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Utilization of *M. sativa* ssp. *caerulea* × *M. sativa* ssp. *sativa* hybridization in improvement of alfalfa aluminium tolerance

Dragan Milić, Ksenija Taški-Ajduković*, Nevena Nagl, Jovanka Atlagić and Đura Karagić
Institute of Field and Vegetable Crops, M. Gorkog 30, 21000 Novi Sad, Serbia

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Abstract

Sensitivity of alfalfa to acidity and aluminium (Al) toxicity in soil is the major limiting factor in broadening of its growing area. Due to lack of Al tolerance in primary alfalfa germplasm, there is a need for transfer of genes for Al tolerance from other *Medicago* germplasm. One of the identified sources of Al tolerance is *M. sativa* ssp. *caerulea* accession PI 464724, which was used as a female parent in our study. The objectives of this study were: (i) obtaining the tetraploid offspring from 2*x*–4*x M. sativa* ssp. *caerulea* – *M. sativa* ssp. *sativa* spontaneous crosses, and (ii) development of a breeding strategy for Al/acid tolerance in alfalfa, using *M. sativa* ssp. *caerulea* as a source of Al tolerance. Out of eleven fully developed plants, five were morphologically similar to *M. sativa* ssp. *caerulae*, while six plants were similar to *M. sativa* ssp. *sativa*. All tested plants were fertile, with the pollen viability ranging from 21.45 to 97.09% and the average number of ovules per plant from 8.80 to 12.29. Eleven SSR primer pairs confirmed the hybrid nature of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa* offspring. Both the Cluster Analysis and the Principal Coordinates Analysis separated plants in the *caerulae* type from plants in the *sativa* type, with one exception. Strategies based on conventional and molecular marker breeding efforts could lead towards development of tolerant alfalfa cultivars and successful crop production on acidic, Al-contaminated soils.

Keywords: acid tolerance, alfalfa, crop relatives, genetic resources, SSR markers

Introduction

Alfalfa (*Medicago sativa* ssp. *sativa*) is a member of the *Medicago sativa* complex, which includes both the diploid ($2n = 2x = 16$) and the tetraploid ($2n = 4x = 32$) forms. The diploid members of the complex include *M. sativa* ssp. *falcata, M. sativa* ssp. *caerulea* and their natural hybrid, *M. sativa* ssp. *hemicycla*. The tetraploid subspecies in the complex include *M. sativa* ssp. *sativa, M. sativa* ssp. *falcata* and the tetraploid hybrid *M. sativa* ssp. *varia* (Quiros and Bauchan, 1988).

Hybridization between taxa of the *M. sativa* complex is possible even across the ploidy levels (Bingham and Saunders, 1974). Gene transfer is more common from the diploid to the tetraploid genome and it usually happens by development of unreduced gametes. Both 2*n* egg and 2*n* pollen producers could be used for the direct gene transfer from the wild diploid relatives into the cultivated alfalfa by means of 2*x*–4*x* and 4*x*–2*x* crosses (Barcaccia *et al*., 2003). Based on more favourable genetic segregation ratios and more robust genetic mapping capabilities, identification of desirable alleles in the diploid germplasm is considerably easier than in the tetraploids (Şakiroğlu and Brummer, 2011). Thus, the use of diploid alfalfa relatives as a source of alleles with higher breeding value is advantageous in alfalfa breeding.

Alfalfa is an important component of sustainable forage systems worldwide. Sensitivity of alfalfa to acidity and aluminium (Al) toxicity in soil limits its productivity and persistence. Yield losses associated with soil acidity are commonly prevented with application of lime fertilizers

*Corresponding author. E-mail: ksenija.ajdukovic@ifvcns.ns.ac.rs

(Gomes *et al.*, 2002; Grewal and Williams, 2003), which are rather expensive (Acharya, 2014). Moreover, liming does not reduce soil acidity in the subsoil, which can substantially reduce the alfalfa yield. Therefore, the most desirable option to improve alfalfa production is the development of plants with Al tolerance (Reyno *et al.*, 2013).

The lack of Al tolerance in primary alfalfa germplasm dictated the need for identifying genes or QTLs (quantitative trait loci) for Al tolerance in *Medicago* germplasm that could be transferred to cultivated alfalfa (Bouton, 1996; Dall'Agnol *et al.*, 1996). Genotypes with Al tolerance have been identified among the diploid *M. sativa* ssp. *caerulea* (Sledge *et al.*, 2002) and *M. truncatula* germplasm (Sledge *et al.*, 2005). Al-tolerant accession *M. sativa* ssp. *caerulea* was later used as the female parent in 2*x*–4*x* hybridization with *M. sativa* ssp. *sativa* (Khu *et al.*, 2013). Although extensive breeding efforts have been made in obtaining the acid-tolerant alfalfa, the progress has been modest in terms of their commercial use (Zhang *et al.*, 2007; Bouton, 2012). According to the currently available data, only one commercial alfalfa cultivar was registered as specifically suitable for cultivation on acidic soil (Acharya, 2014).

The objectives of this study were: (i) obtaining the tetraploid offspring from 2*x*–4*x M. sativa* ssp. *caerulea*–*M. sativa* ssp. *sativa* crosses, by utilizing the occurrence of 2*n* gametes in natural populations of diploid *M. sativa* ssp. *caerulea*; and (ii) development of a breeding strategy for obtaining Al/acid tolerance in alfalfa, using *M. sativa* ssp. *caerulea* as a source of tolerance.

Material and methods

Plant material

Accession PI 464724 of wild diploid alfalfa *M. sativa* ssp. *caerulea* that has shown tolerance to Al (Sledge *et al.*, 2002; Narasimhamoorthy *et al.*, 2007) was used as the female parent. Plants of PI 464724 were grown in the experimental field of the Institute of Field and Vegetable Crops in Novi Sad, Serbia, surrounded by the tetraploid *M. sativa* ssp. *sativa*. Plants were put in open pollination, aimed at utilizing the production of 2*n* unreduced gametes in the diploid *M. sativa* ssp. *caerulea* in field conditions and favouring the 2*x*–4*x* crosses. One *M. sativa* ssp. *caerulea* plant survived and produced 75 seeds that were harvested and sown in the greenhouse during the winter.

Cytogenetic analysis

Fertility of plants was estimated by examining male (size and vitality of pollen grains) and female (number of ovules per ovary) gametophytes. Pollen vitality was determined according to the method of Atlagić *et al.* (2012), while the number of ovules was determined as in Dattee (1972). Pollen grains and ovule number were observed with the stereo microscope Stemi 2000 and photographed with the digital camera Power Shot G5. Light microscope (Amplival 30-G048C, Carl Zeiss, Jena; ocular 12.5 × 16 and objective 16/0.40) was used for pollen viability analysis. Microphotographs were made with the video camera CCD Sony DXC151AP, video card and software Win-Fast PVR2. The size of pollen grains was estimated using light microscope. Microphotographs were made with the digital camera (Canon Power shot A80) and the data were analysed using the AxioVision LE software, Rel.4.3.

DNA analysis

Total genomic DNA was extracted from the leaves of 11 potential hybrid plants and *M. sativa* ssp. *caerulea* PI 454756 plant, according to the protocol of Somma (2004). Eleven SSR primer pairs were used to confirm the hybrid nature of the offspring (Table 1).

PCR was carried out in a 25-µl reaction volume containing 2.5 µl buffer, 0.2 mM of each dNTP, 0.8 µM of each primer (Metabion international AG, Steinkirchen, Germany), 1.5 units of Taq polymerase (Thermo Scientific, Waltham, MA USA) and 50 ng of DNA. PCR reactions were carried out in the Tpersonal PCR thermocycler (Biometra GmbH, Göttingen, Germany) using the following 'touchdown' PCR profiles: an initial denaturation of 2 min at 94°C, followed by a cycle with denaturation at 94°C for 30 s and extension at 72°C for 50 s. The annealing temperature was decreased 0.8°C per cycle in subsequent cycles from 55°C until reaching 47°C and was kept constant for the remaining 30 cycles. PCR ended with a final extension at 72°C for 5 min. Exceptions were the PCR protocol for primers AfCa1, AFCT45, AFCT 60 and MTIC299. These protocols consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 50°C (MTIC299) or 52°C for 40 s and 72°C for 40 s, with final elongation at 72°C for 5 min. PCR products were separated on the 3% Metaphore agarose (Lonza Group Ltd, Basel, Switzerland) gels containing 0.005% ethidium bromide and visualized under the UV light. The band size was calculated by comparison with GeneRuler 50 bp DNA Ladder (Thermo Scientific, Waltham, MA USA).

The SSR profiles were scored based on the size (bp) of the fragments amplified across the tested plants, based on the visual inspection of electrophoretic patterns. Genetic distance between the plants was calculated using the equation from Nei (1978). Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) in PopGene software version 1.32 (Yeh *et al.*, 1997) and visualized using the TreeView software version 1.6.6. (Page, 1996). In addition, relationship among tested

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Table 1. SSR primers used to confirm the hybrid nature of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa* offspring

Locus	Sequence	Amplification profile	LG	Reference
AfCa1	F:CGTATCAATATCGGGCAG R:TGTTATCAGAGAGAGAAAGCG	SSR52	/	Diwan *et al.* (2000)
AFCT45	F:TAAAAAACGGAAAGAGTTGGTTAG R:GCCATCTTTTCTTTTGCTTC	SSR52	/	Diwan *et al.* (2000)
AFCT60	F:CCTCCCTAACTTTCCAACA R:TGGATCAACGTGTCTTTCA	SSR52	/	Diwan *et al.* (2000)
BI4BO3	F:GCTTGTTCTTCTTCAAGCTC R:ACCTGACTTGTGTTTTATGC	SSR55TD	5	Julier *et al.* (2003)
MTIC93	F:AGCAGGATTTGGGACAGTTGT R:ACCGTAGCTCCCTTTTCCA	SSR55TD	6	Julier *et al.* (2003)
MTIC232	F:TAAGAAAGCAGGTCAGGATG R:TCCACAAATGTCTAAAACCA	SSR55TD	7	Julier *et al.* (2003)
MTIC247	F:TTCGCAGAACCTAAATTCAT R:TGAGAGCATTGATTTTTGTG	SSR55TD	1	Julier *et al.* (2003)
MTIC299	F:AGGCTGTTGTTACACCTTTG R:TCAAATGCTTAAATGACAAAT	SSR50	8	Julier *et al.* (2003)
MTIC343	F:TCCGATCTTGCGTCCTAACT R:CCATTGCGGTGGCTACTCT	SSR55TD	6	Julier *et al.* (2003)
MTIC365	F:ATCGGCGTCTCAGATTGATT R:CGCCATATCCAAATCCAAAT	SSR55TD	2	Julier *et al.* (2003)
MTIC451	F:GGACAAAATTGGAAGAAAAA R:AATTACGTTTGTTTGGATGC	SSR55TD	2	Julier *et al.* (2003)

LG, linkage group; SSR55TD, SSR touchdown PCR profile with annealing at 55°C.

plants was examined by the Principal Coordinates Analysis (PCoA) implemented in the GenAlex 6.41 software package (Peakall and Smouse, 2006), based on the genetic distance calculated for co-dominant markers.

Results

Eleven plants fully developed out of 75 seeds sawn in the greenhouse. The developed plants showed differences in their morphology: plants 2, 3, 9, 10 and 11 were similar to *M. sativa* ssp. *caerulae*, being short, with small leaves and thin branches (Fig. 1a), while plants 1, 4, 5, 6, 7 and 8 looked much like *M. sativa* ssp. *sativa*, with bigger habitus, larger leaves and thicker branches (Fig. 1b).

Cytological analysis showed that all 11 plants were fertile. Pollen viability (Fig. 2a) ranged from 21.45 to 97.09%, while the average number of ovules (Fig. 2b) per plant ranged from 8.80 to 12.29 (Table 2). The size of vital pollen grains varied: plants 2, 3, 9, 10 and 11 had smaller pollen, while plants 1, 4, 5, 6, 7 and 8 had larger pollen grains (Table 2).

Eleven SSR primer pairs were used to confirm the hybrid nature of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa*

Fig. 1. Morphology of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa* offspring (a) similar to *M. sativa* ssp. *caerulea* and (b) similar to *M. sativa* ssp. *sativa*.

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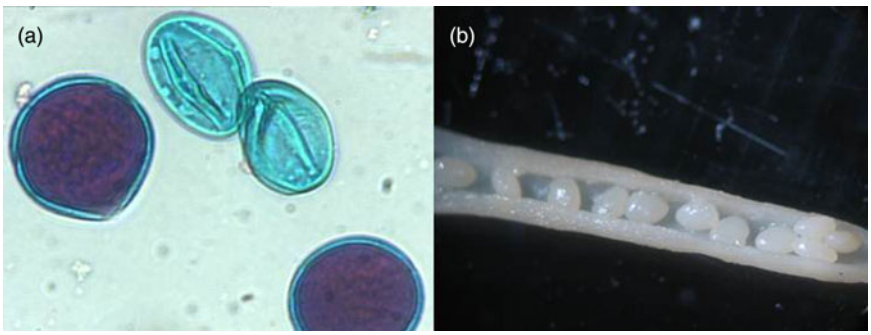

Fig. 2. Cytological analysis of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa* offspring (a) vitality of pollen grains (vital – red, non-vital – green) and (b) ovules in ovary.

offspring (Table 3). In 10 plants, the presence of three alleles was detected after amplification with at least one primer pair. Plant number 7 had four alleles after multiplication with primers AFCT 45, AFCT60 and MTIC 299. Four alleles were detected after multiplication with MTIC 299 in plants 1 and 3, as well as in plant 11 after multiplication with primer AfCa1. Amplifications with the tested primers in plant 10 resulted in only one or two amplification products.

Based on the cluster analysis, the hybrid plants were grouped into two major clusters. The first cluster consisted of plants 1, 4, 6, 7 and 8; plants 2, 3, 5, 9, 10 and 11 were in the second cluster, while *M. sativa* ssp. *caerulea* plant was separated (Fig. 3a).

PCoA was performed as a type of ordination method in order to check the results of the cluster analysis. The first principal coordinate accounted for 26.6% of the total variation and separated the offspring in two groups (Fig. 3b), identically as in the dendrogram. The second principal coordinate accounted for 21.42% of the total variation and indicated that the plants most related to *M. sativa* ssp. *caerulea* were 9, 10 and 11.

Table 2. Pollen grain size and vitality, number of ovules per ovary in offspring of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa*

Plant	Pollen grain width (μm)	Pollen grain length (μm)	Pollen viability (%)	No of ovules in ovary
1	37.24	39.46	84.18	10.12
2	31.81	33.64	88.95	9.20
3	31.44	33.73	92.45	8.90
4	35.23	39.69	22.98	8.80
5	34.75	38.43	59.07	9.50
6	37.26	39.96	26.06	9.50
7	37.04	41.01	40.94	10.60
8	36.05	39.15	47.99	10.20
9	33.10	34.63	97.09	9.20
10	32.39	34.87	21.45	12.29
11	33.20	35.69	78.89	10.30

Discussion

Interspecific hybridization between cultivated species of alfalfa and their wild relatives offers a potential opportunity for introgression of desired traits from the wild to the cultivated forms (Barcaccia *et al.*, 2003). Utilization of diploid alfalfa genetic resources is of special importance, since identification of desirable alleles in the diploid germplasm is considerably easier than in the tetraploids.

In the present study, 11 hybrid plants were obtained through hybridization between the diploid *M. sativa* ssp. *caerulae* and the tetraploid *M. sativa* ssp. *sativa*. In terms of their morphology, the hybrid plants segregated in comparison with the parent species: plants 1, 4, 5, 6, 7 and 8 in the *sativa* type and plants 2, 3, 9, 10 and 11 in the *caerulae* type. The occurrence of characteristics from both progenitors in the hybrids indicates that the hybrid plants inherited genomes from both parental species used for crossing. Since the correct identification of hybrid recognition plant by morphological traits is often be difficult, the molecular approach may be useful for the assessment of the hybrid status. The SSR markers used in this study clearly identified the hybrid origin of the tested plants, identifying three or four alleles for certain SSR markers. In plant 10, all tested markers had one or two alleles, indicating that the plant was either the result of *M. sativa* ssp. *caerulae* self-pollination or the hybrid with still undetected part of the *M. sativa* ssp. *sativa* genome. The results of cluster and PCoA, based on SSR data, agreed with morphological observations of hybrid offspring, segregating plants in the *caerulae* type from plants in the *sativa* type. The only exception was plant 5 that was placed in the *sativa* type according to the morphological observations, but was put among the *caerulae*-type plants by both the cluster analysis and the PCoA. The UPMGA clearly indicated a separate position

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Table 3. Multiple alleles obtained by SSR primers in offspring of *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa*

Primer/Plant	1	2	3	4	5	6	7	8	9	10	11
AFCT45							++++				+++
AfCa1								+++	+++		++++
AFCT60			+++				++++	+++			
MTIC451							+++				
MTIC299	++++	+++	++++	+++		+++	++++		+++		+++
BI4BO3					+++						
MTIC232						+++					
MTIC93		+++			+++	+++					
MTIC343	+++										
MTIC365	+++			+++							
MTIC247							+++				

of *M. sativa* ssp. *caerulae* plant compared with hybrids, but PCoA indicated a different pattern. It is known that the cluster analysis groups individuals in such a way that most similar individuals are first grouped together and progressively similar group are merged (Hair *et al.*, 2006). PCoA results in two- or three-dimensional scatter plot of individuals in such a way that the genetic distances among the individuals are reflected in the distance between the points in the plot (Mohammadi and Prasanna, 2003). Therefore, it is not unusual that results of PCoA has varying degree of effectiveness in capturing cluster structure which, in our case, resulted in the different placement of *M. sativa* ssp. *caerulea* plant on dendrogram and PCoA plot.

The presented results support the findings of Havananda *et al.* (2011), who pointed out that there was a potential for gene flow in virtually all combinations of the *Medicago* subspecies both within and between ploidies. Our results are in agreement with the previous findings considering gene flow in the *M. sativa* gene pool between the diploid and the tetraploid subspecies (Bingham and Saunders, 1974; Veronesi *et al.*, 1986), but they also indicate that this process can happen spontaneously in natural, field conditions. Cytogenetic analysis showed that the hybrid plants were fertile, which is essential for introgression of valuable traits from wild relatives into cultivated germplasm (Barcaccia *et al.*, 2003).

Having in mind that *M. sativa* ssp. *caerulea* accession PI 464724 has shown tolerance to Al (Sledge *et al.*, 2002; Narasimhamoorthy *et al.*, 2007), it is to be expected that a certain amount of genes for Al tolerance was transferred to some hybrid plants. Therefore, the hybrid offspring was crossed with the tetraploid *M. sativa* ssp. *sativa* to produce F_2 plants and possible Al-tolerant progenies. The resulting seed will be planted in the field in soil with low pH, where it will be tested for Al tolerance and agronomic performance in field conditions. The surviving plants will be further

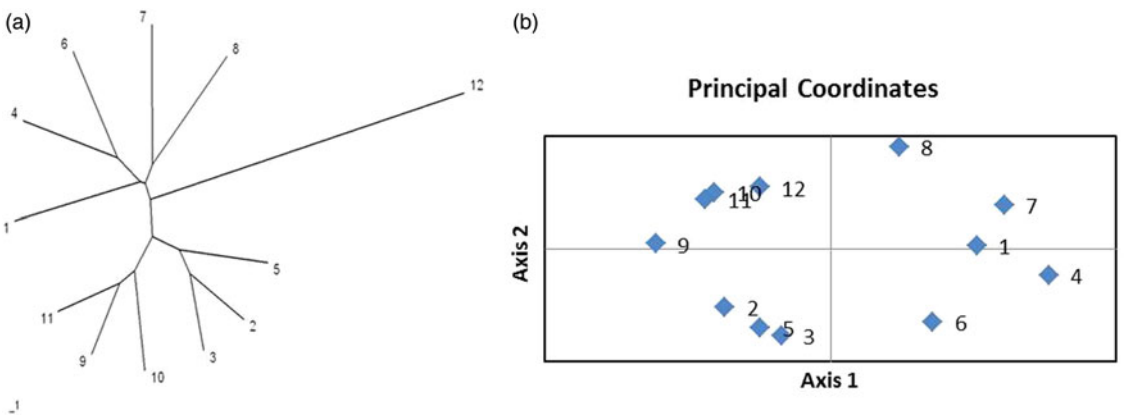

Fig. 3. Relationships between the *M. sativa* ssp. *caerulae* (12) and *M. sativa* ssp. *caerulae* × *M. sativa* ssp. *sativa* offspring (1–11) revealed by (a) UPGMA and (b) PCoA calculated from the SSR data.

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multiplied in the field for development of Al-tolerant alfalfa germplasm.

The information generated in this study can be used for alfalfa acid/Al tolerance improvement and understanding of alfalfa's genetic base, as well as for development of cost-reduced and efficient methods for creating improved alfalfa germplasm. Future results with strategies based on conventional and molecular marker breeding efforts could lead towards the development of more tolerant alfalfa cultivars and more prosperous utilization of this crop on Al-contaminated soils.

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