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Production of *Trifolium pratense* L. and *T. hybridum* L. tetraploid populations and assessment of their agrobiological characteristics

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Abstract

The current study was set out to establish the efficacy of different mitosis inhibitors (colchicine, amiprophos-methyl, trifluralin and oryzalin) used in the production of red clover (*Trifolium pratense* L.) and alsike clover (*Trifolium hybridum* L.) polyploids and to assess the agrobiological traits of tetraploid populations. Four cultivars and five wild populations of *T. pratense*, one cultivar and three wild populations of *T. hybridum* were used for polyploidization. The effect of conventional soaking of germinating seeds in a colchicine solution was compared with that of colchicine treatment of embryos in an *in vitro* culture. Colchicine treatment of *T. pratense* embryos resulted in 3.3 times higher production of tetraploids (teraploid yield 55.0%) compared with colchicine treatment of seedlings, and the production of chimeric plants was 1.9 times lower.

The different mitosis inhibitors were characterised by a diverse polyploidization effect. Tetraploid yield depended not only on the mitosis inhibitor used but also on the plant species. Treatment of *T. pratense* embryos with colchicine, amiprophos-methyl and oryzalin yielded similar results in terms of tetraploid production (31.3–40.7%) and chimeras (14.3–22.4%). The concentration of trifluralin used for the production of *T. pratense* tetraploids was ineffective. Colchicine solution was more efficient for the development of *T. hybridum* tetraploids whose yield was 2.5 times as high as that obtained having treated the embryos with amiprophos-methyl.

Induced tetraploid populations were compared to reference cultivars in field trials. The two *T. pratense* populations 'Radviliai 4n' and 'Arimaičiai 4n' stood out in this respect – their plants accumulated higher dry matter contents, produced more inflorescences than those of the reference cultivar and exhibited prolific seed yield capacity. In relation to the agrobiological traits, the tetraploid populations of *T. hybridum* did not surpass the reference cultivar.

Key words: agrobiological characteristics, mitosis inhibitor, tetraploids, *Trifolium hybridum*, *Trifolium pratense*.

Introduction

Clover (*Trifolium* spp.) is one of the major legumes grown in a field crop rotation in Lithuania. Many of the Lithuanian clover cultivars, including red clover (*Trifolium pratense* L.) and alsike clover (*Trifolium hybridum* L.) have been developed using conventional breeding techniques, such as mass and group family selection, intervarietal hybridization, selection of synthetic populations, polycross, etc. A few cultivars have been produced using experimental ploidy. This method is still considered effective in plant breeding and has been effectively applied up till now in the development of novel cultivars of food, forage and industrial plants. Polyploidy has been an important factor in plant evolution and is often associated with speciation and development of novel adaptations. Tetraploid plants have long been considered as a valuable breeding material characterised by superior agrobiological characteristics. Tetraploids are more productive than diploids, accumulate higher contents of dry matter and crude protein and exhibit better digestibility properties (Algan, Bakar Buyukkartal, 2000). Moreover, it is very important that polyploid populations exhibit increased heterozygosity, thanks to which it is possible to produce populations possessing greater genetic variability.

Experimental development of polyploid plants was started back in 1940 when colchicine, a natural alkaloid with an antimitotic activity was started to be used. Yemets and Blume (2008) have reported that already in 1979 tetraploid populations of as many as 150 plant species were produced by colchicine treatment. However, it was found that colchicine not only doubles the number of chromosomes but also causes undesirable mutations and is poisonous to humans. Furthermore, colchicine often induces chimeric and aneuploid individuals, which are unfit for the development of productive unique genotypes. Around the 1990's, first attempts were made to search for an alternative to the traditional colchicine, attention was drawn to other agents for induction of polyploidy – antimicrotubular herbicides, belonging to dinitroanilines (oryzalin, trifluralin), phosphorothiomines (amiprophos-methyl) and other groups of compounds. These mitosis inhibitors are less noxious and cheaper, since markedly lower doses are required to achieve the effect. A substantial body of research evidence now exists, which details polyploidy production using alternative mitosis inhibitors (Yemets, Blume, 2008). The various biotechnological techniques perfected over the last century have been also used for the polyploidization of different plant species in the *in vitro* systems. Various plant structures characterised by a high morphogenetic potential, including apical meristems, young inflorescences and embryos, microspores, ovaries, embryogenic callus, somatic embryos are treated with mitosis inhibitors (Hansen, Andersen, 1996; Cheng et al., 2012). Each *in vitro* polyploidization technology needs to be adapted to a specific plant species. The data on clover (*Trifolium* spp.) polyploidization using alternative mitosis inhibitors are scarce.

The aim of the current study was to compare the effect of different mitosis inhibitors on tetraploid yield of clover and to assess the agrobiological properties of tetraploid populations.

Materials and methods

For the polyploidization we used red clover (*Trifolium pratense* L.) cultivars 'Liepsna', 'Vyčiai', 'Arimaičiai' and 'Radviliai', wild populations catalogue Nos. 2156, 2196, 2295, 2352 and 2211 (collected in Lithuania), and alsike clover (*Trifolium hybridum* L.) cv. 'Lomiai', wild populations catalogue Nos. 281, 326 (collected in Latvia) and 284 (collected in Sweden). Experiments were carried out at Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry during 2010–2014.

Colchicine treatment of clover seedlings. The trial was conducted with the seed of the red clover cv. 'Radviliai'. The seeds were germinated for 6–8 days in Petri dishes on an agarised medium (7 g agar 1 l^{-1} distilled water). After cotyledons had opened, 0.05% colchicine was poured on the seedlings. The treatment time was 30 h (at 8°C, in the dark). Then the seedlings were removed from the agar, rinsed with distilled water (3 times for 10 min) and planted in the soil in pots. The pots were covered with agro-film and kept in a greenhouse at 20–24°C. After 3–4 weeks, dead plants were counted. When the plants started flowering, plant ploidy level was established according to the shape of dry pollen (Anderson et al., 1991).

Production of clover polyploids in the in vitro culture of embryos. The plants were cultivated in pots in a greenhouse. During flowering they were transferred to a temperature-controlled chamber at 24°C, 16 h photoperiod, light intensity of 9 000 lux, 60% relative air humidity.

Twelve-fourteen days after crossing, the embryos, excised in sterile conditions, were transferred to test tubes on a nutrient Gamborg B-5 (Duchefa Biochemie, the Netherlands) medium supplemented with 0.1 mg l^{-1} kinetin and giberrelin (Vogt, Schweiger, 1983) and cultured for 3–4 days. Later they were soaked in the solution of mitosis inhibitors at 28°C in the dark. The following mitosis inhibitors were used for the development of tetraploids: colchicine (0.4%, treatment time 4 h), oryzalin (50 µM, treatment time 6 h), trifluralin (50 µM, treatment time 6 h) and amiprophos-methyl (APM) (100 µM, treatment time 6 h). Afterwards the embryos were rinsed three times for ten minutes in sterile water and transferred to test tubes on B-5 medium and were cultured for 8 weeks in a cultivation room at 25°C, a light intensity of 9 000 lux, 16 h photoperiod, 60% relative air humidity. The developed plants were transplanted to soil in pots and were placed in a temperature-controlled growth chamber or a greenhouse. When plants started flowering (C_0), ploidy level was determined according to the shape of dry pollen (Anderson et al., 1991). Tetraploid inflorescences were crossed, F_1 population seeds were collected, the number of chromosomes was determined in the preparations of rootlets of emerged seeds. Having proved that F_1 population is composed of tetraploids, they were planted in the field trials for the determination of agrobiological characteristics.

Meteorological conditions. At the end of May 2012, the weather was warm and dry. The moisture reserves in the soil were approaching the critical level. Cool and wet weather prevailed in June. There was enough moisture during the rest of the growing season and periods of cool weather alternated with warm/hot spells.

The spring of 2013 arrived very late. The last snow melted on April 14. In May, the weather was warm and very warm (temperature rose to 28°C for several days). There was enough moisture. June was characterised by warm and very warm, windy, cloudy and dry weather. Productive moisture reserves in the soil were nearing the critical level. Warm, windy weather with a few hot days prevailed in July. Soil moisture during the greater part of this month was sufficient. The weather in August was warm, dry and windy. Productive moisture reserves in the soil in the first and third ten-day periods were too low. Conditions for plant growth and development were favourable during the entire growing season.

The winter of 2013–2014 was unusual for Lithuania's climate. The vegetation season continued until the first ten-day period of January 2014. The snow layer was uneven and thin (up to 5 cm); it formed on 13 January, 2014 and melted on 8 February. The 17–26 January period was the coldest: the lowest air temperature ranged from 14.2°C to 18.5°C below zero (in some places up to −20°C). On 4 February, the air temperature became positive and continued to be such in the daytime until the spring. In terms of air temperatures, the spring was changeable. Spells of warmer weather alternated with colder ones until the second ten-day period of May. There was little precipitation in April; however, the rainfall that fell in the second half of May replenished moisture reserves in the soil. Warm, windy weather predominated during the summer period. There was enough moisture in the soil. In July, there were a few dry spells which slowed down plant re-growth after cuts.

Assessment of tetraploid clover populations. For agrobiological analyses, clover plants were grown in a nursery of field experiments, 20 plants per 50 × 50 cm nutrition area. Red and alsike clover cultivars and tetraploid populations: 'Liepsna 4n', 'Vyčiai 4n', 'Arimaičiai 4n', 'Radviliai 4n', Nos. 2156 4n, 2352 4n, 2211 4n, 2196 4n, 2295 4n, 'Lomiai 4n', Nos. 281 4n and 326 4n. The reference cultivars were 'Vyčiai' and 'Lomiai'. The data were collected in the first year of harvest at the stage of mass flowering (BBCH 65). The plants were assessed for the following agromorphological traits and characteristics: plant height cm, number of stems per plant, growth habit (E – erect, sE – semi-erect, P – prostrate), fresh mass yield per plant (g), dry matter yield per plant (g), number of inflorescences per plant, seed yield per plant (g), maturity (date when 50% of plants are in bloom), over winter survival (%). A near infrared spectrometer NIRS-6500 was used to quantify crude fibre, crude protein and water-soluble carbohydrates (WSC) (Butkutė et al., 2003).

Statistical analysis of data was performed using the software *ANOVA*, *STAT* from the packages *SELEKCIJA* and *IRRISTAT* (Tarakanovas, Raudonius, 2003).

Results and discussion

Comparison of colchicine treatment methods. To compare different polyploidization methods, shoots and immature embryos of the red clover cv. 'Radviliai' were treated with colchicine. Three indicators were assessed: survival rate of explants after colchicine treatment, tetraploid yield and formation of chimeras.

In our study we found that embryos were more sensitive to colchicine treatment (Table 1). Colchicine treatment of clover seedlings resulted in 2.2 times higher survival rate compared with *in vitro* colchicine treatment of embryos. However, tetraploid yield was as many as 3.3 times higher for colchicine treated embryos. There is still no consensus on which colchicine treatment method is the most efficient. The success of polyploidy induction depends on several variables: plant species, antimitotic agents, explant types, exposure times and concentrations (Dhooghe et al., 2011). Traditionally, colchicine treatment is performed on germinating seeds (Wu et al., 2010) and 7-day old seedlings (Abd El-Naby et al., 2012), apical meristems (Schifino, Morales Fernandes, 1987), apical buds (Wang et al., 2009; Talukdar, 2010), axilary meritems (Anderson et al., 1991), epicotyls (Bliznyuk, Filipovich, 2007) and cotyledons (Stanys et al., 2006).

The data on colchicine treatment of isolated embryos are rather limited. Pasakinskiene (2000) has reported that treatment of perennial ryegrass isolated embryos with 0.3% colchicine for 4 h resulted in a polyploid production rate of 65.8% from the total treated embryos, which is a six-fold increase compared with the traditional treatment of germinated seeds. The data obtained by Schifino and Morales Fernandes (1987) suggest that the optimal method to produce *T. riograndense* Burkart polyploids was soaking of seedlings in 0.3% colchicine for 6 hours, the yield of "pure" tetraploids was 3.5% from the number of treated seedlings and 21.4% from the number of survived seedlings. Treatment of *Vigna angularis* germinating seeds with 0.4% colchicine solution for 12 hours resulted in a survival rate of as low as 3.5% and a high incidence of mutations. The 0.2% colchicine solution proved to be optimal (Wu et al., 2010). Treatment of *Lathyrus* apical buds with 0.3% colchicine produced a tetraploid yield of 18.2% (from the number of survived regenerants) (Talukdar, 2010). Three colchicine treatment methods were used to produce *Cyamopsis tetragonoloba* L. polyploids: seed treatment, cotton swab method and method of immersion of cotyledonary leaves. The diploid seedlings of *C. tetragonoloba* of these accessions were treated with different concentrations of aqueous colchicine using the cotton-swab method for 10–18 hours within 2–3 days. The highest percentage of success was recorded when the seedlings were treated with 0.2% colchicine for 10 h within two days (Bewal et al., 2009). According to Omran and Mohammand (2008), the safest method is colchicine treatment of seeds. For the production of *Gossypium herbaceum* L. polyploids they propose treatment of germinated seeds with 4–7 mm hypocotyls with 0.9% colchicine for 16 hours. *In vitro* treatment of embryos can be conducted in a little space and needs a little amount of colchicine. However, high risk of contamination and high sensitivity of embryos and meristems are the drawbacks of this method. The optimum dose of colchicine and the incubation time should be adjusted for each cultivar under various environmental conditions (Omran, Mohammand, 2008).

Table 1. Assessment of colchicine treatment efficacy of *Trifolium pratense* seedlings and embryos

Explants and polyploidization method	Number of treated explants	Survival rate		Tetraploids produced %		Chimeras produced %	
		number	%	from the number of explants treated	from the number of plants grown	from the number of explants treated	from the number of plants grown
Seedlings, 0.05% colchicine, 30 h	190	90	47.4	7.9	16.7	8.9	18.9
Embryos, 0.4% colchicine, 4 h	93	20	21.5	11.8	55.0*	2.2	10.0
SD			13.0	2.0	19.2	3.4	4.5

SD – standard deviation

Already in the initial research on polyploidization it was found that cells exhibit a different response to colchicine, as a consequence of which cytochimeras (mixoploids) with various ploidy segments are produced (Wit, 1958). The data of multiple researchers suggest that myxoploids in legume plants are formed irrespective of the plant organ which has been exposed to colchicine (Schifino, Morales Fernandes, 1987; Barufaldi et al., 2001). Our research evidences that colchicine treatment of embryos yields 1.9 times fewer chimeric individuals.

Assessment of the effect of different inhibitors of mitosis on the production of tetraploids. The effect of different inhibitors of mitosis on the survival of embryos was determined by treating the embryos of red clover (cv. 'Radviliai') and alsike clover (cv. 'Lomiai'). The survival rate of the regenerates in the soil (from the total embryos treated) was established (Table 2).

Of the mitosis inhibitors used in our study, the most toxic were oryzalin and colchicine, under the effect of which 29.2% and 32.7% of red clover regenerants survived and were transferred to the soil. The embryos exhibited an approximately equal response to APM and trifluralin and yielded 50.6% and 47.8% of regenerates. Compared to alsike clover, red clover embryos were more sensitive to colchicine and APM effect, significantly fewer red clover plants survived grown in the soil.

The tested mitosis inhibitors were characterised by a different polyploidization effect (Table 3). Tetraploid yield depended not only on the mitosis inhibitor used but also on the plant species. Treatment of red clover embryos with colchicine, APM and oryzalin yield similar numbers of both tetraploid (31.3–40.7%) and chimeric (14.3–22.4%) individuals. The concentration of trifluralin used

Table 2. Survival rate of embryos (%) after treatment with mitosis inhibitors

Trifolium spp.	Colchicine	Amiprophos-methyl (APM)	Trifluralin	Oryzalin
T. pratense	32.7	50.6	47.8	29.2
T. hybridum	44.4	59.5	–	–
LSD_{05}	9.61	8.81	–	–

LSD – least significant difference, significant at the 0.05 level

Table 3. The effect of mitosis inhibitors on tetraploid production

Trifolium spp.	Colchicine		Amiprophos-methyl (APM)		Trifluralin		Oryzalin	
	1	2	1	2	1	2	1	2
T. pratense	38.0 // 12.7	19.6	33.8 // 13.8	22.4	6.0 // 3.1	7.2	36.0 // 15.0	14.3
T. hybridum	45.2 // 19.9	26.9	18.5 // 10.9	30.2	–	–	–	–
LSD_{05}	9.78 // 7.32	8.38	8.53 // 6.55	8.74	–	–	–	–

LSD – least significant difference, significant at 0.05 level; 1 – % tetraploids produced from the number of plants grown // % tetraploids produced from the number of explants treated, 2 – % chimeras produced from the number of plants grown

in our study for the development of red clover tetraploids proved to be ineffective since it yielded only 6.0% of tetraploids. Colchicine solution was more efficient for alsike clover tetraploid production; it yielded 2.5 times more tetraploids compared with APM.

It is difficult to compare our results with those obtained by other researchers since different concentrations of mitosis inhibitors, exposure times and plant species were used (Hansen, Andersen, 1998; Barufaldi et al., 2001; Talukdar, 2010; Wu et al., 2010). Numerous research publications have indicated that colchicine is the most common mitosis inhibitor used for the production of polyploids of legumes (Anderson et al., 1991; Algan, Buyukkartal, 2000; Wang et al., 2009; Wu et al., 2010; Chaisan et al., 2013). One µM colchicine (24 h) was best suited for *Brassica napus* L. polyploidization. APM was less toxic than oryzalin and trifluralin. In wheat breeding, high potential of APM and trifluralin as chromosome-doubling agents in isolated microspore cultures was identified (Hansen, Andersen, 1998).

A study on the use of colchicine and dinitroanilines (ethalfluralin, oryzalin, cobex and amex) herbicides for polyploidy induction in watermelons (Nasr et al., 2014) showed ethalfluralin to be the most effective antimitotic agent. Pintos and Manzanera (2007) have indicated that compared with colchicine and APM, oryzalin induces more cells with a double chromosome number in the cell cultures of potato and *Quercus suber* L., whereas in *Beta vulgaris* ovule culture the best results were shown by APM (Hansen, Andersen, 1998). In the experiment where *Rosa* spp. apical meristems were treated with trifluralin and colchicine, better results were obtained in the treatment with trifluralin (Zlesak et al., 2005). In another experiment, oryzalin proved to be best suited for polyploidy induction in *Rosa* (Kermani et al., 2003). For the fertility restoration in interspecific currant hybrids, embryos and microshoots were treated with colchicine and oryzalin solutions. Oryzalin was more effective – polyploids were formed by 19.3% embryos and 32.3% microshoots (Stanys et al., 2004).

In the development of tetraploid plant cultivars it is very important to select "pure" tetraploid individuals. In our study, mixoploid (chimeric) individuals were determined in all treatments both in red clover (7.2–22.4%) and alsike clover (26.9–30.2%). When using colchicine and APM, more chimeric plants were identified in alsike clover populations. The success of polyploidization depended not only on the treatment method and substance used but also on the populations' genetic origin (Table 4).

Tetraploid yield (%) for clover populations varied within a wide range. The highest tetraploid yield (71.4%) was obtained having treated the embryos of cv. 'Vyčiai' with colchicine, while the lowest yield (0.0%) was produced having treated the embryos of red clover cultivars with trifluralin. The embryos of cv. 'Liepsna' were the most resistant to colchicine polyploidization effect (tetraploid yield 10.0%); while the most sensitive were embryos of cvs. 'Arimaičiai' (50.0%), 'Radviliai' (63.7%) and 'Vyčiai' (71.4%). Colchicine treatment of wild populations of clover produced a tetraploid yield ranging from 24.0% to 35.0%. In the populations of red clover APM treatment the highest tetraploid yield gave in the cv. 'Liepsna' (47.4%), the lowest yield – in cv. 'Radviliai' (13.6%). Oryzalin induced from 10.0% ('Radviliai') to 60.0% ('Vyčiai') tetraploids. Trifluralin effect was the weakest, only in cv. 'Vyčiai' tetraploid regenerants made up 15.2%. The populations of alsike clover also differed in tetraploid yield. Colchicine treatment induced 2.4 times more tetraploids compared with soaking in APM solution. The highest (58.1%) tetraploid yield was produced by cv. 'Lomiai', the lowest (31.6%) – in the wild population No. 326. Under the effect of APM, the highest (29.0%) tetraploid yield was obtained in the population No. 281, the lowest (8.8%) – in the population No. 284.

Assessment of tetraploid clover populations. Having verified that F_1 populations are composed of

Table 4. Tetrapoid yield (% from the number of survived plants) in different populations of *Trifolium* spp.

Population	Colchicine	Amiprophos-methyl (APM)	Trifluralin	Oryzalin
Trifolium pratense				
2196 4n	31.3	16.7	–	–
2295 4n	24.0	36.4	–	–
Liepsna 4n	10.0	47.4	0.0	26.7
2156 4n	35.0	41.4	0.0	27.8
Vyčiai 4n	71.4	39.5	15.2	60.0
Arimaičiai 4n	50.0	41.4	0.0	55.6
Radviliai 4n	63.7	13.6	0.0	10.0
2352 4n	30.8	–	–	–
2211 4n	26.0	–	–	–
Average	38.0	33.8	6.0	36.0
SD	18.7	12.2		18.9
Trifolium hybridum				
Lomiai 4n	58.1	25.6	–	–
281 4n	55.0	29.0	–	–
284 4n	36.0	8.8	–	–
326 4n	31.8	11.9	–	–
Average	45.2	18.5	–	–
SD	11.5	8.6		

SD – standard deviation

tetraploid plants, we planted them in field trial nurseries. One of the major indicators according to which forage grasses are assessed is dry matter yield (g plant^{-1}) (Table 5). In 2013 and 2014, five red clover teraploid populations (Nos. 2156 4n, 2211 4n, 'Radviliai 4n', 'Vyčiai 4n' and 'Arimaičiai 4n') outyielded the reference/control cultivar. Significantly ($P < 0.05$) higher dry matter yield was produced by populations 'Radviliai 4n' (2.6 times) and 'Arimaičiai 4n' (2.0 times) compared to cv. 'Vyčiai 2n'. Mehta et al. (1964) indicated that *T. alexandrinum* teraploids were more productive in the 1st cut; however, they exhibited poor re-growth and lower herbage yield of the 2nd and 3rd cuts. The populations of both ploidy levels produced similar dry matter and seed yields.

Table 5. Yield-related traits of *Trifolium* spp. cultivars and populations

Cultivar and population	Fresh mass per plant g		Dry matter per plant g		Plant height cm	
	mean ± SE	min–max	mean ± SE	min–max	mean ± SE	min–max
			Trifolium pratense, 2013			
Vyčiai 2n	294.36 ± 74.08	119.3–528.3	74.4 ± 17.44	32.8–126.3	54.60 ± 3.80	41.0–63.0
2156 4n	486.83 ± 96.39	200.9–621.7	92.2 ± 19.7	41.6–134.6	59.25 ± 6.43	43.0–73.0
2352 4n	248.96 ± 27.56	164.1–310.5	56.8 ± 6.6	41.9–75.7	54.80 ± 4.08	43.0–68.0
2211 4n	536.49 ± 95.84	303.9–711.5	122.1 ± 20.3	62.8–171.3	67.75 ± 4.52	60.0–77.0
Radviliai 4n	617.03 ± 185.40	203.8–1087.2	194.9 ± 26.9	108.6–274.6	69.00 ± 4.92	60.0–78.0
LSD_{05}	222.22	–	69.69	–	11.76	–
			Trifolium pratense, 2014			
Vyčiai 2n	489.60 ± 74.20	353.7–634.0	118.2 ± 20.3	82.0–166.6	85.0 ± 7.71	70.0–102.0
Vyčiai 4n	549.88 ± 79.34	369.8–709.2	131.2 ± 22.8	84.2–189.3	92.00 ± 5.99	76.0–105.0
2196 4n	351.88 ± 31.35	270.0–422.3	72.3 ± 6.17	55.6–85.1	63.33 ± 2.72	56.0–69.0
2295 4n	393.63 ± 41.93	314.1–509.6	85.3 ± 11.5	61.2–116.4	50.68 ± 1.25	48.0–54.0
Arimaičiai 4n	1087.08 ± 183.75	673.1–1558.0	240.9 ± 54.3	122.5–384.7	74.25 ± 1.11	72.0–77.0
Liepsna 4n	261.03 ± 41.21	194.7–376.7	51.1 ± 5.9	37.8–66.4	50.25 ± 1.55	46.0–53.0
LSD_{05}	262.24		81.29		13.1	
			Trifolium hybridum, 2014			
Lomiai 2n	553.30 ± 84.14	390.3–723.6	119.8 ± 22.19	73.8–171.4	67.25 ± 5.94	54.0–80.0
Lomiai 4n	360.10 ± 79.32	220.3–574.9	74.85 ± 18.35	44.8–128.2	57.5 ± 4.79	49.0–71.0
281 4n	337.83 ± 40.42	160.8–387.0	80.95 ± 9.57	28.1–77.6	51.68 ± 2.49	45.0–57.0
326 4n	307.33 ± 51.87	220.4–402.0	59.23 ± 11.07	53.8–97.8	59.75 ± 2.39	53.0–64.0
LSD_{05}	214.59	–	52.4	–	14.71	–

LSD – least significant difference, significant at the 0.05 level; SE – standard error

According to the plant height, significantly ($P < 0.05$) taller stems were recorded for the populations No. 2211 4n, 'Radviliai 4n' and 'Vyčiai 4n'. The plants of the population No. 2211 4n produced significantly ($P < 0.05$) more inflorescences and stems compared with the reference plants (Table 6).

The seed yield of clover is largely dependent on the weather conditions. Either deficiency or excess of moisture and a shortage of pollinators during flowering result in poor seed setting and consequently, low seed yield. Red clover populations 'Radviliai 4n' and 'Arimaičiai 4n' produced significantly ($P < 0.05$) larger number of inflorescences, which consequently determined a relatively higher seed yield. However, it did not exceed the seed yield of the reference cultivar. Red clover populations 'Radviliai 4n' and 'Arimaičiai 4n' produced significantly ($P < 0.05$) larger number of inflorescences, which consequently determined a relatively higher seed yield. However, it did not exceed the seed yield of the reference cultivar. The seed yield

Table 6. Seed yield-related traits of *Trifolium* spp. cultivars and populations

Cultivar and population	Number of stems per plant		Number of inflorescences per plant		Seed yield per plant g		Seed setting %
	mean ± SE	min–max	mean ± SE	min–max	mean ± SE	min–max	
			Trifolium pratense, 2013				
Vyčiai 2n	42.00 ± 7.85	20.0–62.0	149.20 ± 26.20	82.0–235.0	8.74 ± 0.92	6.55–10.48	34.4
2156 4n	46.75 ± 8.34	22.0–58.0	228.75 ± 44.34	115.0–310.0	2.93 ± 0.49	1.71–3.88	9.3
2352 4n	40.80 ± 4.41	24.0–48.0	108.20 ± 11.43	69.0–137.0	4.22 ± 1.74	1.78–9.34	13.5
2211 4n	65.00 ± 5.05	51.0–75.0	289.50 ± 38.46	215.0–392.0	8.37 ± 3.86	1.87–18.60	11.3
Radviliai 4n	27.25 ± 7.36	6.0–40.0	287.25 ± 79.64	89.0–472.0	5.08 ± 0.82	3.0–7.23	5.6
LSD_{05}	18.05		108.03		6.25		4.26
			Trifolium pratense, 2014				
Vyčiai 2n	30.50 ± 3.40	22.0–36.0	167.5 ± 15.9	124.0–198.0	19.81 ± 5.6	9.56–36.2	54.2
Vyčiai 4n	29.00 ± 6.24	14.0–44.0	162.0 ± 21.68	106.0–208.0	10.84 ± 4.6	4.26–24.23	40.8
2196 4n	17.33 ± 0.94	16.0–20.0	101.33 ± 4.99	88.0–112.0	3.8 ± 1.3	1.80–7.36	11.4
2295 4n	37.33 ± 4.03	26.0–44.0	139.3 ± 28.11	90.0–218.0	8.0 ± 1.6	3.60–11.0	10.4
Arimaičiai 4n	34.50 ± 6.55	26.0–54.0	345.0 ± 103.85	148.0–638.0	8.3 ± 3.2	1.82–16.62	17.0
Liepsna 4n	20.50 ± 3.40	14.0–30.0	84.5 ± 8.66	72.0–110.0	1.5 ± 0.7	0.52–3.36	11.5
LSD_{05}	13.01		136.95		10.72		4.49
			Trifolium hybridum, 2014				
Lomiai 2n	48.0 ± 8.04	34.0–68.0	367.5 ± 28.03	284.0–404.0	3.1±1.0	1.14–5.62	40.8
Lomiai 4n	28.5 ± 5.32	16.0–40.0	165.5 ± 27.94	116.0–240.0	0.3±0.1	0.08–0.44	4.5
281 4n	33.33 ± 3.68	24.00–42.0	181.5 ± 40.02	104.0–262.0	0.2±0.1	0.02–0.38	7.6
326 4n	33.0 ± 1.29	30.0–36.0	222.0 ± 14.76	182.0–248.0	0.1±0.1	0.01–0.30	3.1
LSD_{05}	17.70		77.78		1.61		4.34

LSD – least significant difference, significant at 0.05 level; SE – standard error of the population No. 2211 4n was almost equal to that of the reference plants. In this population, the more abundant seed yield was influenced by the significantly ($P < 0.05$) higher number of stems. Our research findings corroborate those found in literature suggesting that poorer seed setting and less abundant seed yield are characteristic of tetraploid populations. De Pablo et al. (2004) have indicated that the induced autotetraploids in ryegrass, rye, clovers and *Lotus pedunculatus* show better establishment, higher in vitro digestibility and forage production, and better performance in response to such adverse factors as disease, frost and drought than the corresponding diploids. However, gamete fertility of induced autotetraploid plants is reduced compared with that of the diploid forms. Barufaldi et al. (2001) suggest that the tetraploid form of *L. glaber* also sets fewer seeds than its diploid counterpart.

In terms of the agromorphological traits, the tetraploid populations of alsike clover did not surpass the reference cv. 'Lomiai 2n'.

Overwinter survival is a major characteristic according to which the longevity/persistence of a species is judged (Table 7). Red clover is characterised by poor overwinter survival (60–78%). Other clover species perform better in this respect (85–100%). According to the data from the Dotnuva Weather Station, the winter 2012–2013 was adverse for clover wintering: cold, with permanent snow cover and a long period with deep-frozen soil. In our experiment, in the red clover reference cv. 'Vyčiai 2n' the overwinter survival rate of plants amounted to 52.4% (2013) and 86.2% (2014). Good overwinter survival rate ($P < 0.05$) was exhibited by the populations No. 2156 4n (100%), No. 2211 4n (95.2%) and 'Vyčiai 4n' (81.3%). The tetraploid populations of alsike clover showed significantly worse overwinter survival (66.7% and 77.8%) than the plants of the reference cv. 'Lomiai 2n' (89.5%). An exception was the population No. 326 4n in which 85.0% of plants survived the winter and the result was significant ($P < 0.05$). For most of the populations investigated, the date of mass flowering (≥50% of inflorescences in flower) was 11–12 June. Red clover population No. 2352 4n stood out by the early maturity (7 June), and populations 'Radviliai 4n', 'Vyčiai 4n' and 'Arimaičiai 4n' – by late maturity (21–27 June).

Table 7. Winter survival, heading date, growth habit and quality traits in *Trifolium* spp. cultivars and populations

Cultivar and population	Over winter survival rate %	Heading date	Growth habit	Crude fibre %	Crude protein %	Water-soluble carbohydrates (WSC) %
			Trifolium pratense, 2013			
Vyčiai 2n	52.4	06.12	E	28.0	16.3	12.1
2156 4n	100	06.12	E and sE	26.8	17.3	11.0
2352 4n	71.4	06.07	sE	34.6	15.7	10.9
2211 4n	95.2	06.12	sE and P	31.2	16.2	12.1
Radviliai 4n	76.2	06.27	E	29.5	15.1	13.9
LSD_{05}	4.61			5.73	4.60	4.08
			Trifolium pratense, 2014			
Vyčiai 2n	86.2	06.11	E	27.1	14.0	13.0
Vyčiai 4n	81.3	06.21	E	31.0	13.3	13.1
2196 4n	41.7	06.11	sE	27.3	14.4	14.5
2295 4n	55.6	06.11	sE	30.4	15.0	14.2
Arimaičiai 4n	66.7	06.24	E	25.4	15.8	14.0
Liepsna 4n	72.7	06.11	E	26.5	15.4	14.3
LSD_{05}	5.06			5.11	4.04	3.94
			Trifolium hybridum, 2014			
Lomiai 2n	89.5	06.11	sE	31.9	14.9	10.5
Lomiai 4n	66.7	06.11	sE	28.2	16.5	11.9
281 4n	77.8	06.11	sE	26.7	17.2	11.4
326 4n	85.0	06.11	sE	31.1	16.7	10.3
LSD_{05}	5.49			6.37	5.17	4.38

LSD – least significant difference, significant at 0.05 level; E – erect, sE – semi-erect, P – prostrate

The growth habit characteristic of perennial species of *Trifolium* genus ranges from prostrate to erect stems (Gillett, Taylor, 2001). In our study, the growth habit of tetraploid red clover populations 'Radviliai 4n', 'Vyčiai 4n', 'Arimaičiai 4n' and 'Liepsna 4n' is erect like that of the reference cv. 'Vyčiai 2n'. The growth habit of wild tetraploid populations was semi-prostrate.

All the tested *Trifolium hybridum* populations of alsike clover as well as the reference cultivars exhibited a semi-prostrate growth habit.

Crude protein is the only nutritional compound of forage which contains nitrogen. High quality forage should contain 14–17% protein. The literature data suggest that protein content in clover varies from 14% to 16% (Zableckienė, Butkutė, 2006). In our study, crude protein content in red clover varied from 13.3% ('Vyčiai 4n') to 17.3% (No. 2156 4n) (Table 8). For the reference cv. 'Vyčiai 2n' it ranged from 16.3% (2013) to 14.0% (2014), for cv. 'Lomiai 2n' – 14.9%. In our tested tetraploid populations the crude protein content was similar or a little higher: No. 2156 4n – 17.3% and No. 281 4n –17.2%. The literature indicates that legumes contain less crude fibre than grasses (Vaičiulytė, Bačėnas, 2004). The optimal crude fibre content in forage is 22–25%. The plants of the reference cv. 'Vyčiai 2n' accumulated 28.0% (2013) and 27.4% (2014), on average 27.7%. A slightly lower content of crude fibre was determined only in the populations No. 2156 4n (26.8%) and 'Arimaičiai 4n' (25.4%).

The concentration of WSC in the red clover populations ranged from 10.9% (No. 2211 4n, 2013) to 14.5% (No. 2196 4n, 2014). The literature data indicate that WSC should range within 8–12%. Comparison of average WSC concentration recorded for the reference plants (12.6%) with that identified in tetraploid populations showed that in six populations WSC content was insignificantly higher. In 2013 population 'Radviliai 4n' accumulated 13.9%, in 2014 populations 'Vyčiai 4n' – 13.1%, No. 2196 4n – 14.5%, No. 2295 4n – 14.2%, 'Arimaičiai 4n' – 14.0% and 'Liepsna 4n' – 14.3% WSC.

Analysis of alsike clover qualitative indicators and comparison with those of the plants of the reference cv. 'Lomiai 2n' suggested that tetraploid populations are characterised by slightly higher quality. They were found to contain higher concentrations of crude protein and WSC (except for No. 326 4n) and less crude fibre.

Almost all the agrobiological traits tested varied within a wide range. The most stable trait for both red clover and alsike clover was stem height. Only for the

Table 8. Coefficients of variation (CV%) of agrobiological traits in *Trifolium* spp. cultivars and populations

Cultivar and population	Fresh mass per plant	Dry matter yield per plant	Plant height cm	Number of culms per plant	Number of inflorescences per plant	Seed yield per plant
			Trifolium pratense, 2013			
Vyčiai 2n	56.27	52.43	15.57	41.82	58.58	21.13
2156 4n	47.01	47.65	22.76	35.68	38.77	33.11
2352 4n	24.75	25.82	16.64	24.16	23.62	82.47
2211 4n	30.46	37.12	11.54	15.54	26.57	92.16
Radviliai 4n	53.04	34.34	12.34	54.05	55.45	37.89
			Trifolium pratense, 2014			
Vyčiai 2n	30.31	34.29	18.03	22.32	18.99	60.24
Vyčiai 4n	28.86	34.8	13.01	43.07	26.76	85.15
2196 4n	17.82	17.08	8.59	10.88	9.85	66.85
2295 4n	21.31	27.03	4.92	21.58	40.35	39.75
Arimaičiai 4n	33.81	45.11	2.99	37.98	60.21	77.80
Liepsna 4n	31.57	23.04	6.13	33.20	20.49	88.23
			Trifolium hybridum, 2014			
Lomiai 2n	30.41	37.04	17.65	33.51	15.25	63.68
Lomiai 4n	44.06	49.03	16.65	37.30	33.37	75.06
281 4n	33.76	33.37	9.65	22.10	44.1	76.75
326 4n	23.93	23.64	8.01	7.82	13.30	98.76

population No. 2156 4n the variation coefficient (CV%) slightly exceeded 20%, for other populations it ranged from 2.9% to 16.6%. The feeding value of the populations was also a relatively stable indicator (Table 8).

Seed yield was found to be the most variable indicator for all populations tested. The coefficient of variation ranged from 21.13% ('Vyčiai 2n') to 98.76% (No. 326 4n). In tetraploid red clover the lowest seed yield per plant was 1.5 g ('Liepsna 4n') and the highest – 8.4 (No. 2211 4n).

Analysis of the indicators of the tetraploid populations revealed that the plants of No. 2196 4n were characterised by the least variation of traits. The values of the coefficient of variation of all traits (except for seed yield per plant) fluctuated within 8.59–17.82% range.

Conclusions

1. Colchicine treatment of red clover (*Trifolium pratense* L.) embryos gave a tetraploid yield 3.3 times higher compared with colchicine treatment of seedlings. Treatment of alsike clover (*T. hybridum* L.) embryos with colchicine, amiprophos-methyl and oryzalin induced a similar number of tetraploid (31.3–40.7%) and chimeric (14.3–22.4%) individuals, while trifluralin was found to be ineffective.

2. Colchicine solution was more suitable for tetraploid production of *Trifolium hybridum* – colchicine treatment yielded 2.5 times more tetraploids compared with amiprophos-methyl treatment.

3. The response of different *Trifolium* spp. populations to treatment with mitosis inhibitors was highly variable, tetraploid yield ranged from 0% (embryo trifluralin treatment, 'Liepsna', No. 2156, 'Arimaičiai' and 'Radviliai') to 71.4% (embryo colchicine treatment, 'Vyčiai').

4. Some of the newly induced tetraploid plant populations exceeded reference populations: population 'Radviliai 4n' were better yielding (both fresh and dry matter yield was significantly higher), produced more inflorescences and the plants were taller. Plants of population No. 2211 4n were taller, produced higher fresh mass yield, higher number of stems and inflorescences and population 'Arimaičiai 4n' produced higher fresh and dry matter yield, formed more inflorescences. Four tetraploid populations (Nos. 2156 4n, 2352 4n, 2211 4n and 'Radviliai 4n') exhibited higher winter survival than the reference population. In terms of indicators of agrobiological traits, *T. hybridum* tetraploid populations did not surpass the reference cv. 'Lomiai'.

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Trifolium pratense L. ir *T. hybridum* L. tetraploidinių populiacijų kūrimas ir jų agrobiologinių savybių vertinimas

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Santrauka

Tyrimo metu siekta nustatyti skirtingų mitozės inhibitorių (kolchicino, amiprofos-metilo, trifluralino ir orizalino) efektyvumą kuriant raudonojo (*Trifolium pratense* L.) bei rausvojo (*Trifolium hybridum* L.) dobilų poliploidus ir įvertinti sukurtų tetraploidinių populiacijų agrobiologinius požymius.

Poliploidinant buvo panaudota raudonojo dobilo 4 veislės bei 5 laukinės populiacijos ir rausvojo dobilo 1 veislė bei 3 laukinės populiacijos. Tradicinio sudygusių sėklų mirkymo kolchicino tirpale poveikis buvo palygintas su gemalų kolchicinavimu *in vitro* kultūroje. Nustatyta, kad raudonojo dobilo gemalus veikiant kolchicinu, gaunama 3,3 karto daugiau (išeiga 55,0 %) tetraploidų nei paveikus daigus, o chimerinių individų formuojasi 1,9 karto mažiau.

Skirtingi mitozės inhibitoriai pasižymėjo nevienodu poliploidinimo efektyvumu. Tetraploidų išeiga priklausė ne tik nuo panaudoto mitozės inhibitoriaus, bet ir nuo augalo rūšies. Raudonojo dobilo gemalus paveikus kolchicinu, amiprofos-metilu ir orizalinu, gauti panašūs kiekiai tetraploidinių (31,3–40,7 %) ir chimerinių (14,3–22,4 %) individų. Raudonojo dobilo tetraploidams kurti panaudota trifluralino koncentracija buvo neefektyvi. Jiems kurti labiau tiko kolchicino tirpalas – juo paveikus gauta 2,5 karto daugiau tetraploidų nei gemalus veikiant amiprofosmetilu.

Įvertinus dobilo veislių kontrolinių augalų ir sukurtų tetraploidinių populiacijų agrobiologinius požymius nustatyta, kad kai kurios jų buvo esmingai produktyvesnės ir geresnės kokybės. Vertingais požymiais pasižymėjo raudonojo dobilo populiacijos ‘Radviliai 4n’, ‘Arimaičiai 4n’, Nr. 2211 4n, Nr. 2156 4n ir Nr. 2352 4n. Jų augalams būdingas geras žiemojimas, didesnis sausųjų medžiagų ir žiedynų kiekis. Rausvojo dobilo tetraploidinės populiacijos agrobiologinių požymių rodikliai neviršijo kontrolinės veislės augalų.

Reikšminiai žodžiai: agrobiologinės savybės, mitozės inhibitoriai, tetraploidai, *Trifolium hybridum*, *Trifolium pratense*.

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