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Hardseededness in two *Medicago* species as affected by water stress during seed development

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SUMMARY – In order to evaluate the effect of water stress on hardseededness during seed development in two *Medicago* species native to a natural pasture in Sicily (south Italy), an experiment was carried out in a growth chamber, where plants were grown in pots and five different water treatments were applied (irrigation at 1, 4, 8, 12 or 16-day intervals). Water treatments were differentiated from the beginning of the fruit-set stage. Leaf transpiration was progressively reduced with the lengthening of the dry interval, from 1.81 to 0.45 μg $cm^{-2}s^{-1}$ on average in *M. rugosa*, and from 2.34 to 0.95 μg $cm^{-2}s^{-1}$ in *M. orbicularis*. Stress degree-day (difference between leaf temperature and chamber temperature) increased with the reduction of irrigation frequency. In the germination trials performed at the end of the growth chamber experiment, the lower germination percentage corresponded to seeds produced in plants irrigated at longer intervals, highlighting a water stress effect during seed filling, promoting hardseededness.

Keywords: *Medicago rugosa*, *Medicago orbicularis*, leaf transpiration, seed germination, hardseededness.

RESUME – *"Dureté des graines chez deux espèces de Medicago affectées par stress hydrique pendant le développement des graines". Une expérience a été menée en chambre de croissance, afin d'évaluer l'effet du stress hydrique au cours du début de la nouaison jusqu'à la maturité de la graine, sur la dureté de celle-ci pour deux espèces de* Medicago *natives d'un pâturage naturel de Sicile (Italie du Sud). On a étudié l'effet de l'irrigation effectuée selon une fréquence de 1, 4, 8, 12 ou 16 jours. La différenciation des traitements de l'eau a été effectuée au début de la nouaison. Le flux de transpiration des feuilles s'est progressivement réduit avec l'allongement de l'intervalle, de 1,81 à 0,45 μg $cm^{2}s^{-1}$ chez* M. rugosa*, et de 2,34 à 0,95 μg $cm^{-2}s^{-1}$ chez* M. orbicularis*. Le stress degrés jours (différence entre la température de la feuille et la température de la chambre) a augmenté avec la diminution de la fréquence d'irrigation. Les valeurs minimales des pourcentages de germination ont été observées pour le traitement qui prévoyait la fréquence maximale, montrant l'effet du stress hydrique pendant le remplissage des graines sur la dureté de la graine.*

Mots-clés : Medicago rugosa*,* Medicago orbicularis*, transpiration de la feuille, germination de la graine, dureté de la graine.*

Introduction

Wrinkled medic (*Medicago rugosa* Desr.) and blackdisk medic (*Medicago orbicularis* L. Bartal) are two annual forage legumes quite common in natural pastures of the Mediterranean basin (Tutin *et al*., 1968; Chebouti and Abdelguerfi, 1999). These species, for different aspects, are very interesting since they are considered potential species for pasture improvement in semi-arid zones.

Both species exhibit dormancy due to the presence of an impermeable seed coat (Jha and Pal, 1992). This coat imposed dormancy, known as "hardseededness", is an ecological mechanism which prevents seed from imbibing and germinating until a set of physiological conditions relating to temperature and moisture availability have been met (Loi *et al*., 2005). Indeed, in Mediterranean environment, annual legumes survive as seed over the dry hot summer, representing an important resource for pasture improvement and soil erosion preservation.

However, hardseededness represents a limit when a prompt and high germination is required. Factors involved in the onset of the seed-coat imposed dormancy during seed development are still not well-known. Hardseededness in other species is believed to be under genetic and environmental

control during seed development, such as soil water content (Clua and Giménez, 2003). In the present research, the level of hardseededness as affected by water stress occurred during seed development and ripening, in two *Medicago* species typical of natural pastures of Ragusa plateau, was investigated.

Materials and methods

Legumes of *Medicago rugosa* Desr. (wrinkled medic) and *Medicago orbicularis* (L.) Bartal. (blackdisk medic) were collected in summertime from a natural pasture of the Ragusa plateau (36°55' Lat N, 14°45' Long E), in the South Eastern area of Sicily (South Italy). Seeds were hand separated from the pods and stored in paper bags at room temperature (15-25°C) under laboratory conditions (RH 40–60%) until used for the experiment.

The experiment was carried out in growth chamber at 20±1°C constant temperature. Light was alternated (day:night = 12:12 h). Three seeds per each species where sown in ∅ 20 cm plastic pots filled with a mixture of soil and sand (3:1) adequately fertilized. The seeds were previously hand scarified with sand paper to remove hardseededness and promote germination (Patanè and Bradford, 1993). After seedling emergence, one plant was left per each pot. Five different water treatments were applied: daily irrigation (control) and irrigation at 4, 8, 12 or 16 days interval, each with three replicates. Irrigations were carried out filling the pots until soil appeared water-saturated, allowing the exceeding water to percolate (up to approximately field capacity). Water treatments were differentiated from the first fruit appearance onwards; until then, water was applied daily in every pot. After the differentiation, in order to monitor soil water content, all pots were daily weighed. The available soil water deficit (ASWD, %), reported as negative value, was calculated for each day as follows: [(PFC-P/(PFC-PWP))x100], where PFC is the weight of pot at soil field capacity (g), P is the daily weight of the pot (g), PWP is the weight of the pot at soil wilting point. During the same period, leaf transpiration ($\mu g\ cm^{-2}s^{-1}$) and leaf temperature (°C) were weekly measured by means of a "steady state" porometer (Model LI-1600, Li-Cor Inc., Lincoln, Nebraska, USA). The measurements were carried out on the lower surface of the three last fully expanded leaves. Thereafter, stress degree-day (°C) was calculated by considering chamber temperature (20°C, constant) and leaf temperature.

At the end of the growth chamber experiment, on the seeds harvested for each water treatment, a germination test was performed. Before the test, the seeds were exposed to a chemical treatment with 95-97% concentrated sulphuric acid solution for 2, 10 or 30 minutes, in order to promote germination (Patanè and Bradford, 1993). The seeds were then thoroughly rinsed with tap water first and distilled water after, and dried at room temperature. For each species, samples of 80 seeds were placed on a single Whatman No.3 filter paper in 9 cm Petri dishes (four replicates of 20 seeds each) moistened with 5 ml of distilled water. The dishes were covered and sealed with Parafilm to prevent evaporation during the germination period, then randomised in a thermostatically controlled incubator in the dark at 20±1°C, which is considered the optimum temperature for other forage legume species similar to those studied (ISTA, 1999). Water was replenished as needed. Radicle protrusion (minimum 2 mm long) was taken as the end point of germination, scored daily until no further visible radicle emergence was observed. At the end of the experiment, the germinated seeds and swollen seeds final percentages and Mean Germination Time were calculated (Scott *et al.,* 1984). Swollen seeds are those that were imbibed but did not germinate.

For each species, the germination percentage values were arcsine transformed and statistically analysed by analysis of variance (ANOVA) using CoStat version 6.003 (CoHort Software). In case of significance, mean values were separated using the LSD test (Snedecor and Cochran, 1989).

Results and discussion

The available soil water deficit, which varied according to the treatments, imposed increasing periods of water stress, trying to mimic the field behaviour (Fig. 1). The ASWD never exceeded -15 - 20% in the daily irrigated treatment (I-1), whereas I-16 treatment achieved wilting point at the first irrigation as well as I-12 treatment, in *M*. *rugosa*. In *M. orbicularis* I-4 never exceeded the -40% of ASWD, I-8 the -50% and I-12 the -60%, while in *M. rugosa* I-4 water treatment attained at -50%, I-8 at -60% and I-12 and I-16 at below -60% of ASWD, just after the first irrigation (Fig. 1).

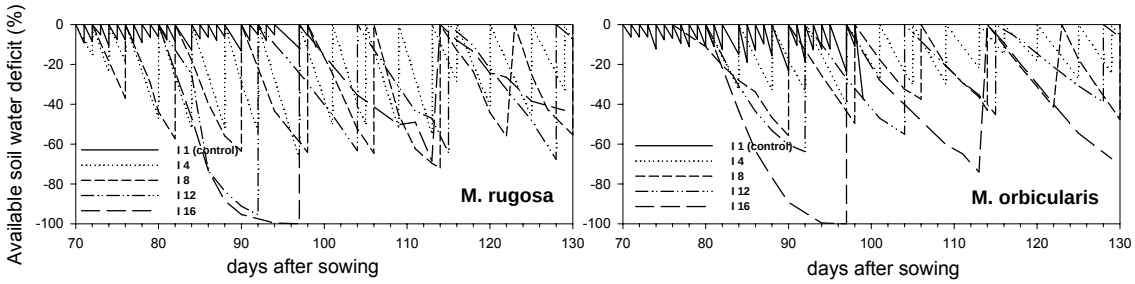

Fig. 1. Available soil water content from the first fruit appearance according to the water treatments.

Leaf transpiration, on average of measurements, decreased according to the level of water stress, from 1.81±0.35 to 0.45±0.16 μg $cm^{-2}s^{-1}$, in *M. rugosa*, and from 2.34±0.65 to 0.95±0.21 μg cm^{-2} s^{-1}, in *M. orbicularis*, with this last always transpiring more than wrinkled medic.

Stress degree-day increased with the lengthening of the dry interval and resulted higher in blackdisk medic at the greatest levels of water stress (I-8 to I-16 treatments) (Table 1).

Table 1. Leaf transpiration and stress degree-days (±σ) on average of values (n=6) recorded during seed development, in the two *Medicago* species in relation to water treatment

Water treatment	Leaf transpiration (μg $cm^{-2}s^{-1}$)		Stress degree-day (°C)	
	M. rugosa	*M. orbicularis*	*M. rugosa*	*M. orbicularis*
I-1	1.81±0.35	2.34±0.65	2.78±0.88	2.57±0.89
I-4	1.31±0.19	1.64±0.48	2.85±1.21	2.74±1.18
I-8	1.04±0.34	1.19±0.14	3.20±1.24	3.50±1.00
I-12	0.77±0.13	0.85±0.30	3.44±1.12	3.88±1.09
I-16	0.45±0.16	0.95±0.21	3.58±1.42	3.73±0.25

Germination percentage of wrinkled medic seeds after 2 min soaking in sulfuric acid ranged between 58.3 and 80.4%, with no statistical differences among water treatments (Table 2). The seeds took on average 6.65 days (MGT) to germinate. With the increase of time exposure, the coat-imposed dormancy was totally removed (100% germination) and seeds germinated promptly (MGT less than 2 days) but with 30 min exposure some seeds produced under I-1 and I-4 water treatments were damaged (43.3 and 1.3% of swollen seeds, respectively) and did not germinate.

Seeds of blackdisk medic failed to germinate after 2 min soaking in sulfuric acid (0% germination); with 10 min of chemical treatment seeds germination occurred and significantly declined (from 85.3 to 30.7%) with the decrease in irrigation frequency during seed ripening; a tendency in MGT increase was recorded. Thirty minutes of chemical treatment induced the full germination (100%) and enhanced MGT, when excluded the seed produced under very water stress conditions (I-16 treatment) which slightly but significantly less germinated (to 89.3%). No seeds apparently injured were observed.

In previous experiments, seeds of wrinkled medic exhibited a 65.0% germination after 35 min soaking in a 70% concentrated sulfuric acid solution (Patanè and Bradford, 1993) whilst seeds of blackdisk medic did not germinate under the same chemical treatment (Patanè and Gresta, 2006). These results are supported by the different thickness of the palisade layer of the seed coat, as previously observed at SEM (Patanè and Bradford, 1993; Patanè and Gresta, 2006), which is more than two-fold greater in blackdisk medic when compared to wrinkled medic. This layer is believed to be responsible for impermeability to water (Bewley and Black, 1978), and apparently made the seed coat of blackdisk medic more resistant to sulphuric acid treatment. Moreover, the increase in water stress during seed ripening seems to induce a thickening in seed coat since longer exposures to sulphuric acid are required by the seeds of both species to remove hardseededness, when produced under very water stress conditions. Similarly, Clua and Gimenez (2003) demonstrated that seed-coat imposed dormancy in *Lotus edulis* largely depends on soil water content during seed development,

and that drought determines a more compact layer of palisade cells in the testa, hence producing an enhancement of impermeability in the seed.

Table 2. Germination percentage, mean germination time (MGT) and swollen seeds in the two *Medicago* species in relation to water treatment, under different time exposures (2, 10 or 30 min) in sulphuric acid solution

Water treatment	Germination (%)*			MGT (days)			Swollen seeds (%)		
	2 min	10 min	30 min	2 min	10 min	30 min	2 min	10 min	30 min
M. rugosa									
I-1	80.4	100.0	56.7[b]	6.25	1.00	1.00	0	0	43.3
I-4	77.3	100.0	98.7[a]	5.73	1.13	1.12	0	0	1.3
I-8	72.0	100.0	100.0[a]	6.82	1.35	1.27	0	0	0
I-12	58.3	100.0	100.0[a]	6.74	1.57	1.38	0	0	0
I-16	58.3	100.0	100.0[a]	7.70	1.65	1.45	0	0	0
M. orbicularis									
I-1	0	85.3[a]	100.0[a]	-	2.59	1.07	0	0	0
I-4	0	59.1[ab]	100.0[a]	-	3.81	1.16	0	0	0
I-8	0	56.4[b]	100.0[a]	-	4.02	1.29	0	0	0
I-12	0	52.0[b]	100.0[a]	-	4.52	1.36	0	0	0
I-16	0	30.7[b]	89.3[b]	-	3.39	2.66	0	0	0

*Values, within column of each species, with the same letter do not differ significantly at p≤0.01.

Conclusions

These results showed that the level of hardseededness in *M. rugosa* and *M. orbicularis* may greatly depend on the intensity of plant water stress occurred during seed development and filling. Therefore, it may be possible to obtain more permeable and promptly germinating seeds by modifying the irrigation management during seed development and ripening.

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