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Assessment of the agronomic utility of interspecific hybrids *Trifolium pratense* L. x *T. diffusum* Ehrh. and confirmation of their hybridity with ISSR markers

Giedrė Dabkevičienė*, Vanda Paplauskienė and Izolda Pašakinskienė
Lithuanian Institute of Agriculture, Instituto al.1, Dotnuva-Akademija, Kedainiai, LT-58344, Lithuania.
e-mail: giedre@lzi.lt, vanda@lzi.lt, izolda@lzi.lt

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Abstract

Hybrids were made between two species of clovers, *Trifolium pratense* L. (2n = 2x = 14) and *Trifolium diffusum* Ehrh. (2n = 2x = 16) and a *T. pratense* (♀) x [*T. pratense* x *T. diffusum*] backcross (BC_1) population was also developed. ISSR (inter-simple sequence repeats) markers were used to tag the hybrid plants. Traits of the BC_1s were compared with diploid and tetraploid Lithuanian cultivars of red clover. BC_1 plants were found to be of considerable agricultural value, despite the difficulties in transferring autogamy from *T. diffusum* into the allogamous *T. pratense*. Improved seed yield in BC_1 plants was achieved due to a significant increase of the number of inflorescences per plant in comparison with diploid red clover. Some of *T. pratense* x *T. diffusum* hybrids expressed high levels of autogamy which provides a valuable resource for studies on the inheritance of autogamy.

Key words: Clover, *Trifolium pratense, T. diffusum,* hybrids, autogamy-introgression, ISSR.

Introduction

Trifolium is a large genus in the family Leguminosae. It is comprised of about 300 species which exhibit variability of amativeness and colonise widely diverse areas globally. Attempts are being made to improve cultivated clovers by transferring valuable agronomic traits from wild species into their genomes. In this approach, *T. ambiquum* Bieb. and *T. medium* L. are donors for persistency and for the ability to form rhizomes, which is associated with drought and disease resistance [11, 19, 25]. *T. diffusum* Ehrh. and *T. nigrescen*s Viv. are the source material for high seed productivity (80% self-fertility), for disease and pest resistance[7, 10, 23]; whereas *T. apertum* L. and *T . constantinopolitanum* Ser. in DC are valued for their high forage quality [13, 22].

Reports on interspecific hybridization of clovers were published as early as 1953 [5], and hybridization has been successfully carried out more recently for the cross-combinations of *T. repens* L. x *T. ambiquum* Bieb. and *T. repens* L. x *T. nigrescen*s Viv., focusing on forage quality traits [1, 15-18]. Hybridization involving various species of clovers has now been undertaken in many countries, such as the UK, Poland, Germany, Sweden, Japan, New Zealand, Canada, the USA and Russia, and has recently been reviewed by Abberton [3].

Red clover (*T. pratense* L.) is a high-yielding legume, and it is also an organic source for nitrogen enrichment in the soil by nitrogen-fixing bacteria. Red clover is a cross-pollinating species where bees and other insects are involved in pollen transfer and floret fertilization, and its seed yield therefore is very much dependent on the weather. The predominance of wet weather during flowering is a major cause of low seed yield for this main legume in the crop rotation, and clover rot (*Sclerotinia trifoliorum* Erikss.) also imposes constraints on its usage in the Baltic countries.

Attempts to improve red clover by hybridization with *T. diffusum* Ehrh. have been made by number of researchers. Taylor *et al.* [27] were the first to develop hybrids between *T. pratense* L. and *T. diffusum* Ehrh. Later these hybrids were produced and studied by Schwer [26], Rubcov and Komkova [23] and Pozdniakov [21]. In clovers, a different DNA marker approach has been applied for the characterization of germplasm, for hybridity assessment and for studying genome rearrangements in these hybrids [2, 4, 28].

The aim of the present study was to make assessments of a number of agronomic traits in hybrids and backcrosses between *T. pratense* and *T. diffusum*, with a view to producing superior clover cultivars more suitable to the environmental conditions prevailing in the Baltic and Nordic countries. Our principal target was to improve seed productivity by transferring the autogamous trait from *T. diffusum* into allogamous *T. pratense*. We also evaluated the utility of ISSR profiling as marker for hybridity.

Materials and Methods

***Hybridization and assessment of interspecific hybrids*:** For the production of hybrids and introgression lines, two clover species, *T. pratense* L. represented by the diploid var. Liepsna (2n = 2x =14) and the tetraploid var. Vyliai (2n = 4x = 28) and *T. diffusum* Ehrh. (2n = 2x =16), were used. Interspecific hybrids were developed by the method of embryo culture. For chromosome doubling and fertility restoration sterile F_1 hybrids (2n = 2x = 15) were treated with the colchicine using embryo and meristem culture and microcloning [6]. Chromosome-doubled hybrids were identified by screening for an irregular shape of dry pollen [20] and further confirmed by chromosome counts in root meristem cells. F_1 allotetraploids (2n = 4x = 30) were tested for seed production by both cross- and self-pollination. BC_1 plants were produced manually by emasculation of the florets of maternal plants, followed by paternal pollen transfer. Two backcross populations were produced reciprocally using F_1C_0 or *T. pratense* as the female plant. These BC_1 populations were defined as F_1 x *T. pratense* and *T. pratense* x F_1, respectively.

BC_1 hybrids were compared with the diploid var. Liepsna (2n = 2x = 14) and the tetraploid var. Vyliai (2n = 4x = 28). For evaluation, 30 seedlings from each group were planted in an experimental plot measuring 50 cm x 50 cm. Plant height, bunch density, forage yield, dry matter content and number of inflorescences were analysed, and an assessment for seed setting by self- and cross-pollination was carried out. To estimate self-fertility inflorescences were bagged before flowering.

ISSR fingerprinting: DNA was extracted from young leaves by a micro-method following the DNA extraction protocol of Doyle and Doyle [9]. PCR were carried out in 25 µl volume in an Eppendorf Master Cycler Gradient thermocycler. Amplification products were analysed in 1.5% agarose gel, and electrophoresis was carried out in 1xTAE buffer. GeneRuler™ DNA Ladder Mix (Fermentas) was used as the DNA fragment size marker. For DNA fingerprint analysis of *T. pratense, T diffusum* and their F_1 interspecific hybrids, 17 microsatellite tetra-, tri- and dinucleotide repeats were used as primers.

Results

Traits of T. pratense x T. diffusum hybrids: All F_1 hybrids of *T. pratense* and *T. diffusum* were annual. Phenotypically they mostly resembled the annual paternal *T. diffusum*, but according to some morphological traits they took an intermediate position. Hybrids partly inherited leaf hairiness specific to *T. diffusum*, and the colour of inflorescences was pink compared with red in *T. pratense* and white in *T. diffusum*. After colchicine treatment the chromosome number was doubled and fertile F_1 hybrids were obtained. Most of the F_1C_0 plants had a chromosome number of 2n = 4x = 30, but there were also some aneuploids with 28 or 29 chromosomes. In order to combine traits of perenniality and seed fertility, backcrosses of the F_1C_0 allotetraploids with tetraploid red clover var. Vyliai (2n = 4x = 28) were performed reciprocally: F_1(♀) x *T. pratense* and *T. pratense* (♀) x F_1 (F_1 stands for F_1C_0). The offspring from these reciprocal backcrosses were different. Two types of plants were produced from F_1(♀) x *T. pratense* cross: 74.4% of them were annual and inherited bunch type and leaf hairiness from the *T. diffusum* parent, whereas others were perennial (25,6%) and mostly resembled *T. pratense* in their morphology. Therefore, in this BC_1 group there were about 3 times more of the *T. diffusum* type hybrids than those of the *T. pratense* type. In another group, produced from the backcross *T. pratense* (♀) x F_1, all of the individuals were perennial and mainly inherited traits of *T. pratense*. The viability of the seeds in these two BC_1 groups was also different, showing germination rates of 39.3% and 88.7% for F_1(♀) x *T. pratense* and *T. pratense* (♀) x F_1, respectively. Further, only *T. pratense* (♀) x F_1 offspring were included for evaluation because of its good seed germination.

Traits were evaluated in *T. pratense* x *T. diffusum* F_1C_0 hybrids and *T. pratense* (♀) x F_1 backcrosses, and compared with those of the standard Lithuanian red clover varieties Liepsna (diploid) and Vyliai (tetraploid). According to plant height and forage yield per plant *T. pratense* (♀) x F_1 hybrids were close to var. Liepsna and significantly exceeded *T. diffusum* (Table 1). They also lagged behind var. Vyliai for forage yield, although dry matter content in the backcross plants was significantly higher than that in var. Vyliai. *T. pratense* (♀) x F_1 plants had the greater number of inflorescences per plant. On average they formed 1.5 times more inflorescences than red clover var. Liepsna and Vyliai and 2 times more than *T. diffusum*.

Special attention was paid to seed setting trait in the hybrids. When fertilized by cross-pollination, *T. pratense* (♀) x F_1 exceeded both var. Liepsna and var. Vyliai for seed yield per plant. This was mainly determined by a significant increase in the number of inflorescences in the backcross hybrids. It is noticeable, that the seed yield per plant varied within a wide range in these BC_1 plants. The variation rate was 1.4 times higher for the BC_1s in comparison with red clover plants of tested varieties. Floret fertility assessed from cross-pollination (free crossing in field) in the BC_1s was significantly higher than in the F_1C_0 hybrids and close to the level of var. Vyliai. However, *T. diffusum* plants were superior for seed setting both by cross- and self-pollination. *T. diffusum* also

Table 1. Comparison of traits between *T. pratense* x *T. diffusum* hybrid*s* and *T. pratense* diploid var. Liepsna and tetraploid var.Vyliai.

Trait	*T. diffusum*	*T. pratense* 'Liepsna' (2n = 2x =14)	*T. pratense* 'Vyliai' (2n = 4x = 28)	F_1C_0 (2n = 4x = 30)	B_1 *T. pratense* (♀) x F_1 (2n = 4x = 28, 29, 30)
Plant height, cm	20.7±0.94	42.7±1.57	54.8±1.16	27.2±2.3	44.7±1.03
Forage yield, g	12.02±2.13	336.8±39.1	1066.7±195.8	110.0±3.64	345.0±43.7
Dry matter, %	-	30.9±1.52	26.0±1.21	23.2±1.39	28.8±1.58
Stem, number	9.3±0.71	38.9±0.53	52.6±4.35	23.3±1.78	29.7±3.27
Inflorescence, number per plant	56.3±3.16	72.1±3.53	77.2±11.06	60.1±6.23	114.8±13.91
Seed yield per plant, g	3.9±0.94	2.1±0.54	3.1±0.44	0.78±0.59	3.3±0.65
Floret fertility by cross - pollination per plant, %	90.8±0.37	37.2±2.19	21.4±2.38	8.0±1.97	17.7±2.63
Floret fertility by self-pollination per plant, %	81.8±0.73	0	0	6.28±0.94	1.9±0.59
Days to flowering	59±2.60	75±2.65	82±2.18	52±2.08	71±2.31
Persistency, by % of survivors on the second year of vegetation	0	64.7±5.36	72.4±4.71	0	70.7±7.13

yielded the highest number of seeds per plant, which is quite striking considering how far this species lags behind others for stem number per plant (Table 1).

Autogamy (seed setting by self-pollination) is an attribute of *T. diffusum* but not of *T. pratense*. F_1C_0 hybrids showed a significant capacity for self-fertility when compared with *T. pratense*, although under the effect of backcrossing with red clover the level declined considerably: floret fertility was at 6.28% on average for F_1C_0 hybrids, whereas for *T. pratense* (♀) x F_1 plants it came down to 1.9%. At the same time seed setting by cross-pollination increased greatly in the backcross plants compared with F_1C_0 hybrids, from 8.0% to 17.7%, respectively. It is likely that after repeated backcrossing with 'Liepsna' and 'Vyliai' fertility can be increased to an even higher level. Our previous studies have revealed self-fertility rate variation from inflorescence to inflorescence, displaying a wide range between 0 and 56.8% of fertile florets [8]. In terms of the earliness trait, the F_1C_0 hybrid plants started to flower as early as *T. diffusum*, and *T. pratense* (♀) x F_1 plants were close to the early harvesting diploid var. Liepsna. *T. pratense* (♀) x F_1 was found to be able to survive for the second year of vegetation, reaching the level of persistency equivalent to 'Liepsna' and 'Vyliai'.

Confirmation of hybridity: DNA fingerprinting of *T. pratense, T. diffusum* and their interspecific hybrids and BC_1 was carried out using microsatellite tetra-, tri- and dinucleotide motif primers. For the interspecific hybrids 4 types of profiles were produced (Table 2). Hybridity was clearly confirmed in two cases: firstly, when patterns were comprised of an integrated set of specific fragments coming from *T. pratense* and *T. diffusum*, and also when this composition was supplemented by novel fragment(s). When assessing hybrids and backcross plants, hybridity was also confirmed if the profile was identical to paternal *T. diffusum*, but not when it resembled the maternal *T. pratense*, which could be regarded as a case of a contaminated offspring produced by cross-pollination with *T. pratense* itself. The ISSR fingerprint revealed genetic polymorphism in hybrid individuals. The highest polymorphism in F_1C_0 hybrids was identified with $(GACA)_4GT$ and $(GAA)_5CG$ primers, where 8 and 5 types of profiles were obtained, respectively. Profiles of F_1C_0 hybrids amplified by the $(CTC)_5$ primer contained fragments of both paternal species, and all individuals tested had an identical DNA fingerprint.

$(GACA)_4GT$ and $(ATG)_4GA$ gave a 100% success rate for confirming hybridity in F_1C_0 hybrids, providing either the pattern comprised of a set of specific fragments deriving from *T. pratense* and *T. diffusum*, or the same, but with some additional novel components. However, these primers were completely invalid for confirming hybridity in BC_1 plants displaying the *T. pratense* profile only. In the most successful screening, using primer $(TC)_8G$, in F_1C_0 plants the fingerprints generated were identical to *T. diffusum*, and the profiles of BC_1 individuals contained fragments of parental species as well as novel ones. Therefore, in revealing ISSR polymorphism the $(TC)_8G$ primer was the most efficient for hybridity confirmation of the F_1C_0s and BC_1s.

Table 2. Inter-SSR profile type frequency (%) in the interspecific *T. pratense* x *T. diffusum* hybrid.

Primer	*T. pratense*, *T. diffusum* and 'novel' fragments		*T. pratense* and *T. diffusum* fragments		Profile identical to *T. pratense*		Profile identical to *T. diffusum*	
	F_1	B_1	F_1	B_1	F_1	B_1	F_1	B_1
$(GACA)_4GT$	60.0	0	40.0	0	0	100	0	0
$(CTC)_5$	0	20.0	100	66.7	0	13.3	0	0
$(GAA)_5$ CG	0	16.7	93.3	23.3	0	60.0	6.7	0
$(ATG)_5GA$	100	0	0	0	0	100	0	0
$(TC)_8G$	0	26.7	0	73.3	0	0	100	0
Mean	32.0	12.6	46.7	32.7	0	54.7	21.3	0

Discussion

In nature autogamous species are often annual, and their self-fertility ensures that they have an advantage as the first colonizers of new biotopes. Autogamy is found in several species of clovers, such as *Trifolium angustifolium* L., *Trifolium aureum* Poll., *Trifolium constantinopolitanum* Ser. in DC., *T. diffusum*, *Trifolium dubium* Sibth., *Trifolium glanduliferum* Boiss., *Trifolium glomeratum* L. and *Trifolium hirtum* L. Our main target was to improve seed productivity by transferring the self-fertility trait from *T. diffusum* into allogamous *T. pratense*. Some of the F_1C_0 plants inherited a distinctly expressed autogamy, where the frequency of self-fertile florets reached as high as 56.8% in some inflorescences. The change in the pattern of reproduction from allogamy into autogamy is not a simple task and, in general, seed setting by cross-pollination inherited from *T. pratense* was more pronounced in the hybrids than the autogamy trait coming from *T. diffusum*.

For the first time, in our study we have combined the traits of *T. pratense* and *T. diffusum* species by producing backcross populations. The plants from the backcross *T. pratense* (♀) x [*T. pratense* x *T. diffusum*] are of considerable value to the breeders, because being perennials (in contrast to *T. diffusum*) they have an improved seed yield, which significantly exceeds that of the maternal diploid red clover and approaches the level found in tetraploid red clover. This gain in fertility was achieved not so much by overcoming the allogamy trait in *T. pratense*, but mostly by an increase of the number of inflorescences per plant. Therefore, we report a case of a new successful combination of traits in interspecific clover hybrids. Similar results were observed in interspecific hybrids of *T. repens* x *T. nigrescens*, where backcross plants developed by crossing *T. repens* x *T. nigrescens* with the paternal species *T. repens* formed more inflorescences and flowers than *T. repens* by itself [14]. Our previous studies have shown that *T. pratense* (♀) x [*T. pratense* x *T. diffusum*] are resistant to clover rot similarly as the plants of tetraploid var. Vyliai, in comparison with the diploid var. Liepsna which is characterized by low resistance [7].

Molecular marker techniques are now highly developed in genetic research of major crops, whereas grasses and legumes have been less well investigated in this respect. DNA fingerprinting can be used for hybridity confirmation and it also provides important information on the disclosure of polymorphisms within individual genotypes. In our study, the simple sequence repeats employed as primers produce specific ISSR patterns which provided robust information for testing hybridity of *T. pratense* x *T. diffusum* hybrids and *T. pratense* (♀) x F_1 plants. The appearance of novel ISSR components in the DNA profiles of the hybrids compared with their parental species provides an evidence of genome changes in these inter-specific clover hybrids.

This could be considered as a result of genome interaction and rearrangements, as previously reviewed in the distant hybrids in gramineae [12].

We have succeeded in producing both *T. pratense* x *T. diffusum* hybrids and the backcross population of *T. pratense* (♀) x [*T. pratense* x *T. diffusum*]. These are newly made allopolyploids which have both practical and theoretical importance. They could be developed further as a new crop for use in mixtures with grasses in newly established pasture lands, providing improvements in the soil nitrogen level. In this respect they have a potential in organic farming. In addition, they could also make a valuable resource for production of the new cultivars for ornamental purposes and could be used alongside amenity grasses in gardens and sports venues.

Focusing on the theoretical side, *T. pratense* x *T. diffusum* hybrids which express high levels of autogamy - in some individuals up to 56.8% provide a valuable resource for studies on the inheritance of autogamy [8]. This prospect now is wide open, with an increased accumulation of knowledge in this field from Arabidopsis, where the *S-LOCUS RECEPTOR KINASE (SRK)* and the *S-LOCUS CYSTEINE-RICH PROTEIN (SRC)* genes are found to drive the mechanism of switching between self-fertility versus self-incompatibility [24].

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