Variation of Morphological and Agronomic Traits in Hybrids of *Trifolium pratense* × *T. medium* and a Comparison with the Parental Species

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Abstract: *Trifolium pratense* L. is a high-yielding and high-quality fodder crop. However, it shows low persistence, which may be overcome by hybridisation with species that produce rhizomes. Hybrids between *T. pratense* L. and *T. medium* L. were previously obtained by embryo rescue. The aim of this study was to evaluate 16 characteristics (stem weight, stem length, internode number, length/width ratio of a central leaflet of the trifoliate leaf on the 4th internode, stem thickness on the 4th internode, average leaf area of the trifoliate leaf, weight of dry plant, plant length, stem and head number per plant, head number per stem, seed weight per plant, seed weight per head and dry matter per stem) in the hybrid plants derived from F_1 hybrids and in both parental genotypes. The characteristics were evaluated in 500, 745 and 112 hybrid plants in the years 2006, 2008 and 2009, respectively. The significance of the morphological differences was determined by the general linear model and generalised linear model with Poisson distribution with two factors, genotype and year. Some of the examined traits were intermediate in the hybrids. There were significant differences between the hybrids and *T. pratense* in nearly all the analysed characteristics, except for the following ones: length/width ratio of a central leaflet of the trifoliate leaf on the 4th internode under the top head, plant weight and head number per plant. The stem number per plant was significantly higher in the hybrids compared to both parental species, which could have a positive impact on the yield. Short rhizomes were observed in the hybrids after the harvest of plants in the second harvest year. The stability of this trait and its impact on the perenniality of the hybrids will be investigated in the next years.

Keywords: interspecific hybrids; morphological traits; persistence; red clover; rhizomes; zigzag clover

The red clover (*Trifolium pratense* L.) is a widespread forage crop in the Czech Republic as well as in the rest of Europe. It is a high-yielding and high-quality fodder crop from the aspect of both nutrient content and ensilage suitability. Red clover has a high content of proteins, water-soluble carbohydrates, tannins, polyphenol oxidase and polyunsaturated fatty acids.

Clover breeding is aimed at the production of fresh and dry matter that is of high quality combined with satisfactory seed yields. Polyploidy induction (tetraploidy $2n = 4x = 28$) in red clover is useful for obtaining varieties with higher fodder production compared to diploids because autoploidy increases the cell size, especially in meristematic tissues, and the autoploids usually have broader leaves. Higher persistence and higher resistance to sugar-beet powdery mildew (*Erysiphe betae* (Vanha) Weltzien) have also been observed in tetraploid clovers, however, they have lower seed yields (Mištinová & Užík 1979). Breeding methods in red clover include many procedures suitable for cross-pollinated crops; above all, the crosses for combining ability and successive population breeding are performed. For this purpose, genotypes with a wide genetic variability are needed for the phenotypic selection of superior plants.

In the framework of breeding efforts directed at increasing persistence and tolerance to diseases, pests and unfavourable environmental effects in promising red clover varieties, attention has been focused on the utilisation of useful genetic resources through hybridisation. The wide hybridisation (mainly interspecific hybridisation) of red clover with a related wild species offers a greater potential for the introgression of useful traits into red clover. Various types of barriers of crossability (Hughes 1986) which make conventional crossing procedures and the obtainment of viable hybrids entirely unsuccessful are the main features of this procedure. In *Trifolium*, post-fertilisation barriers are of greater importance (Kazimierska 1978; Taylor *et al.* 1980; Řepková *et al.* 2006). To overcome these barriers, various *in vitro* methods are necessary, such as embryo culture or protoplast fusion (Taylor & Quesenberry 1996). An alternative procedure, currently in use, is the incorporation of foreign genes by genetic transformation via *Agrobacterium tumefaciens*.

From the 1970s to the 1990s, the method of embryo culture seemed to be a promising way of overcoming the interspecific, post-fertilisation barriers in *Trifolium*, even though its success was unreliable. It provided the means to overcome the incompatibility caused by abnormal endosperm development and insufficient embryo nutrition that result in abortion (Kazimierska 1980). The efforts for interspecific hybridisation in *T. pratense* and an interest in hybrid evaluation have continued to date. So far, *T. pratense* has been successfully crossed with five species (reviewed by Abberton 2007): *T. sarosiense* Hazsl. (Collins *et al.* 1981; Phillips *et al.* 1982), *T. medium* L. (Collins *et al.* 1981; Vogt & Schweiger 1983; Merker 1984; Sawai *et al.* 1990; Nedbalkova *et al.* 1995), *T. alpestre* L. (Collins *et al.* 1981; Merker 1988; Phillips *et al.* 1992), *T. ambiguum* M.Bieb. (Vogt & Schweiger 1983), and *T. diffusum* Ehrh. (Schwer & Cleveland 1972). These reports mostly described the intermediate morphological appearance of the F_1 hybrids compared to both parents. More recent advances are focused on the evaluation of morphological and agronomic traits in the F_1 progeny and on the improvement of methods of incorporating superior traits into the elite germplasm.

The hybrids between *T. pratense* ($2n = 4x = 28$) and *T. medium* (zigzag clover; $2n = 6x$ to $8x = 48$ to 80) were obtained by embryo rescue at the Research Institute for Fodder Crops, Ltd., Troubsko (Czech Republic) in 1991 (Řepková *et al.* 1991). Cytological studies performed between 2000 and 2003 by the flow cytometric analysis of the progeny after interspecific hybridisation revealed plants with a different DNA content, as compared to the tetraploid *T. pratense*. Thirty somatic chromosomes were observed in 442 plants; furthermore, 42 and 44 somatic chromosomes were always observed in three plants (Řepková *et al.* 2003). The objective of the research presented here was to compare the morphological, agronomic and reproductive traits of both parental species with the plants derived from the former F_1 hybrids. Because the main disadvantage of red clover varieties is their low persistence, which may be overcome by hybridisation with the more persistent *T. medium*, we focused our attention on the evaluation of a characteristic linked with this trait (e.g. persistence).

MATERIALS AND METHODS

Plant material

T. pratense × *T. medium* F_3 generation hybrid plants were planted in nurseries and open to repeated pollination within the population and with the *T. pratense* tetraploid variety Amos for five generations (in the years 1992–2004). Consequently, plants (hybrids, *T. pratense* and *T. medium* clones named 10/8, used for the original interspecific cross) were transplanted to a breeding nursery (Švancerovo, Hladké Životice) in 2005. The second breeding nursery (Válkovo) was established in 2007 from the seeds of the best plants arising from each family of the Švancerovo breeding nursery during 2005–2006. The morphological and agronomic traits were evaluated in the first harvest year (2006 at Švancerovo breeding nursery and 2008 at Válkovo breeding nursery) and in the second harvest year (2009 at Válkovo breeding nursery).

Experiments in 2006, 2008 and 2009

2006 and 2008 were the first harvest years of the plants (for the breeding nurseries established in 2005 and 2007, the years of sowing), and 2009 was the second harvest year of the plants (for the breeding nursery established in 2007).

In 2006, 500 *T. pratense* × *T. medium* hybrids, 60 plants of red clover and 36 plants of zigzag clover were evaluated. In 2008, 746, 60 and 65 plants of hybrids, red clover and zigzag clover, respectively, were evaluated. In 2009, 112, 65 and 65 plants of hybrids, red clover and zigzag clover, respectively, were evaluated.

Morphological and agronomic characteristics

Traits were measured on single-spaced plants in the breeding nurseries (4.95 × 45 m), where plants were grown at a spacing of 0.45 × 0.45 m. Each plant was taken into account as the replication. The number of replications is mentioned thereinafter.

The following morphological traits were evaluated for each of the hybrid plants in 2006 and 2008 (i.e. the first years after years of sowing) on green plants, 14 days after full flowering: stem weight (g), stem length (cm) of the longest stem, number of internodes, length and width of a central leaflet (mm) of the trifoliate leaf on the 4^{th} internode under the top head, length/width ratio of a central leaflet (mm) of the trifoliate leaf on the 4^{th} internode under the top head, stem thickness on the 4^{th} internodes (mm), and average leaf area of the trifoliate leaf (cm^2). Various numbers of the largest trifoliate leaves on the plant longest stem were measured, i.e. from four to ten. Further traits were evaluated after the whole mature plant harvesting and air drying, as follows: plant weight (g), plant length (cm), stem number per plant, head number per plant, head number per stem, seed weight (g), seed weight per head (g), seed weight per plant (g), and dry matter per stem (g). In 2009, the characteristics were measured and evaluated only on green plants. The same measurements were performed for the parental genotypes, *T. pratense* and *T. medium* (clone 10/8), in 2006 and plants from the breeding nurseries in 2008 and 2009. Additionally, the rhizomatous habitus of *T. medium*, *T. pratense* and the hybrids was assessed. After the harvest of plants in the second harvest years, 2007 (for which only subterranean parts of plants were analysed) and 2009, strong and healthy plants were dug up from the soil, and the roots were cleaned mechanically and washed in water to examine the shape of roots and to identify the rhizomes.

Statistical analysis

The measured data on all characteristics were analysed by the general linear model (GLM) with two factors (year and genotype), with the exception of the number of internodes. First, the global hypothesis regarding the equality of the means of all three clover genotypes (hybrid, red clover and zigzag clover) was tested. When it was rejected, statistically significantly different pairs of clover genotypes were found by pairwise comparisons using the least significant differences. For the characteristic of the number of internodes, the values were not normally distributed (nor were they approximate). The generalised linear model with the Poisson distribution and logarithm link function was used for an analogous analysis in this case. All calculations were realised in the computational system GenStat 13. Predictions as estimates of factor level effects and the least significant differences at a 5% level of significance were calculated for all characteristics in the specified years. The genetic character of the individual traits in the analysed population of hybrid plants, as compared with their parental genotypes, was determined.

RESULTS

The statistical values of predictions and the least significant difference of predictions for the individually analysed traits are summarised in Table 1. The following conclusions were drawn after the statistical evaluation of the obtained measurements. In green plants (14 days after full flowering), there were significant differences between the hybrids and both control genotypes of red clover and zigzag clover in the data on stem weight, stem length, length and width of a central leaflet, stem thickness and average leaf area. The data on dry plants (after harvest) were significantly different among all the analysed genotypes for plant length, stem number per plant, head number per stem and seed weight per plant and head. There were

Table 1. Results of the evaluation of morphological and agronomic traits in interspecific hybrids *Trifolium pratense* × *T. medium* and in their parents *T. pratense* 4*n* and *T. medium*

Character		Hybrid	*T. pratense*	*T. medium*
Green plants				
Stem weight (g)	P	20.33	25.15*	8.79*
	LSD		1.704	1.631
Stem length (cm)	P	67.37	73.93*	32.02*
	LSD		1.679	1.613
Internode number	P	1.881	1.954*	1.844
	LSD		0.06573	0.06674
Length of a central leaflet[1] (mm)	P	42.82	44.91*	32.86*
	LSD		1.306	1.255
Width of a central leaflet[1] (mm)	P	27.19	28.01*	16.00*
	LSD		0.7963	0.765
Ratio length/width	P	1.598	1.637	2.153*
	LSD		0.04773	0.04586
Stem thickness (mm)	P	4.164	4.530*	2.449*
	LSD		0.2155	0.2071
Average leaf area[2] (cm^2)	P	13.42	16.32*	8.06*
	LSD		0.6821	0.6529
Dry plants				
Plant weight (g)	P	179.6	183.7	111.00*
	LSD		6.59	13.20
Plant length (cm)	P	65.68	66.35*	30.42*
	LSD		1.280	2.580
Stem number/plant	P	70.66	44.48*	40.30*
	LSD		2.253	4.511
Head number/plant	P	241	247.6	86.9*
	LSD		10.74	21.47
Head number/stem	P	3.54	5.733*	2.027*
	LSD		0.1822	0.3709
Seed weight/head (g)	P	0.02879	0.03822*	0.00623*
	LSD		0.001534	0.003122
Seed weight/plant (g)	P	7.025	9.251*	0.295*
	LSD		0.4768	0.9549
Dry matter/stem (g)	P	2.644	4.382*	2.751
	LSD		0.1249	0.2541

[1]Central leaflet of the triple leaf on the 4th internode under the top head; [2]leaf area of the triple leaf
*Significance P = 0.05; P – prediction – estimate of factor level effect
LSD – least significant differences of predictions (P = 0.05) between hybrids and *T. pratense*, hybrids and *T. medium*

significant differences only between the hybrids and red clover in the internode number and dry matter per stem. There were significant differences between the hybrids and zigzag clover only in the length/width ratio of a central leaflet, dry plant weight and head number per plant. Nearly all the examined traits were on an intermediate level in the hybrids; they reached higher values than in the zigzag clover and lower values than in the red clover (Figure 1A, 1B, 1C, 1D, 1E, 1G, 1M, 1N, 1O). The stem number per plant was significantly higher in the hybrids compared with both parental genotypes (Figure 1K). There were significant differences between the years examined (2006, 2008 and 2009) in all the traits analysed, with the exception of leaf area, where no significant differences were found out between 2008 and 2009 (Table 1). The traits of dry plants were not evaluated in 2009 because of flooding.

The first observation of roots was carried out in 2007. Different root structure compared to the maternal species *T. pratense* was found out in

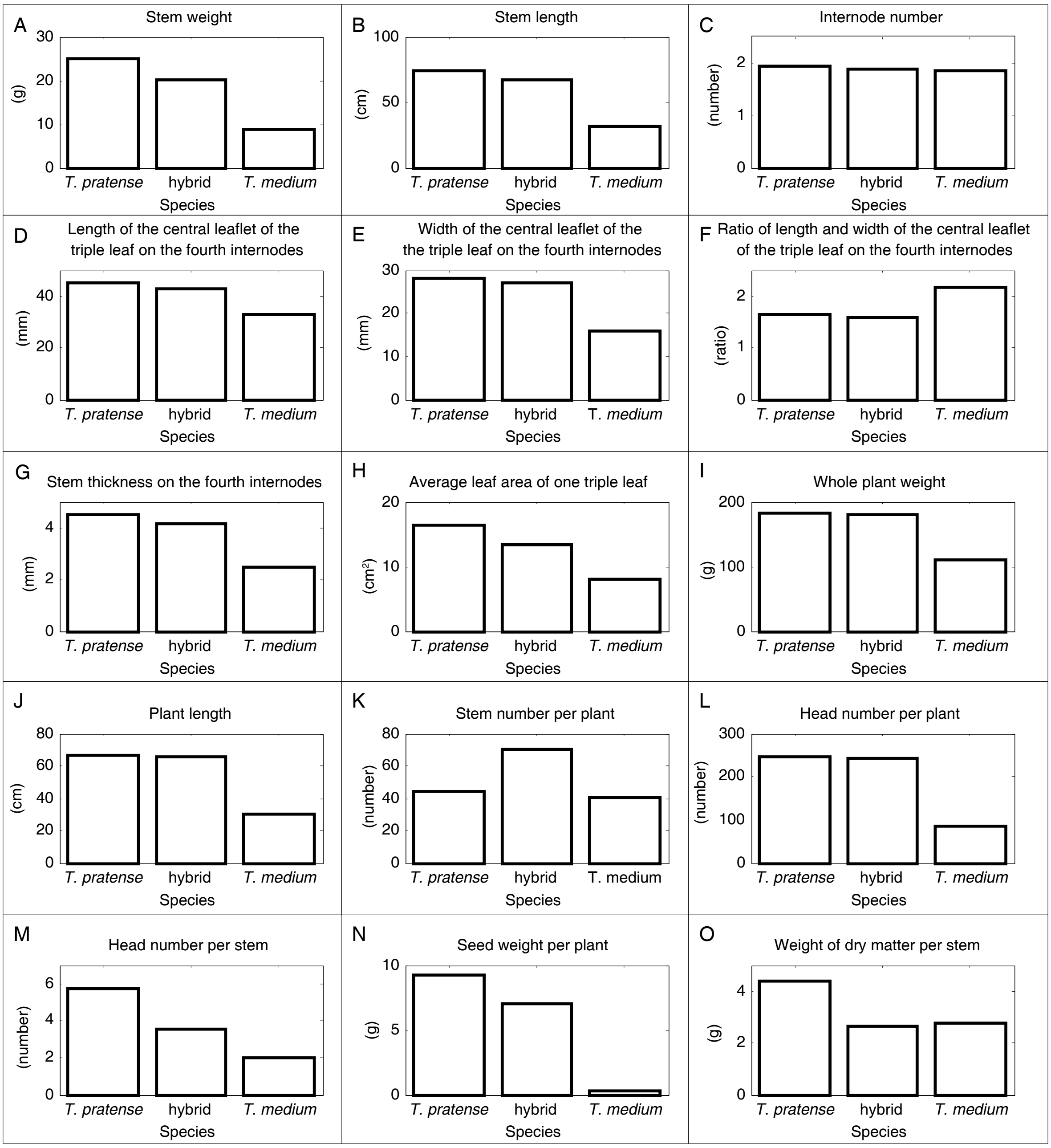

Figure 1. Distributions of data on 15 morphological and agronomic traits in *Trifolium pratense* × *T. medium* hybrids and parental genotypes obtained in three years

Figure 2. Root system of *Trifolium pratense* 4n with a compact root system (A) and *Trifolium pratense* × *T. medium* hybrids – young plant (B), plants after the first (C) and second (D) year harvest. Arrows indicate loose root branching

30 hybrid plants out of 102 analysed ones. Characteristic loose root branching under the soil surface (Figure 2) was observed in the hybrids repeatedly in 2007 and 2009. In *T. pratense*, roots of 85 plants were analysed in the years 2007–2009, but the branching of root crowns was not observed, they were compact.

DISCUSSION

Red clover is an intensively exploited agricultural species. However, it has a low resistance to diseases and cold, and proper breeding donors are required for persistence traits. In this respect, *T. medium* is a prospective fodder crop. Compared to *T. pratense*, *T. medium* is more tolerant to the damage of its underground organs by heavy agricultural mechanisation, and to powdery mildew, *Erysiphe polygoni*, and it exhibits an extraordinary resistance to viral diseases (Smrž *et al.* 1985). The F_1 interspecific hybrids mostly display a low level of fertility and insufficient vigour. For this reason, the efforts to improve seed productivity are aimed at the repeated backcrossing of hybrids with the cultured species, as it has been shown that backcrossing is an effective way of increasing both fertility and vigour. Interspecific hybridisation with *T. repens* has predominantly been performed in order to improve fertility and reproductive traits. Anderson *et al.* (1991) produced third or fourth backcross progenies of hybrids between *T. ambiguum* and *T. repens* and succeeded in increasing their fertility to a sufficient level. Marshall *et al.* (1998, 2002, 2008) produced backcrossed hybrids of *T. repens* × *T. nigrescens* with a higher potential seed yield than the control varieties. Indeed, in our experiments, the repeated open pollination helped to improve the level of fertility and vigour of the *T. pratense* × *T. medium* hybrids. Only the hybrids with higher chromosome numbers (42 and 44) were unsuccessful in establishing a population of fertile hybrids as a source for further selection of useful traits.

Introgressions in the genus *Trifolium* have been performed with the related species, *T. ambiguum* M. Bieb., for the desirable traits such as rhizomatous habitus (Paplauskiene *et al.* 2004), persistence and tolerance to moisture stress (Marshall *et al.* 2001), clover cyst nematode resistance (Hussain *et al.* 1997) and *T. repens* and *T. hybridum* for reproductive traits (as a means of improving the seed yield) presented by Marshall *et al.* 1995, 1998, 2002). However, there have been no reports on hybrid plants being used as germplasm in conventional breeding.

Our data showed significant differences between the progeny of the interspecific hybrids and the parental species in the majority of the analysed traits, such as stem length, stem weight, stem thickness, number of internodes, leaf shape and plant length, which are traits that are monitored by the tests for the evaluation of distinctness, uniformity and stability in red clover by UPOV (International Union for the Protection of New Varieties of Plants). The hybrids in our study showed a typically intermediate level of trait for three characters, stem weight, average leaf area of one trifoliate leaf and head number per stem. Resemblance of the hybrids to *T. pratense* was observed in the majority of the analysed traits. In the only trait, the weight of dry matter of stem, the hybrids showed similarity to *T. medium*. In our opinion, the prevailing dominance of *T. pratense* traits in hybrids is a consequence of repeated backcrosses. This corresponds to the results of Isobe *et al.* (2001), who observed different leaf morphologies between BC_4 plants and *T. pratense* in the same cross combination in comparison with the parental species. The intermediate morphological appearance of interspecific hybrids was also observed in other species combinations, such as *T. alexandrinum* × *T. apertum* (Malaviya *et al.* 2004), *T. alexandrinum* × *T. constantinopolitanum* (Roy *et al.* 2004) and *T. alexandrinum* × *T. resupinatum* (Kaushal *et al.* 2005). A few hybrids of *T. alexandrinum* × *T. apertum* also showed better growth than either parent (Malaviya *et al.* 2004).

The yield of fresh matter is influenced by tillering, stem number, branching, foliage, plant height and dry matter content. The number of stems per plant is a promising trait with a good prediction for the agronomical value of fresh matter yield and seed productivity; the hybrid progeny surpassed both parental genotypes in this trait in our study. The reproductive characteristics, as documented by seed weight, were satisfactory, even though they were lower compared to the red clover.

Persistence is a discrete, complex characteristic with great selectable possibilities, even though the productivity in the first harvest year may be decisive. The persistence in *T. medium* is ensured by the rhizomatous growth habit to a great extent. Therefore, additional tests for this specific character were carried out in the hybrids, and root branching characteristic of rhizomes was observed in some hybrids. In addition, a 3:1 ratio ($\chi^2 = 1.059$, $P > 0.05$) that resulted from the segregation of 72 plants with compact roots and 30 plants with root branching indicates that this result is consistent with a model of one gene determining the root character with a recessive allele for root branching. Sawai *et al.* (1995) obtained first and second backcross progenies of the hybrid, *T. medium* × *T. pratense*, to *T. pratense* to introduce the rhizomatous growth habit and perenniality from *T. medium* to *T. pratense*. However, neither the resulting fertility nor vigour was adequate for the use in a breeding programme. In addition, a higher ploidy level (higher DNA content) could be a promising trait in hybrids, as it is known that tetraploids display an increase in winter hardiness, which means a complex trait that results in higher persistence and yield stability in the second harvest year (Užík 1975).

We suggest that it is necessary to repeat this experiment in the next years to verify the results in the first and second harvest years and to include an evaluation of the resistance to viral and fungal pathogens.

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