	0.62	-	-	-	0.84	0.93	1.17	0.89
	SDW	15.66	2.95	4.49	4.61	-	-	-	3.88	4.03	4.50	14.68
O. biebersteinii	RDW	-	1.55	1.35	0.96	-	-	-	2.38	1.81	1.26	0.71
	SDW	-	8.14	10.42	6.60	-	-	-	9.95	5.37	-	3.46
O. viciifolia	RDW	0.06	2.04	1.35	1.58	-	-	-	3.37	2.29	2.73	1.70
	SDW	0.66	8.14	8.19	9.58	-	-	-	9.65	-	8.67	3.11
O. arenaria	RDW	-	0.54	0.58	2.15	1.54	3.46	1.04	2.26	1.31	1.89	2.31
	SDW	-	2.42	0.80	9.66	6.05	5	8.35	6.68	7.68	5.70	4.40
O. persica Sirj	RDW	1.12	11.04	1.11	1.72	1.46	0.99	1.72	3.41	2.26	2.20	8.23
	SDW	19.61	7.38	8.36	8.28	8.48	5.88	13.58	10.71	7.29	9.18	13.70

in peat (P+SC) increased seed germination in species, except O. arenaria and O. persica ; also, in O. montana and O.vaginalis which was a significant increase in comparison to the SC alone. Basaran et al. reported that sowing in path and the combination of mechanical scarification and sowing in peat significantly increased seed germination in all Vicia species [32]. Though scarification of seed with chilling (CL) improved germination to some extent, it could be due to physiological dormancy in addition to physical dormancy. Van Assche et al. reported that in V. aphaca, the lower temperature was optimum compared to the higher temperature for germination [33]. According to Bewley and Black, cooling can raise the ambient oxygen levels by making less oxygen available for citric acid [34]. However, in the present study, the CL had no effect on seedling characters in O. vaginalis, O. biebersteinii, O. vassilczenkoi, O. inermis, O. arenaria and O. persica, while it significantly improved the seedling characters of other species.

In some plant species, acid scarification has caused damage to embryo, with the extent of damage being varied in different species depending on the duration of acid scarification [29, 35]. The results of present study indicated that Acid scarification for 7 minutes (SA7) resulted in the increase in germination percentage and rate of O. caput-galli, O. inermis, O. vassilczenkoi and O. persica while the application of sulfuric acid for 15 minutes (SA15) increased germination in O. montana, O. caput-galli, O. argyrea and O. arenaria much more than other applications. Immersion in sulfuric acid for 15 minutes almost completely eliminated hardseedness and longer immersion had a positive effect on germination percentage. Results of this study indicated that the effects of treatments on germination time depend on the species. UP treatment highly accelerated germination time as compared to other treatments. Moreover, with the treatment of UP, a peak of germination and also, a high portion of the total germination were observed in the first 3 days for most of the species. The CL treatment highly accelerated the germination time in all species except O. biebersteinii and O. vaginalis, in comparison to other species, probably due to physiological dormancy. On the other hand, in O. vaginalis, O. chorassanica and O. vassilczenkoi, the highest germination ratio in the first 7 days was obtained from seeds used for UP+P treatment. This showed that treatment conditions in peat were good for germination and reduction of hardseededness of the studied species. Majidi and Barati reported that acid scarification significantly increased germination percentage and rate although the best duration of acid scarification was highly dependent on species and the presence or absence of seed pods [18].

To conclude, among treatments, seed pod removal was

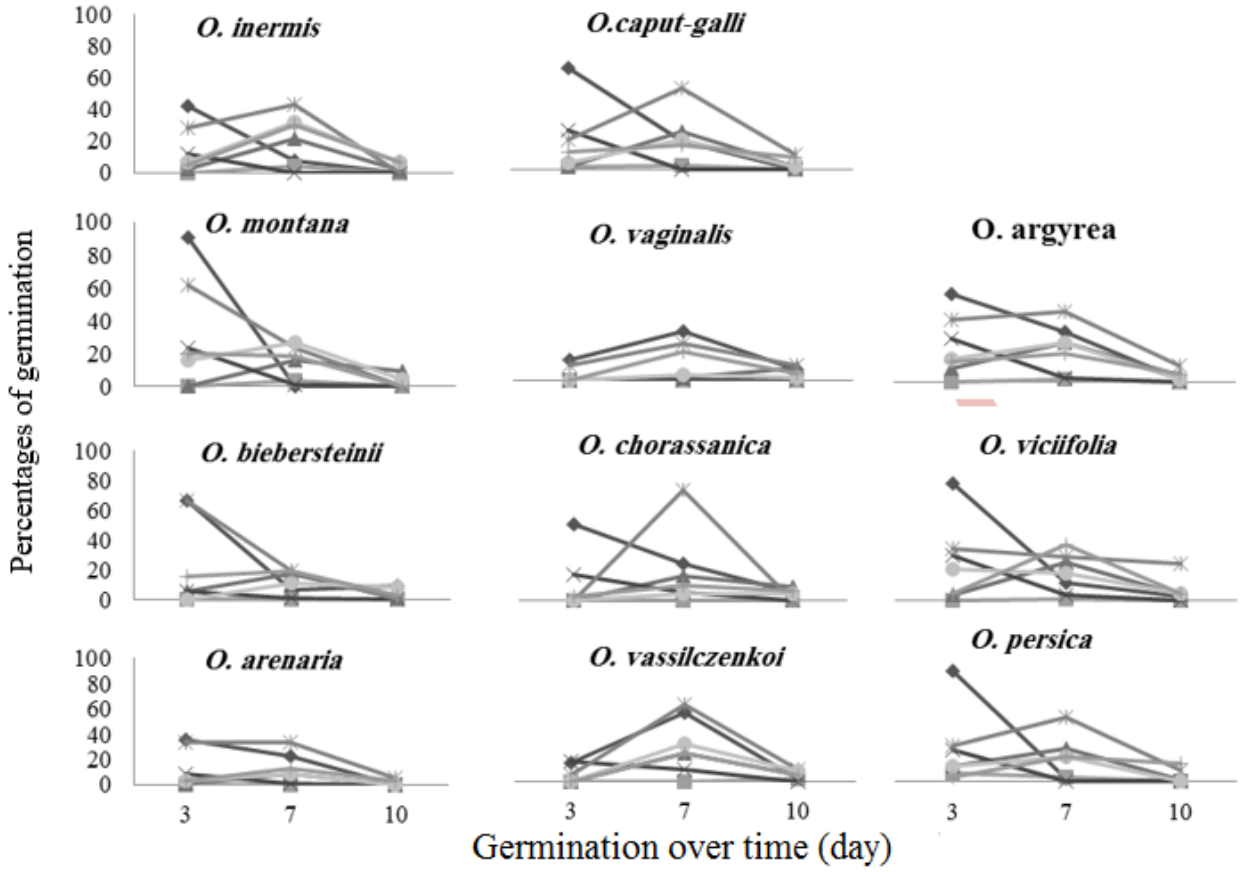

Figure 3. The germination over time of 11 species in response to treatments.: −♦−: removing the seed pod (UP), −■−: control (C), −▲−: scarification (SC), − × −: chilling (CL), − • −: seeds with pods sowing in peat (P+P), − ı −: scarification+sowing in peat (P+SC), − ∗ −:seeds without pods sown in peat (P+UP).

found to be the best for improving seed germination and rate in the sainfoin species. Additionally, scarification and chilling were effective in removing hardseedness and they could be suitably used for increasing germination in the species studied. The effect of seedbed was significant and some species reached to a significant increase in germination when their seeds were only sown in peat while all species reached to a high percentage of germination when their seed pod removal was sown in peat. Also, our results showed that acid scarification significantly increased germination percentage and rate; however, it had no effect in the case of O. viciifolia, O. biebersteinii, O. chorassanica, and O. vaginalis species. Generally, our results showed that all species reached to a high percentage of germination when pod removal and sowing were used in peat treatments.

Conflict of interest statement:
The authors declare that they have no conflict of interest.

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