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CHARACTERIZATION OF LATVIAN ALFALFA *MEDICAGO SATIVA* GENETIC RESOURCES

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Genus *Medicago* contains about 50 species, majority of them are either diploid (2n=16) annuals or tetraploid (2n=32) perennials. *Medicago sativa* complex includes two subspecies growing in Latvia – *M. sativa* subsp. *sativa* and *M. sativa* subsp. *falcata*. Diploid and tetraploid forms exist in both subspecies. If the same ploidy level, crosses of subsp. *falcata* and subsp. *sativa* produce viable hybrids (*M. varia*). In Latvia, both subspecies are cultivated as forage crops but could also be found growing naturally. Ploidy level of accessions of Latvian alfalfa genetic resources, including semi-wild populations, accessions repatriated from the N. Vavilov All-Russian Institute of Plant Industry (VIR), and commercial varieties, were investigated. To determinate possible gene flow controlled crosses between subsp. *sativa* and subsp. *falcata* plants were made. Variability of ploidy level between and within different alfalfa accessions of the Latvian origin was investigated using both flow cytometry (Partec flow cytophotometer CyFlow Space) and root tip chromosome count. Most of accessions were tetraploid (a commercial variety, breeder lines). Three accessions were mixoploids (a commercial variety with wide genetic background and two semi-wild populations). Crossing between subspecies *sativa* and *falcata* give viable seeds, what prove possibility to transfer important genetic material from semi-wild populations of subsp. *falcata* to breeding material of cultivated forms. Nevertheless, there are differences in reciprocal crosses: fertility is higher if plants of subsp. *sativa* are used as the female parent. Several plants morphological properties were determinate in field trials.

Key words: *Medicago sativa*, ploidy level, flow cytometry.

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INTRODUCTION

Conservation and evaluation of plant genetic resources is important from both scientific and practical point of view, especially in variable climate conditions. Nowadays special attention is paid to genotypes that have good ability to adapt to different climatic growing conditions. From this point of view big interest is attracted by populations of wild crop relatives. Involving accessions from such populations in breeding programs is rather urgent for many species, including alfalfa.

Alfalfa is one of the most important perennial forage crops in the world due to its high protein

content and digestibility. It is used in pellets as forage supplements and also for grazing by all types of domestic livestock. The ability of alfalfa to fix atmospheric nitrogen makes it ideal for crop rotation systems. Because of high biomass production, perennial nature, its symbiotic relationship with specific bacteria for producing own nitrogen, and valuable co-products, alfalfa has the potential of being one of leading crop in the production of cellulosic ethanol. Another potential use of alfalfa is as the source of pulp for paper manufacturing and as the "factory" for production of industrial enzymes (Bouton 1996, Woodward 2008).

Alfalfa belongs to genus *Medicago* which consists of more than 50 different species. Subspecies composing the *Medicago sativa* complex includes diploid (2n=2x=16) subspecies *coerulea* and *falcata* and tetraploid (2n=4x=32) subspecies *sativa* and *falcata*. Diploid *M. sativa* subsp. *coerulea* and tetraploid *M. sativa* subsp. *sativa* are characterized by purple flowers and coiled pods (Quiros & Bauchan, 1988). Plants of the subspecies *falcata* are characterized by yellow flowers with straight to sickle shape pods. *M. varia* is a hybrid naturally occurring between subsp. *sativa* and subsp. *falcata,* it has variegated flowers with C-shaped to spiraled pods.

For breeding purpose it is very important to broadening the genetic background of initial material, therefore it would be very favorable to involve wild or semi-wild accessions in this process. To design successful controlled crosses between these cultivated and semi-wild accessions it is necessary to know their ploidy level.

Cytogenetic research on alfalfa is difficult due to several factors: chromosomes are very small (2-3 μm in root tip cells), they are morphologically very similar, cultivated alfalfa usually are tetraploids with a relatively high number of chromosomes (Bauchan and Hossain 1997). The alternative is flow cytometry that offers an accurate and rapid method to asses ploidy of single plants or plant populations. In the last two decades use of flow cytometry in plant biology have increased rapidly because it has several advantages comparing with other methods: rapid sample preparation, non-destructive sampling, rapid detection of mixed samples or endopolyploidy and relatively low operating costs (Ochatt 2006, Suda et al. 2007). Flow cytometry is widely used to determinate ploidy level in different plant species such as maize, blueberry, bananas (Wan et al. 1992, Costich et al. 1993, Roux et al. 2003) and also for *Medicago* species (Brummer et al. 1999). They detected ploidy level of accessions of *M. sativa* subsp. *falcata* and subsp. *sativa*, *M. prostrate* and *M. carstiensis* originated from different countries. The only, in this investigation, sample originated from Latvia was tetraploid *M. sativa* subsp. *falcata*.

At present, there is only one alfalfa (*M. sativa* subsp. *varia*) variety *Skrīveru* officially registered for commercial growing in Latvia. It was bred using hybridization between 8 varieties of different origin: *Mežotnes* (Latvia), *Alfa* (Sweeden), *Jogeva-118* (Estonia), *Warotte* (France), *Ostsaat Kurmark* (Germany), *N 102 Brand*, *Saranac* (both USA) and *Ladak* (Canada) (Jansons 1980). In Latvia breeding of alfalfa is continued in the Research Institute of Agriculture of the Latvia University of Agriculture. For broadening of initial breeding material several expeditions were carried out to collect wild forms in different regions of Latvia.

For planning of crossing combinations knowledge of the ploidy level is very important. Not always subspecies *sativa*, *falcata* and *varia* could be distinguished by morphology. Therefore the objective of this research was to determine ploidy of different accessions of Latvian alfalfa genetic resources, including commercial varieties, breeding lines and plants from semi-wild populations, as well to test crossability of different alfalfa subspecies to evaluate the potential ability of use of semi-wild populations us as genetic resources for breeding.

MATERIALS AND METHODS

Plant material

Nine *Medicago* accessions (Table 1) were includ-

Table 1. Analyzed accessions of the Latvian alfalfa genetic resources

Accession name	Accession type	Species	Source collection	Year of reproduction	Country of origin
Antane	commercial variety	*M. sativa*	RIA	2006	Lithuania
Skrīveru	commercial variety	*M. varia*	RIA	2007	Latvia
Mentu kalna	semi-wild	*M. sativa*	RIA	1989	Latvia
Lucerna Nr.2	semi-wild	*M. sativa*	RIA	1993	Latvia
Mežotnes	semi-wild	*M. sativa*	RIA	1994	Latvia
Aizkraukles	semi-wild	*M. falcata*	RIA	2004	Latvia
Dzelmes	semi-wild	*M. falcata*	RIA	2008	Latvia
Local k-31068	unknown	*M. sativa*	VIR	1964	Latvia
Local k-31069	unknown	*M. sativa*	VIR	1964	Latvia

Table 2. Results of controlled crosses between plants of *M. satviva* subsp. *sativa* and *M. Sativa* subsp. *falcata*

Hybrid combination	Subspecies of *M. sativa*	Number of crossed inflorescence	Number of crossed flowers	Percent of formed pods
Antane (♀) x Aizkraukles (♂)	*sativa* x *falcata*	9	69	17.39±0.50
Aizkraukles (♀) x Antane (♂)	*falcata* x *sativa*	9	67	8.96±0.36
Antane (♀) x Dzelmes (♂)	*sativa* x *falcata*	10	82	17.07±0.50
Dzelmes (♀) x Antane (♂)	*falcata* x *sativa*	7	52	7.69±0.33

ed in the analysis: i) commercial varieties grown in Latvia, ii) plants from semi-wild populations collected in different regions of Latvia and iii) accessions of unknown Latvian origin. Seeds of commercial varieties and semi-wild populations are received from the Research Institute of Agriculture of the Latvia University of Agriculture (RIA), two accessions of unknown Latvian origin were repatriated from the N. Vavilov All-Russian Institute of Plant Industry (VIR).

Flow cytometric analysis

Flow cytometry analysis was performed using the Partec flow cytophotometer CyFlow Space. Tissue samples were taken either from *in vitro* germinated 4 weeks old plants (*Antane*, Local k-31068, Local k-31069, Lucerna Nr.2, *Mentu kalna*, *Mežotnes*, *Skrīveru*) or from a greenhouse-grown plants (*Aizkraukles*, *Dzelmes*). Approximately 50 mg of tissue from leaves (Fig. 1) and 50 mg roots were collected from each plant (12 plants per accession). Samples were chopped with razor blade in a Petri dish in 0.5 ml of CyStain UV ploidy solution. After that 1.5 ml CyStain UV ploidy solution were added and incubated at room temperature for 5 minutes. Suspension of isolated nuclei was filtered through the Partec 50 µm CellTrics disposable filter and analyzed in flow cytometer. Ten thousand nuclei per sample were measured. Tissue of *M. lupulina* (grown in a greenhouse) leaves (2n=16) were used as the

Fig. 1. Four weeks old plant of alfalfa. Arrow indicated trifoliate leaf that was used in analysis by flow cytometer.

reference standard.

Chromosome counts

Several accessions (*Aizkraukle*, *Antane*, *Dzelmes* and *Skrīveru*) additionally were analyzed cytogenetically. Chromosome counts were performed on root tips of newly germinated, approximately two weeks old alfalfa plants using 0.1% colchicine pretreatment. After 2 hours root tips were rinsed and placed in Clarc's fixator (ethanol 96% : glacial acetic acid, 3:1). Fixed material was rinsed with water and placed in 0.1 M HCl at 60 °C for 7 minutes and transferred to aceto-carmine stain (5% carmine in 45% acetic acid). Counts were made microscopically for two to ten cells per plant at magnification 400 to 1000.

Field evaluation

Four accessions *Aizkraukles*, *Antane*, *Dzelmes* and *Skrīveru* were sown in the field (5 g seeds/1 m^2) in 2009. Next year, at the time of full flowering growth habit, time of flowering, stem length, color of flowers and the shape of pods were determined.

Crossing

Several reciprocal crosses of *M. sativa* subspecies *sativa* (*Antane*) and *falcata* (*Dzelmes* and *Aizkraukles*) were performed on plants growing in a greenhouse in 2009 (Table 2). Four plants of each accession were used for hybridization. Emasculation and pollination were done by tweezers. After pollinations inflorescences were covered by isolators. Two month after pollination formation of pods was recorded.

RESULTS AND DISCUSSION

Flow cytometric analysis give a possibility to distinguish of ploidy level of cells in different plants (Fig. 2). In six of the analyzed accessions only tetraploid cells were found, both in leaves and roots (Table 3). Among them a commercial

Table 3. Ploidy level of evaluated accessions of the Latvian alfalfa genetic resources

Accession name	Root tip counts	Number of plants tested by flow cytometry	Ploidy level	
			Leaf cells	Root cells
Local k-31068		12	4x	4x
Local k-31069		12	4x	4x
Mentu kalna		12	4x	4x
Lucerna Nr.2		12	4x	4x
Mežotnes		12	4x	4x
Antane	2x; 3x	12	4x	4x
Dzelmes		12	2x; 4x; 2x/4x	2x; 4x; 2x/4x
Aizkraukles	2x; 3x; 3x/4x	12	2x/3x; 2x/4x	2x/3x/4x
Skrīveru	2x; 3x; 4x 2x/3x	12	4x	2x/4x; 4x

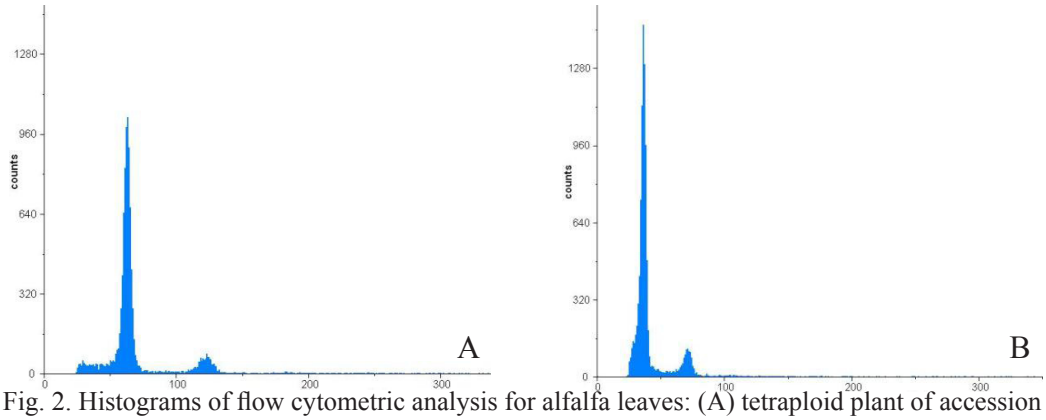

Fig. 2. Histograms of flow cytometric analysis for alfalfa leaves: (A) tetraploid plant of accession Skrīveru and (B) diploid plant of accession Dzelmes.

variety (*Antane*), breeder lines and both accessions repatriated from the VIR (Local k-31068 and Local k-31069). In they turn, some plants of accessions *Aizkraukles, Dzelmes* and *Skrīveru* were mixoploids: cells with different ploidy were found. Distribution of ploidy levels in plants from those accessions are shown in Fig. 3. For accessions *Aizkraukles* and *Dzelmes* cells with different ploidy level (di-, tri- and tetraploid) were found both in leaves and roots. In opposite, all tested leaf cells of variety *Skrīveru* were tetraploid while roots had different ploidy level (Table 3). Only one diploid plant according both leaves and root was found in *M. sativa* subsp. *falcata* accession

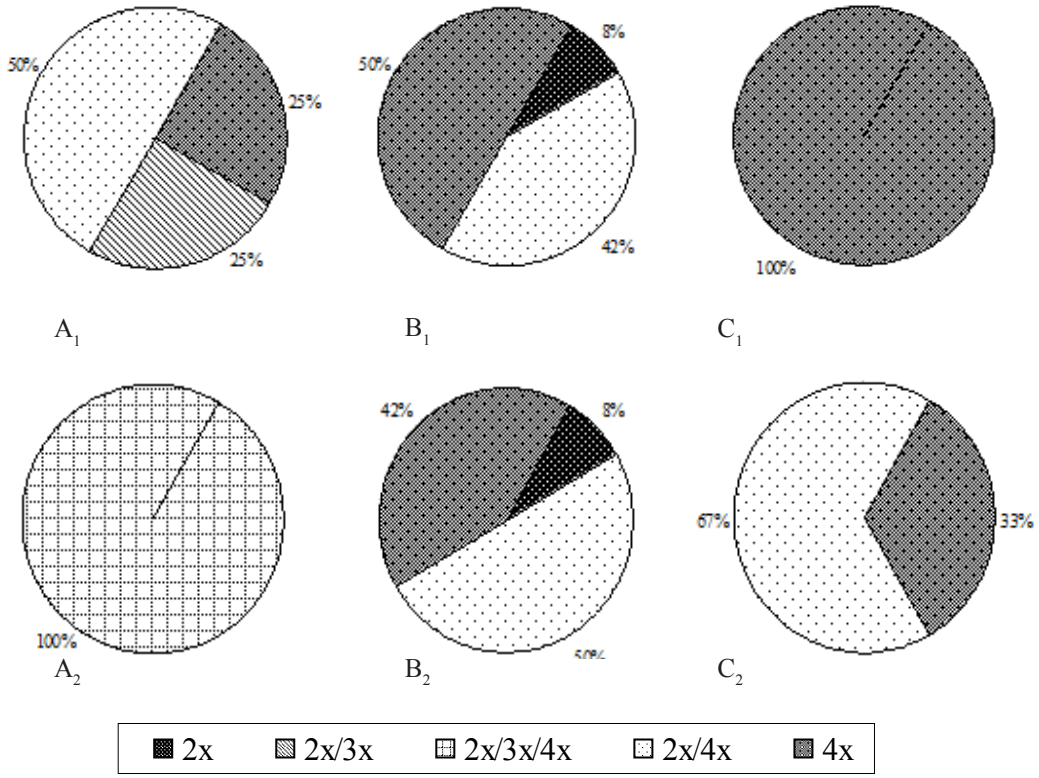

Fig. 3. Percent of plants with different ploidy level in leaves (A1, B1, C1) and roots (A2, B2, C2) for accession *Aizkraules* (A), *Dzelmes* (B) and *Skrīveru* (C).

Table 4. Field evolution of some accessions of the Latvian alfalfa genetic resources

Accession	Full flowering	Flower color	Pod shape	Growth habit	Stem length (cm)
Skrīveru	Beginning of July	Violet, light blue, light violet, green-yellow	Coiled (2.5-3 turns)	Erect	118.15 ± 4.05
Antane	Beginning of July	Light violet, light blue	Coiled (2.5-3 turns)	Erect	115.65 ± 4.11
Dzelmes	Beginning of July	Yellow, light violet	Sickle	Prostrate	101.10 ± 3.76
Aizkraukles	Beginning of July	Yellow, light violet, green-yellow	Sickle/ coiled	Prostrate, erect	104.90 ± 4.30

Dzelmes. Variability of ploidy level of these three accessions could be related with origin of accessions: *Aizkraukles* and *Dzelmes* are semi-wild populations, *Skrīveru* – a commercial variety with very broad genetic background.

Performed direct chromosome count showed variability in chromosome numbers between plants of the same accessions, as well as mixoploid cells in several roots. In accession *Skrīveru* we observed two different sizes and shape of cells – diploid cells were smaller and square-shape while triploid cells were bigger and longer. For root cells existence of endopolyploidy is well known (Brummer et al. 1999) therefore chromosome numbers in roots and leaves could differ. In accession *Dzelmes* (subsp. *falcata*) were found plants with diploid roots but only small number of metaphases could available for analysis.

Accession *Skrīveru* and *Antane* have erect growth habit with about 115-118 cm plant height while *Dzelmes* and *Aizkraukles* had only 101-105 cm long stems (Table 4). It is possible because *Antane* and *Skrīveru* were bred as varieties and plant height was one of important agronomical trait. These two accessions also have similar pod shape with 2.5-3 turns while *Dzelmes* have sickle pod shape. Observations of the growth habit (prostrate), flower color (yellow) and pod shape (sickle) class accession *Dzelmes* as subsp. *falcata*. Aizkraukle could be classified as subsp. *falcata* but some traits are indicating on *M. varia* – flower color, pod shape (Bolton 1962, Quiros & Bauchan 1988).

Percent of developed pods (approximately 17%, Table 2) was higher when plants of subsp. *sativa* were used in the cross as the female parent. When reciprocal crosses were made – plants of subsp. *falcata* were the female parent, only 7.69-8.96% of pollinated flowers developed pods. In alfalfa crossing for breeding programs it is advisable to use plants with inconsistent ploidy (mixoploids) as the male parent.

CONCLUSIONS

High number of mixoploid plants was detected, although most of evaluated accessions were tetraploid. Crossing between subspecies *sativa* and *falcata* produced viable seeds what allows to transfer genetically controlled favorable traits from plants of semi-wild populations of subspecies *falcata* to breeding material. Differences in reciprocal crosses were found, fertility is higher if plants of subsp. *sativa* is used as the female parent.

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