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CALLICULTUREINDUCINGFROMREDCLOVER(*TRIFOLIUM PRATENSE* L.) WILD ACCESSIONS AND RESULTS OF DNA EXTRACTION

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Red clover is the most popular fodder legume suitable for growing in agro-ecological conditions of Latvia, and is used as summer- and winter-forage for cattle. Latvia is rich with wild and semi wild forms of red clover. The objective of this work was to extract genomic DNA from the red clover (*Trifolium pratense* L.) wild accessions leafs for microsatellite analysis in the future, and study possibilities of obtaining calli culture from Latvian red clover wild accessions. Latvian red clover wild accessions were never analysed in this aspect before. Fifty *Trifolium pratense* L. accessions from the Herbarium of Daugavpils University Institute of Systematic Biology were used in this investigation, 16 of them were used in calli culture establishing.

The genomic DNA was isolated from all accession samples. DNA concentrations varied from 3.6 till 58.3 ng/mkl. It was found out, that calli could be obtained from different parts of red clover plants (stem segments, leaf petiole segments and leaf segments). The influence on calli development and growth of genotype, explant type und size of explant were observed.

Key words: red clover, *Trifolium pratense*, calli, DNA extraction.

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INTRODUCTION

Red clover (*Trifolium pratense*) is one of the most cultivated forage legumes in the world (Bowley et al. 1984, Dias et al. 2008) and its centre of origin is located in Southern Europe (Ulloa et al. 2003). Red clover has important medicinal properties due to the presence of antioxidants, which are thought to protect against breast cancer, offer relief from menopausal symptoms and have cardioprotective and bone sparing effects (Atkinson et al. 2004, Marioara & Savatti 2007). Red clover is a most popular fodder legume suitable for growing in agro-ecological conditions of Latvia, and is used as summer- and winter-forage for cattle (Jansone 2008). The main task for red clover breeders is to create new varieties, suitable for local circumstances, with high yield, disease- and pest-resistance, good wintering qualities. The basic breeding material is important for the outcome

of the breeding effort (Marum 1997). Use of red clover wild or semi wild accessions in breeding schema are typical praxis in red clover breeding. Red clover is a diploid (2n=14) cross-pollinated (insects pollination is typical) species, the genome size is approximately 440 Mb (Sato et al. 2005) with a gametophytic self-incompatibility system (Taylor and Quesenberry, 1996), however tetraploid (2n=4x=28) type is known (Jansone, 2008). Tetraploid clover generally is characterised by higher yield, better winter-hardiness and better resistance to diseases (Taylor & Quesenberry 1996), accordingly the interest of breeders is taken by tetraploid type clover. In Latvia tetraploid variety 'Agra' on base of 130 varieties and forms of tetraploidal red clover has been created (Jansone 1997, Jansone 2008).Traditional methods of obtaining red clover initial breeding material, by crossing and following selection are time and handwork consuming. Therefore, it is important to use *in vitro* methods in red clover breeding programs (Taylor & Quesenberry 1996). One of the methods for obtaining increased variability is use of somaclonal variation, which is typical for calli culture and give row material for *in vitro* or traditional selection (Grauda et al. 2004, Vicas 2009).

The important task in evolution of plant variability different aspects, including somaclonal variation, is use of molecular methods, based on DNA isolation and analysis. Various studies based on molecular markers provided initial insight into genetic variability within and among ecotypes, landraces and cultivars as well as on the relationship of cultivateded clover clover populations (Campos-De-Quiroz & Ortega-Klose 2001, Kölliker et al. 2003, Herrmann et al. 2005, Kölliker et al. 2006, Isobe et al. 2009).

The objective of this work was: i) to create extraction of genomic DNA from the red clover (*Trifolium pratense* L.) leafs wild accessions, detect DNA quality and quantity for microsatellite analysis in the future; ii) to study possibilities of obtaining calli culture from Latvian red clover wild accessions. Latvian red clover wild accessions were never analysed in this aspect before.

MATERIAL AND METHODS

Fifty *Trifolium pratense* L. accessions (Table 1) from the Herbarium of Daugavpils University Institute of Systematic Biology were used in this investigation. The herbarium plants were collected in time of flowering. Total DNA isolation of all 50 accessions was performed from plant leafs by using of the Macherey-Nagel DNA purification kit. The NucleoSpin® Plant II protocol based on CTAB lyses method with some modifications was used. For increasing the final DNA yield in the eluate, 200 mkl elution buffer was used. For DNA quantification, the spectrophotometer NanoDrop 1000 was used. Estimation of DNA quality was done by measuring the 260:280 absorbance ratios.

From 16 accessions that have seeds (Table 1) the calli culture were initiated. Donor plants were grown on MS medium (Murashige & Skoog 1962). Earlier elaborated method (Grauda et. al. 2004, Kokina et. al. 2005) was used for calli culture establish. From each accession plants 30 explants from leaves, stem segments and leaf petiole segments with different length from 2 to 20 mm were put on the basal MS medium (3 % sucrose, 0.7 % agar, pH 5.8) supplemented with 2 mg/l 2,4 D and cultivated under 24 ^{0}C and 16 h photoperiod. Calli formation frequency and diameter of obtained calli were evaluated.

For ploidy detection, leaves, stems and roots were used. Approximately 50 mg of plant tissue were placed onto a plastic petri dish, added 0.5 ml of *CyStain UV Ploidy* and choped the tissue with a sharp razor. 1.5 ml of *CyStain UV Ploidy* were added and incubated at room temperature for 5 minutes. Then samples were filtered through a Partec 50 mkm CellTrics disposable filter and analysed in Partec Flow Cytometer using UV-laser and blue emission was measured.

RESULTS AND DISCUSSION

The genomic DNA was isolated from all accessions samples. DNA concentrations varied from 3.6 till 58.3 ng/µl. The dependence of DNA quantity from sampling year of accession was not

Table 1. Latvian origin wild Trifolium pratense L. accessions

Nº	**Sample ID Nº**	**Originated**	**Year of collecting**	**Number of seeds in collection/ Number of seeds used in calli culture formation**
1.	A001	Naujenes parish, Stropi	1979	2/2
2.	A002	Skrudalienas parish, Nature reserve "Ilgas"	1994	-
3.	A003	Daugavpils, Križi	1993	-
4.	A004	Daugavpils	1986	-
5.	A005	Daugavpils, Stropi	1990	-
6.	A006	Jelgava	1990	-
7.	A007	Rīga, Daugavgrīva	2007	-
8.	A008	Mārupes parish, Tīraine	2007	-
9.	A009	Rīga, Mežciems	2007	-
10.	A010	Eglaines parish, Eglaine	2009	10/8
11.	A011	Valmiera	2008	8/8
12.	A012	Ilūkste	2008	7/7
13.	A013	Kalkūnes parish, Randene	2008	10/10
14.	A014	Medumu parish, Medumi	1974	-
15.	A015	Zilupe	2002	12/8
16.	A016	Rēzekne	2004	-
17.	A017	Dagda	2000	-
18.	A018	Daugavpils, Mežciems	1984	-
19.	A019	Daugavpils, Mežciems	1994	-
20.	A020	Medumu parish, Medumi	1987	-
21.	A021	Bebrenes parish, Bebrene	1971	-
22.	A022	Daugavpils, Mežciems	1982	-
23.	A023	Daugavpils, Kalkūni	1983	5/5
24.	A024	Daugavpils, Kalkūni	1973	-
25.	A025	Bebrenes parish,	2002	-
26.	A026	Skrudalienas parish, Nature reserve "Ilgas"	2000	10/9
27.	A027	Madona	2001	-
28.	A028	Rēzekne	1986	-
29.	A029	Daugavpils, Kalkūni	1983	-
30.	A030	Krauja	1992	-
31.	A031	Preiļi	2002	15/13
32.	A032	Medumi	1974	-
33.	A033	Medumi	1980	-
34.	A034	Skrudalienas parish, Nature reserve "Ilgas"	1995	-
35.	A035	Skrudalienas parish, Nature reserve "Ilgas"	1988	-
36.	A036	Daugavpils, Kalkūni	2001	-
37.	A037	Daugavpils	1984	-
38.	A038	Skrudalienas parish, Ilgas	1995	5/5
39.	A039	Malta	1995	-
40.	A040	Jēkabpils	1999	2/2
41.	A041	Daugavpils	2007	-
42.	A042	Daugavpils, Mežciems	1988	-
43.	A043	Skrudalienas parish, Nature reserve "Ilgas"	1995	-
44.	A044	Daugavpils, Judovka	1984	-
45.	A045	Cēsis	2006	5/5

46.	A046	Krāslava	2006	3/3
47.	A047	Rēzekne	2009	-
48.	A048	Koknese	2009	16/13
49.	A049	Preiļi	2009	10/9
50.	A050	Skrudalienas parish, Nature reserve "Ilgas"	2009	5/4

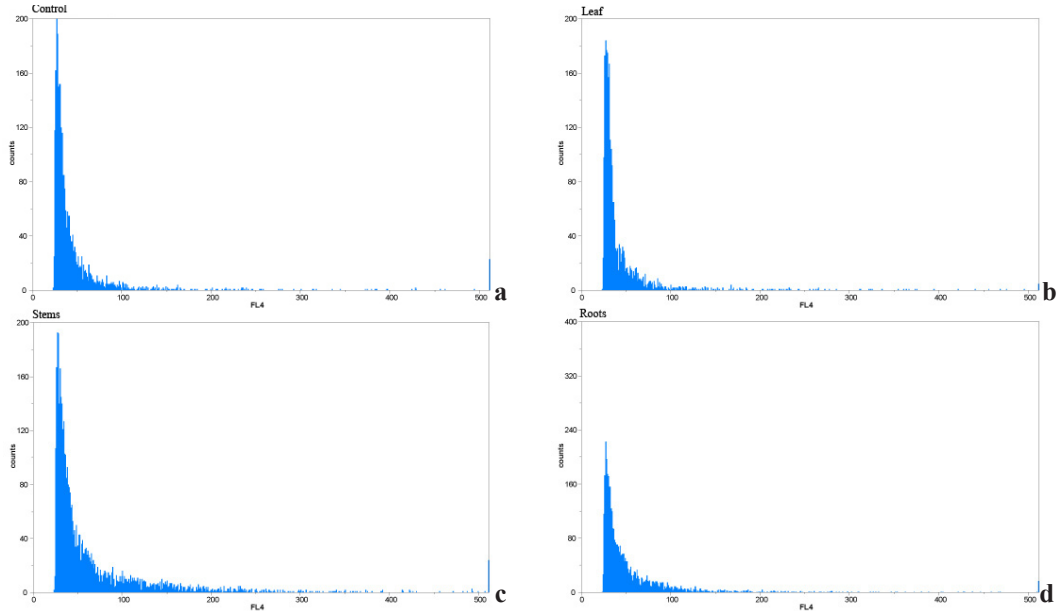

Fig. 1. Results of flow cytometry: a) 2n ploidy control, b) the typical result for leaves, c) the typical result for stems, d) the typical result for roots.

found. The purity of DNA solution varied from 1.20 to 1.90, it means that the samples contain pure DNA. Thirty eight tested samples are suitable for further using in genetic diversity detection revealed by microsatellite markers in future. Approximately 200 cells were measured with a minimum of double analyses performed for each sample (Fig. 1). All samples tested from different parts of plants (leaves, stems and roots) were similar and corresponded diploid level.

It was found that calli could be obtained from different parts of red clover plants (stem segments, leaf petiole segments and leaf segments. Results of calli formation on different explants from different accessions and explants are presented in Table 3. All leaf segment explants formed calli. From other explants calli formation frequency varied from 50.0% to 96.6%, depending on accession. Calli formation frequencies (%) on leaf petiole segments also varied according to size of explants. The most frequently (96.6 %) calli were developed on shorter explants, the longest explants (20 mm) formed calli in 93.3 %. On leaf petiole explants with length 10 mm calli formation frequency was 90 %. The diameter of calli from leaves varied from 8.0 mm to 8.6 mm. The largest calli were observed on leaf petiole segments with length 10 mm. Analysis of variance showed that calli size was significantly depending on from length of explants and does not depend on the type of explants. Previously (Grauda et. al. 2004, Kokina et. al. 2005) when calli culture of tetraploid variety 'Agra' was established, the result were similar: all leaf segment explants produced calli, stem segments, calli formation frequency was 87.5%, leaf petiole segments, calli formation capacity varied from 70% till 97.5%. Shorter (2mm) explants develop calli better than long (20 mm) segments. Analogical results were obtained in process of establishing calli culture from tetraploid wild Turkey red clover *(Trifolium*

Table 2.DNA extraction from different *Trifolium pratense* L. accessions

Sample ID Nº	DNA concentration ng/mkl	Ratio 260/280
A001	12,05	1,6
A002	9,54	1,3
A003	18,03	1,7
A004	24,30	1,8
A005	7,36	1,3
A006	3,6	1,5
A007	29,15	1,8
A008	14,32	1,7
A009	16,52	1,9
A010	32,64	1,8
A011	48,52	1,8
A012	7,52	1,7
A013	24,56	1,7
A014	19,99	1,8
A015	29,42	1,8
A016	16,45	1,7
A017	19,30	1,6
A018	16,70	1,7
A019	15,34	1,3
A020	9,04	1,4
A021	18,54	1,8
A022	19,56	1,7
A023	16,72	1,8
A024	5,3	1,2
A025	45,32	1,8
A026	19,48	1,4
A027	13,25	1,7
A028	23,15	1,3
A029	14,56	1,8
A030	6,54	1,7
A031	7,32	1,6
A032	8,54	1,7
A033	4,21	1,4
A034	14,57	1,7
A035	17,15	1,8
A036	36,25	1,7
A037	6,32	1,5
A038	13,15	1,5
A039	14,57	1,8
A040	23,65	1,9
A041	34,75	1,8
A042	31,02	1,6
A043	6,52	1,7
A044	3,6	1,2
A045	42,65	1,8
A046	23,74	1,8
A047	32,47	1,6
A048	36,48	1,8
A049	16,95	1,7
A050	58,32	1,8

Table 3. Calli formation on different explants of the Latvian red clover wild accessions

Sample ID Nº	Type of explant	Number of calli obtained	Frequency (%) of calli formation	Mean diameter (mm) of calli
A001	Leaf segments	30	100,0	8.0±0.1
	Stem segments	25	83,3	4.5±0.2
	Leaf petiole segments (2 mm)	29	96,6	3.6±0.1
	Leaf petiole segmants (10 mm)	19	63,3	7.5±0.3
	Leaf petiole segments (20 mm)	15	50,0	5.2±0.1
A010	Leaf segments	30	100,0	8.2±0.1
	Stem segments	16	53,3	7.3±0.1
	Leaf petiole segments (2 mm)	23	76,6	6.3±0.2
	Leaf petiole segmants (10 mm)	25	83,3	7.3±0.1
	Leaf petiole segments (20 mm)	27	90	5.2±0.1
A011	Leaf segments	30	100,0	8.4±0.1
	Stem segments	16	53,3	7.6±0.2
	Leaf petiole segments (2 mm)	23	76,6	4.5±0.2
	Leaf petiole segmants (10 mm)	19	63,3	6.4±0.1
	Leaf petiole segments (20 mm)	16	53,3	7.5±0.1
A012	Leaf segments	30	100,0	8.2±0.1
	Stem segments	25	83,3	7.4±0.1
	Leaf petiole segments (2 mm)	29	96,6	6.2±0.2
	Leaf petiole segmants (10 mm)	22	73,3	5.4±0.1
	Leaf petiole segments (20 mm)	18	60,0	3.5±0.1
A013	Leaf segments	30	100,0	8.2±0.1
	Stem segments	24	80,0	6.3±0.2
	Leaf petiole segments (2 mm)	23	76,6	5.3±0.2
	Leaf petiole segmants (10 mm)	21	70,0	4.2±0.1
	Leaf petiole segments (20 mm)	19	63,3	6.2±0.2
A015	Leaf segments	30	100,0	8.5±0.1
	Stem segments	23	76,6	2.3±0.1
	Leaf petiole segments (2 mm)	25	83,3	3.8±0.3
	Leaf petiole segmants (10 mm)	27	90,0	5.6±0.1
	Leaf petiole segments (20 mm)	16	53,3	6.4±0.2
A023	Leaf segments	30	100,0	8.4±0.1
	Stem segments	24	80,0	6.5±0.2
	Leaf petiole segments (2 mm)	23	76,6	5.5±0.2
	Leaf petiole segmants (10 mm)	18	60,0	3.6±0.2
	Leaf petiole segments (20 mm)	15	50,0	6.5±0.2
A026	Leaf segments	30	100,0	7.4±0.2
	Stem segments	29	96,6	3.6±0.3
	Leaf petiole segments (2 mm)	16	53,3	6.5±0.1
	Leaf petiole segmants (10 mm)	25	83,3	7.4±0.1
	Leaf petiole segments (20 mm)	23	76,6	6.5±0.3
A031	Leaf segments	30	100,0	8.3±0.1
	Stem segments	26	86,6	2.3±0.2
	Leaf petiole segments (2 mm)	21	70,0	5.6±0.2
	Leaf petiole segmants (10 mm)	16	53,3	3.6±0.2
	Leaf petiole segments (20 mm)	19	63,3	5.4±0.1
A038	Leaf segments	30	100,0	8.4±0.1
	Stem segments	29	96,6	2.6±0.1
	Leaf petiole segments (2 mm)	16	53,3	5.6±0.2
	Leaf petiole segmants (10 mm)	15	50,0	4.6±0.3
	Leaf petiole segments (20 mm)	19	63,3	1.5±0.1

A040	Leaf segments	30	100,0	8.6±0.2
	Stem segments	25	83,3	8.4±0.2
	Leaf petiole segments (2 mm)	29	96,6	3.5±0.2
	Leaf petiole segmants (10 mm)	26	86,6	6.4±0.2
	Leaf petiole segments (20 mm)	24	80,0	5.4±0.2
A045	Leaf segments	30	100,0	8.6±0.2
	Stem segments	26	86,6	5.4±0.4
	Leaf petiole segments (2 mm)	24	80,0	2.8±0.1
	Leaf petiole segmants (10 mm)	19	63,3	4.5±0.1
	Leaf petiole segments (20 mm)	28	93,3	1.5±0.3
A046	Leaf segments	30	100,0	8.4±0.1
	Stem segments	29	96,6	5.6±0.3
	Leaf petiole segments (2 mm)	26	86,6	4.2±0.1
	Leaf petiole segmants (10 mm)	24	80,0	5.6±0.1
	Leaf petiole segments (20 mm)	19	63,3	4.6±0.2
A048	Leaf segments	30	100,0	8.6±0.3
	Stem segments	29	96,6	4.5±0.1
	Leaf petiole segments (2 mm)	15	50,0	6.5±0.2
	Leaf petiole segmants (10 mm)	26	86,6	3.2±0.1
	Leaf petiole segments (20 mm)	26	86,6	4.3±0.1
A049	Leaf segments	30	100,0	8.6±0.1
	Stem segments	28	93,3	6.4±0.1
	Leaf petiole segments (2 mm)	27	90,0	3.6±0.3
	Leaf petiole segmants (10 mm)	16	53,3	5.4±0.1
	Leaf petiole segments (20 mm)	19	63,3	6.4±0.1
A050	Leaf segments	30	100,0	8.8±0.2
	Stem segments	26	86,6	3.6±0.1
	Leaf petiole segments (2 mm)	19	63,3	8.1±0.1
	Leaf petiole segmants (10 mm)	15	50,0	3.6±0.2
	Leaf petiole segments (20 mm)	16	53,3	5.6±0.1

Hum. pratense L.) (Çölgeçen & Toker 2008) and diploid variety 'Roxana' and tetraploid varieties 'Napoca' and 'Apollo-tetra' calli cultures (Vicas 2009). It follows that the choice of explant type are main terms for successful red clover calli culture establish.

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