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Characterization of Algerians populations of *Medicago* for cold tolerance by morphological traits and molecular markers

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Abstract. The study was carried on 16 accessions of annual *Medicago* species (*M. truncatula* Gaertn. *M. ciliaris* Krocker., *M. aculeata* Wild. and *M. polymorpha* L.). Seedlings of different accessions of *Medicago* collected from sites of contrasting altitudes (10 to 1170 m) were subjected to different durations of low temperature regimes. Root to shoot ratios of acclimated and non acclimated plants was compared. 12 accessions among 16 studied were used to assess the degree of genetic polymorphism by SSR microsatellites. Results show that accessions originated from high altitude have a better root to shoot ratios (higher ability to cold acclimation) than accessions originated from low altitude (lower ability to cold acclimation).Tests differentiation between species by Fisher pair show that all species are different from each other. Results show the high level of homozygosity for all species (> 80%). There are differences between populations of the same species of cold acclimation, which is encouraging for a study of association between cold acclimation and molecular polymorphism.

Keywords. Cold acclimation – Root:shoot ratios – Molecular polymorphism – Annuals populations – *Medicago*.

Caractérisation des populations algériennes de* Medicago *pour la tolérance au froid à partir de traits morphologiques et de marqueurs moléculaires

Résumé. *L'étude a porté sur 16 accessions de différentes espèces de* Medicago *(*M. truncatula *Gaertn*., M. ciliaris *Krocker*., M. aculeata *Wild. et* M. polymorpha *L.), appartenant à des altitudes contrastées. Des plantules de différentes accessions de* Medicago *ont été soumises à différentes durées de régimes de basse température. Les ratios racines/tiges des plantes acclimatées et non acclimatées (control) ont été comparés.12 accessions parmi les 16 étudiées ont fait l'objet d'une caractérisation moléculaire à l'aide de marqueurs de type SSR. Les résultats montrent que les accessions tolérantes affichent des ratios plus importants par rapport aux sensibles. Le test de Fisher de différentiation entre les espèces montre que les espèces sont différentes les unes des autres. Les résultats montrent aussi le haut niveau d'homozygotie pour toutes les espèces (> 80%). Le fait qu'il existe des différences entre les différentes populations étudiées pour la tolérance au froid est encourageant pour une étude d'association entre l'acclimatation au froid et le polymorphisme moléculaire.*

Mots-clés. *Acclimatation au froid – Ratios racine/tiges – Polymorphisme moléculaire – Populations annuelles* – Medicago.

I – Introduction

The production of a crop is challenged by abiotic and biotic stresses. Temperature is one of the most important environmental factors controlling seed germination, development of seedlings growth and limiting crop distribution. During crop establishment, extreme temperatures can decrease plant emergence and lead to drastic losses in crop yield and quality (Dias *et al*., 2010; Avia *et al*., 2013). Acclimation also is known as cold hardening or cold tolerance (Baruah *et al*., 2011; Pirzadah *et al*., 2014). Many morphological changes have been documented during the acquisition of cold tolerance in different species (Thapa *et al*., 2008; Baruah *et al*., 2011). Cold

tolerance can be evaluated by changes in morphological indices such as root/shoot ratios (Hekneby *et al*., 2001; Thapa *et al*., 2008; Hund *et al*., 2008). In natural environments, the spatial distribution of individual plants within populations often depends on environmental factors that affect seedling establishment, such as temperature. Phenotypic assessment can provide a direct an easy estimation of variability for cold stress adaptations. Microsatellite markers, freeing this constraint, are often used in combination with phenological traits to characterize populations and their adaptation to constraint environments (Avia *et al*., 2013). It would be useful to develop morphological and genetic markers to detect genotypes with best degree of acclimation at low temperatures and consequently freezing tolerance using a screening tests in controlled conditions to avoid interference with other limiting factors. The major objective of this study was to develop a laboratory screening procedure to quantify cold acclimation (CA). CA was quantified by measuring morphological cold tolerance indices such as root to shoot ratio treated at different duration in comparison with the control, estimating the genetic diversity of natural *Medicago* accessions using SSR markers and answering the question is there a relationship between the classification of populations through cold tolerance indices as well as their site of origin (low or high altitude) and SSR markers used?

II – Materials and methods

The study was carried on four annual *Medicago* species. A set of 16 accessions (Table 1) was tested for degree of cold acclimation. Ten seeds for each accession were germinated, after scarification, at temperature room in Petri dishes containing universal compost imbibed with distiller water. At three days growth stage, seedlings were divided into two lots. Cold acclimation (CA) lot at 4 °C for three durations 5, 8 and 11 days (T1, T2 and T3) and control lot non-acclimated (NA) lot kept at 23 °C (T01, T02 and T03). CA was quantified by measuring root to shoot ratios at different durations in order to compare with the control.

Table 1. Accessions analyzed for cold tolerance with their origin and ecological description

Species	Accessions	Origin	Latitude	Longitude	Altitude (m)
M. aculeata Willd.	cv. Ac 15678	Australia	–	–	–
	cv. Ac 15679	Australia	–	–	–
	cv. Ac 14821	Australia	–	–	–
	cv. Ac 80	Syria	–	–	–
M. ciliaris Krocker.	Cil 123	Algeria	36°46'02''N	8° 18' 9.57'' E	16
	Cil 124	Algeria	36°17'15'' N	7° 57' 14.77'' E	565
	Cil 125	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Cil 126	Algeria	36° 28' 0'' N	7° 26' 0'' E	290
M. polymorpha L.	Poly 57	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Poly 54	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Poly 136	Algeria	36°49'0'' N	5° 46' 0'' E	10
	Poly 213	Algeria	35°23'17''N	1° 19' 22'' E	1170
	Poly 42	Algeria	36°54'15''N	7°45'07''E	200
M. truncatula Gaertn.	Tru 210	Algeria	34°6' 50'' N	2° 5' 50.14'' E	1150
	Tru 216	Algeria	34° 6' 50'' N	2° 5' 50.14'' E	1150
	Tru 62	Algeria	36° 28' 0'' N	7° 26' 0'' E	290
	Tru 26	Algeria	35° 23' 17'' N	1° 19' 22.16'' E	1170

Among 16 accessions studied, 12 populations have been characterized using 14 SSR microsatellites. DNA is extracted from 200 mg samples of fresh young leaves material, in the presence of liquid nitrogen and 3 ml of CTAB buffer (Doyle and Doyle, 1990). PCR amplification was performed with fourteen SSR microsatellites. Extraction was carried out at INRA Lusignan France. PCR products were separated using polyacrylamide gel 6.5% in the LI-COR IR2 automated DNA sequencer (LI-COR Inc.). The different parameters are calculated using the Genetix software (version 5.0.4).

III – Results and discussion

1. Ability of cold acclimation

During cold treatment of 4 °C, cold-acclimated plants had reduced stem length and root length (data not shown). This reduction differs from one accession to one another. The values of root to shoot ratios are also different from one accession to another. Differences in root to shoot ratios between acclimated and non acclimated lots were significant at the tree durations of treatment time (Figs 1, 2 and 3). Tolerant accessions have better ratios (root to shoot) than the sensitive one. Ac 80, Ac and 15678 of *M. aculeata,* two populations of *M. polymorpha* Poly 136 and Poly 57, Cil 125 and Cil 126 of *Medicago ciliaris* and Tru 62 and Tru 216 of *M. truncatula* have been found that their degree of acclimation is more efficient for tree durations of treatment. Janska *et al.* (2010) showed that cold tolerant species –herbs, grasses and ground shrubs– have a low leaf surface area and a high root:shoot ratio and cold-adapted plants tend to be slow growing. Thapa *et al*. (2008), observed that growing of *M. truncatula* under low temperature regimes comparatively to control conditions, resulted in an increase of the root:shoot ratio.

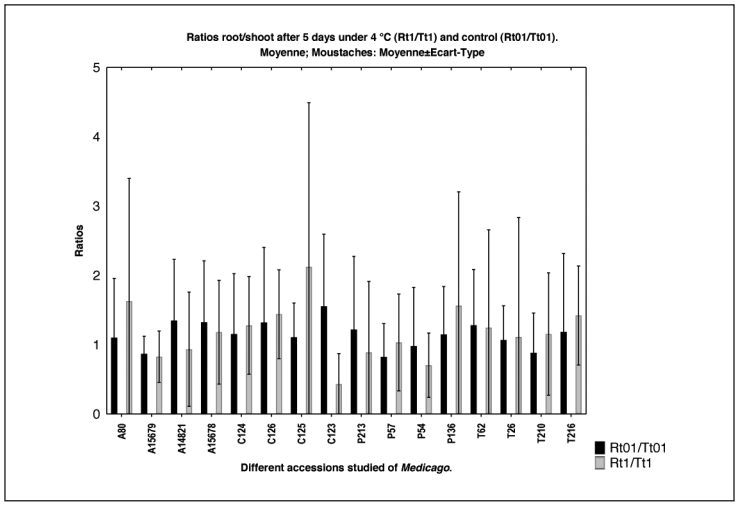

Fig. 1. Ratios root to shoot after 5 days under 4 °C (Rt1/Tt1) and control (Rt01/Tt01), for different accessions studied of *Medicago*.

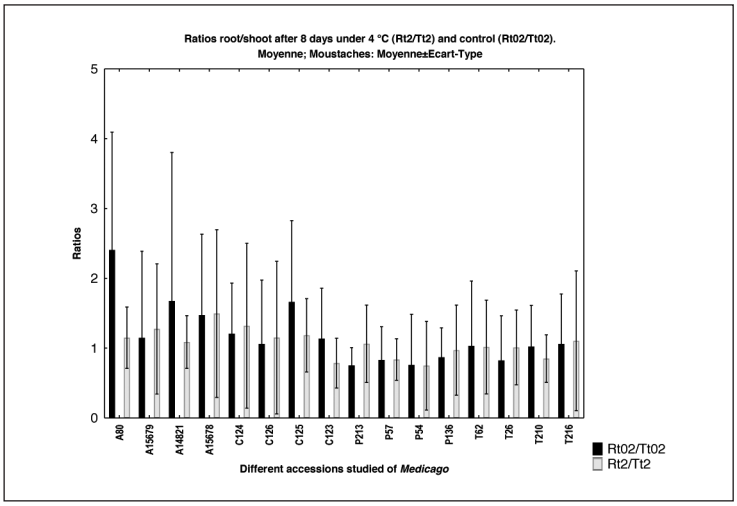

Fig. 2. Ratios root to shoot after 8 days under 4 °C (Rt2/Tt2) and control (Rt02/Tt02), for different accessions studied of *Medicago*.

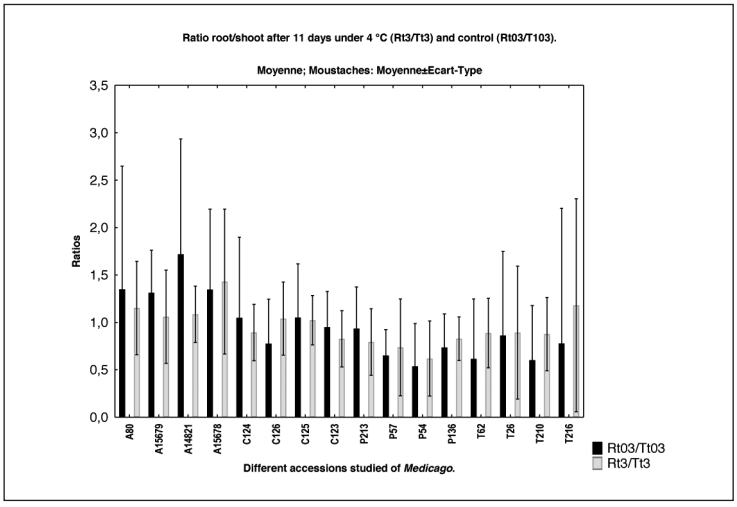

Fig. 3. Ratios root to shoot after 11 days under 4 °C (Rt3/Tt3) and control (Rt03/Tt03), for different accessions studied of *Medicago*.

2. SSR Marker

On 14 SSR markers used, nine were selected (not missing data, Table 2). Differentiation tests between species by Fisher pair; show that all species are different from each other. Results show the high level of homozygosity for all species (> 80%). The overall level of polymorphism, of *M. ciliaris* is lower *than M. polymorpha* and *M. aculeata*. So it is possible to differentiate between four species with nine microsatellite markers. It is possible to differentiate between populations for *M. aculeata* and differences between populations *M. aculeata* are similar to those between species. Some alleles detected at marker loci MTIC-131-432 and MTIC-079 seem to have a relationship with cold tolerance and the geographical origin of accessions (data not shown).

Table 2. Number of alleles and percentage of homozygosity

Loci	*M. ciliaris*	*M. truncatula*	*M. aculeata*	*M. polymorpha*	All species
ATPase456	1 (100%)†	1(100%)	5 (77%)	3 (50%)	6 (79%)
Mtic338	3 (85%)	1 (100%)	4 (92%)	5 (100%)	7(93%)
Mtic082	1 (100%)	1 (100%)	2 (100%)	4 (67%)	7 (91%)
mtic451	5 (100%)	3 (100%)	5 (85%)	2 (75%)	12 (88%)
B14B03	1 (100%)	1 (100%)	3 (100%)	5 (92%)	10 (98%)
mtic135	3 (92%)	2 (100%)	3 (100%)	2 (100%)	8 (98%)
mtic343	2 (100%)	2 (100%)	6 (92%)	6 (83%)	12 (93%)
mtic131	2 (92%)	2 (100%)	4 (100%)	4 (83%)	06 (93%)
Mtic432	1 (100%)	2 (80%)	2 (100%)	6 (83%)	13 (93%)
Total	19 (97%)	15 (98%)	34 (94%)	37 (81%)	81 (92%)

† The percentage of homozygosity is indicated in parenthesis.

IV – Conclusion

The effect of cold treatment was investigated in different populations of annual *Medicago*, and revealed a high genetic variability for cold tolerance. The degree of cold acclimation increased with the duration of treatment. It appears that it does not seem to be structuring between populations for *M. polymorpha* and *M. ciliaris* (to be confirmed with more individuals and markers) while there are differences between populations of the same species of cold acclimation. This is encouraging for a study of association between cold acclimation and molecular polymorphism.

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