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IN VITRO METHODS IN CLOVER ALLOPOLYPLOIDY

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Abstract

In clover breeding, besides increasing the yield of vegetative mass, it is very important to develop varieties with a higher seed yield and disease resistance. For the development of such forms wild clover species can be used for crossing with cultivated clover species. It was found expedient to use embryo culture for the production of interspecific hybrids *T. pratense* L. x *T. diffusum* Ehrh. and *T. ambiguum* Bieb. x *T. hybridum* L. The yield of allodiploids reached 15.7% and 9.6%, respectively. Fertility of interspecific hybrids *T. pratense* L. x *T. diffusum* Ehrh. was restored by chromosome doubling. Biotechnological (*in vitro*) methods, such as embryo and meristem culture, and micropropagation were combined with colchicine treatment. According to the yield of fertile allotetraploids, all fertility restoration methods were more or less equivalent (48.7–61.1%). A slightly more effective (on average 8.3%) was polyploidisation of embryos. However, all our attempts to restore fertility of *T. ambiguum* x *T. hybridum* allodiploids did not give any positive results.

Key words: *Trifolium* spp., interspecific hybridization, embryo and meristem culture, micropropagation, colchicine.

Introduction

Red clover *Trifolium pratense* (2n = 2x = 14) is one of the main legumes grown in field crop rotations. In terms of herbage yield, it is a productive crop but insufficiently productive in terms of seed yield, and is noted for a low resistance to diseases. Wild clover *Trifolium diffusum* (2n = 2x = 16) is an annual plant, but produces a high seed yield and is characterised by self-pollination (up to 80%). Crosses between these species are aimed at transferring self-pollination trait and thus increasing seed yield in cultivated red clover. Alsike clover *Trifolium hybridum* (2n = 2x = 16) is fairly widely grown on acid or wet soils. By hybridising alsike clover with wild *Trifolium ambiguum* (2n = 2x, 4x, 6x = 16, 32, 48), characterised by persistency, ability to spread vegetatively (form rhizomes) and drought resistance, it is expected to transfer into its genome the genes responsible for these characters /Marshall et al., 1995; Meredith et al., 1995; Hussain et

al., 1997/. The first hybrids between *T. ambiguum* and *T. hybridum* were developed by W. F. Keim (1953) and A. M. Evans (1962), and the first hybrids between *T. pratense* and *T. diffusum* were developed by N. L. Taylor with co-workers /Taylor et al., 1963/. Later on, the same hybrids were grown and comprehensively tested by Russian researchers E. K. Firsova, M. I. Rubcov and T. N. Komkova, V. A. Pozdniakov /Фирсова и др., 1980; Комкова, 1981; Рубцов, Комкова, 1983; Поздняков, 1985/. No clover varieties developed by hybridisation between species have been registered so far, therefore all research contributing to interspecific clover hybridisation are of great relevance. Interspecific hybrids of clover (allodiploids) are most often sterile. The reasons of their sterility could be as follows: they do not form inflorescences, have non-dehiscent anthers, either do not produce pollen or it is inviable. Colchicine polyploidization is the most common technique used for fertility restoration. After chromosome doubling in hybrids, meiosis occurs normally, and fertile plants – allotetraploids are produced. Polyploidization can be performed with parental forms or by intercrossing the resulting tetraploid forms. Fertile hybrids between *T. repens* and *T. nigrescens*, *T. pratense* and *T. diffusum* have been obtained in this way /Taylor et al., 1963; Комкова, 1981/. This method of fertility restoration does not always work. Hybridization of tetraploid *T. pratense* L. with *T. medium* L. or *T. hybridum* results in sterile allotetraploids /Merker, 1984; Hyrkas et al., 1986/. Another method to restore fertility is to transfer the developed sterile allodiploids into tetraploid level. Different organs and tissues can be exposed to colchicine treatment: embryos, apical points, lateral meristems, inflorescences, zygotes, and callus. Polyploidization at early development stages shortens development time of breeding material, and polyploids are characterised by a lower chimericity /Anderson et al., 1990; Basiulienė, Dabkevičienė, 1996; Hussain, Williams, 1997; Stanys, 1997 a; Dabkevičienė, 1999/.

To preserve and multiply the population of interspecific hybrids, that are difficult to produce, is possible by the application of microcloning (microvegetative propagation). Plants can be regenerated from callus or, having skipped callus stage, directly from embryos, apical tips, lateral meristems, and cotyledons /Anderson et al., 1990; Marshall et al.,1995; Stanys, Stanienė, 2001; Вовк и др., 1999/. Microvegatitive propagation of apical points and lateral meristems in combination with colchicine treatment is successfully applied in the production of red clover polyploids /Близнюк и др., 1992/. The objective of the experimental work was to restore the fertility of interspecific hybrids between *T. pratense* x *T. diffusum* and *T. ambiguum* x *T. hybridum* using various *in vitro* methods.

Materials and methods

For interspecific hybridisation two combinations were chosen, in which progamic and postgamic incompatibility is relatively easier to overcome and a sufficient number of interspecific hybrids can be produced: *T. pratense* x *T. diffusum* and *T. ambiguum* x *T. hybridum*. The materials used were: *T. pratense* 2n = 2x = 14, 'Liepsna', which is high-yielding in terms of forage, low in seed production, and susceptible to diseases, *T. diffusum*, 2n = 2x = 16, wild form, which is self-pollinating, low-yielding, disease resistant, annual, *T. ambiguum*, 2n = 2x, 4x, 6x = 16, 32, 48, which is rhizo-

matous, persistent, disease and drought resistant, *T. hybridum*, 2n = 2x = 16, 'Daubiai', which is high-yielding, susceptible to diseases, tolerant of acid soils.

Production of interspecific hybrids. The plants used for interspecific hybridisation were cultivated in pots in a greenhouse, and during flowering in a temperature-controlled chamber at +24 ºC temperature, 16 h photoperiod, a light intensity of 9 thousand lux, 60% relative air humidity. The unopened flowers of female *T. pratense* or *T. ambiguum* plants were emasculated by removing anthers manually with a pair of pincers /Williams, 1954/ and after three days were pollinated with the pollen of male species *T. diffusum* or *T. hybridum.* Seeking to increase the yield of hybrid embryos, one day after emasculation the flowers were sprayed with proline (200 mg l^{-1}) and 2–3 days after pollination were sprayed with giberrelin (75 mg l^{-1}) and boric acid (15 mg l^{-1}) solutions /Dabkevičienė, 2000/. 12–14 days after crossing the embryos, excised in sterile conditions, were transferred to test tubes on nutrient Gamborg B5 medium (B5) with the addition of 0.1 mg l^{-1} kinetin and giberrelin /Linsmaier, Skoog, 1965; Vogt, Shweiger, 1983/ and were cultured for 8 weeks in a cultivation room at +25 ºC temperature, 16 h photoperiod. The developed plants were transplanted to soil in pots and were transferred to a controlled climate chamber or a greenhouse.

Microcloning (microvegetative propagation). Allodiploid embryos isolated in sterile conditions or lateral meristems of adult hybrids were transferred to B5 medium with increased content of 6-benzylaminopurine (BAP) 10 mg l^{-1} /Близнюк и др., 1990/. After 4–6 weeks cultivation at 22–24 ºC temperature, light intensity of 9 thousand lux, 16 h photoperiod, 60% relative air humidity embryogenic tissue, able to regenerate shoots, was formed. The developed shoots were separated and transferred to test tubes with ½ rate of B5 medium with 0.2 mg l^{-1} alpha-naphthalenacetic acid (NAA) for rooting /Dabkevičienė, 2000/. The regenerants that had formed roots were transferred to soil. The remaining embryogenic tissue was planted again to test tubes with B5 + 10 mg l^{-1} BAP medium for further subcultivation and regeneration of hybrid shoots. Four subcultivations were performed. Coefficient of regenerant multiplication i. e. the number of regenerants per one explant that formed embryogenic tissue was determined.

Polyploidy in vitro. Fertility of the hybrids between *T. pratense* L. and *T. diffusum* Ehrh. (2n = 2x = 15) was restored by combining biotechnological methods with polyploidysation (Table 1).

1. Colchicine treatment of embryos. Embryos were cultured for 3–4 days on B5 medium with the addition of 0.1 mg l^{-1} kinetin and giberrelin /Vogt, Shweiger, 1983/, later they were soaked for 4 hours in sterile 0.4% colchicine solution at +28 ºC temperature in the dark. Afterwards the embryos were rinsed three times for ten minutes in sterile water, then were transferred to test tubes on B5 medium and were cultured for 8 weeks in a cultivation room at +25 ºC temperature, a light intensity of 9 thousand lux, 16 h photoperiod, 60% relative air humidity. The plants developed were transplanted to soil in pots and were placed in a temperature-controlled growth chamber or a greenhouse.

2. Colchicine treatment and microcloning of embryos. Embryos were cultured for 3–4 days on B5 nutrient medium then were transferred for 3 days on the same nutrient medium supplemented with 0.1% colchicine (+25 ºC, darkness). The colchicine-treated embryos were transplanted for 4–6 weeks into test tubes on B5 multiplication

medium supplemented with 10 mg l^{-1} BAP. The developed shoots were separated from the embryogenic tissue and were cultivated in root-inducing medium ½ rate of B5 supplemented with 0.2 mg l^{-1} NAA. Four subcultivations were performed.

Table 1. Combinations of methods used for the polyploidisation of interspecific hybrids
1 lentelė. *Metodai, taikyti poliploidizuojant tarprūšinius hibridus*

Treatment *Poveikio kolchicinu būdas*	Crossing combination *Kryžminimo kombinacija*	
	T. pratense x *T. diffusum*	*T. ambiguum* x *T. hybridum*
1. Colchicine treatment of embryos *Gemalų kolchicinavimas*	+	+
2. Colchicine treatment and microcloning of embryos *Gemalų kolchicinavimas ir mikroklonavimas*	+	+
3. Colchicine treatment of meristems *Meristemų kolchicinavimas*	+	–
4. Colchicine treatment and microcloning of meristems *Meristemų kolchicinavimas ir mikroklonavimas*	+	+
5. Microcloning of meristems and colchicine treatment of embryogenic tissue *Meristemų mikroklonavimas ir embriogeninio audinio veikimas kolchicinu*	–	+

3. Colchicine treatment of lateral meristems. Lateral meristems of F_1 hybrids were isolated and sterilised for 3 min in diocide ($C_2H_5OHgCl : C_{21}H_{37}ClN$ = 1:2) solution, then were rinsed 3 times for 10 min in sterile water and placed for 21 days in test tubes with B5 nutrient medium supplemented with 0.2 mg l^{-1} BAP (+25 ºC temperature, 9 thousand lux light intensity). Afterwards the meristems were transferred to B5 nutrient medium with 0.1% colchicine for 3 days (+25 ºC temperature, darkness). After colchicine treatment, seeking to induce rhizogenesis, lateral meristems were transplanted on ½ rate B5 medium (½ of macro and micro salts according to that of standard B5) supplemented with 0.2 mg l^{-1} NAA. After 4–6 weeks the hybrids that had formed leaves were transplanted to soil.

4. Colchicine treatment and microcloning of lateral meristems. Microcloning of 0.1% colchicine-treated lateral meristems of hybrids was performed using citokinin BAP. Meristems treated with colchicine were transferred on B5 nutrient medium, supplemented with 10 mg l^{-1} BAP. After 6–8 weeks of culturing, embryogenic tissue was formed, which could directly regenerate shoots. The shoots were separated and transferred to test tubes with ½ rate of B5 medium supplemented with 0.2 mg l^{-1} l NAA for root formation. Four subcultivations were carried out.

5. Colchicine treatment of embryogenic tissue. Lateral meristems, taken from F_1 hybrids were sterilised for 3 min in diocid solution, were rinsed 3 times for 10 min in sterile water and were planted for 21 days in test tubes containing B5 medium supplemented with 10 mg l^{-1} BAP. The embryogenic tissue formed was cut into 200–300 mg pieces and was placed on B5 medium supplemented with 0.05% colchicine.

After 72 hours the embryogenic tissue was transferred to B5 medium with 10 mg l^{-1} BAP for microcloning.

Assessment of ploidy and identification of hybrids. The ploidy of interspecific hybrids, grown using various methods, was initially verified in flowering plants according to dry pollen shape /Parrot, Smith, 1986; Anderson et al., 1990/. Since chimeras occur among F_1 plants, all inflorescences were checked. Final identification of hybrids was done having counted chromosomes in young leaves /Слесаравичюс, Станене, 1985/ and having conducted analysis of electrophoretic spectra of isoenzymes superoxiddismutase and peroxidase in the leaves of hybrids /Dabkevičienė, Paplauskienė, 2001/. Pollen fertility was identified by staining with acetocarmine dye.

Results

The allodiploids between *T. pratense* and *T. diffusum* (2n = 15) grown by us, like those obtained by other researchers, inherited intermediate quantitative and qualitative traits from the parental forms. According to height, shape of bush, number of stems and inflorescences, allodiploids F_1 excelled *T. diffusum*, but lagged behind red clover. The inflorescences of hybrids were light red in colour (*T. pratense* inflorescences are purple, *T. diffusum* – white). From the wild parental form the allodiploids inherited characteristic stem and leaf hairness and annuality trait. The allodiploids were sterile, completely inviable pollen of irregular shape was found in their hard non-dehiscent anthers.

In the combination *T. pratense* (2n = 14) x *T. diffusum* (2n = 16) interspecific hybrids can be developed without the use of embryo culture. However, the allodiploids grown in this way were rare (5.4% from the number of pollinated flowers). Application of embryoculture enabled us to grow 2.9 times more hybrid clover (Table 2).

Table 2. Efficiency of the use of embryoculture in interspecific crossings
2 lentelė. *Embriokultūros panaudojimo tarprūšinių kryžminimų metu efektyvumas*

Crossing combination *Kryžminimo kombinacija*	Application of embryoculture *Embriokultūros panaudojimas*	Pollinated flowers, number *Apdulkinta žiedų vnt.*	Allodiploids produced *Gauta alodiploidų*	
			number *vnt.*	%
T. pratense x *T. diffusum*	–	1 845	74	5.4
T. pratense x *T. diffusum*	+	1 525	240	15.7
T. ambiguum x *T. hybridum*	+	2 499	241	9.6
LSD_{05} / R_{05}				4.86

In the combination *T. ambiguum* x *T. hybridum* embryo isolation at early development stages is indispensable. In our experiment allodiploid yield amounted to 9.6%. According to adventitious root formation, shape of bush and inflorescences the hybrids were more similar to maternal species *T. ambiguum*. However, according to leaf morphology, they took an intermediate position. When allodiploids started flowering, they were found to be completely sterile. Although the plants flowered, the pollen that

had formed in their anthers was inviable. The hybrids *T. ambiguum* x *T. hybridum* did not set seed when intercrossed or backcrossed.

Seeking to keep and propagate clover interspecific hybrids, that are difficult to develop, immature embryos after colchicine treatment were propagated by the micro-vegetative method (Table 3). In the combination *T. pratense* x *T. diffusum*, out of 50 embryos, planted on B5 nutrient medium supplemented with 10 mg l^{-1} BAP, embryogenic tissue was formed by 66.0% of embryos. After 4 subcultivations 269 allodiploids were obtained, which contained 15 chromosomes in somatic cells and were sterile.

Table 3. Formation of regenerants of interspecific hybrids applying embryo micro-cloning and colchicine treatment
3 lentelė. *Tarprūšinių hibridų regenerantų formavimas, atliekant gemalų mikroklonavimą ir kolchicinavimą*

Treatment *Veikimo kolchicinu būdas*	Embryos, number *Gemalai vnt.*	Survival *Išgyveno*		Embryos, forming embryogenic tissue *Gemalai, formuojantys embriogeninį audinį*		Regenerants produced *Gauta regenerantų*	Propagation coefficient *Pasidauginimo koeficientas*
		number *vnt.*	%	number *vnt.*	%	number *vnt.*	
T. pratense x *T. diffusum*							
Embryo microcloning (control) *Gemalų mikroklonavimas (kontrolinis variantas)*	50	41	82.0	33	66.0	269	8.2
Embryo microcloning and colchicine treatment (0.1%) *Gemalų mikroklonavimas ir kolchicinavimas (0,1 %)*	122	43	35.2	15	12.3	18	1.2
T. ambiguum x *T. hybridum*							
Embryo microcloning (control) *Gemalų mikroklonavimas (kontrolinis variantas)*	93	38	40.9	17	18.3	71	4.2
Embryo microcloning and colchicine treatment (0.1%) *Gemalų mikroklonavimas ir kolchicinavimas (0,1 %)*	44	13	29.5	6	13.6	0	0
LSD_{05} / R_{05}			6.36		5.42		

The propagation coefficient (number of regenerants per explant that formed embryogenic tissue) was on average 8.2, for different genotypes it varied from 0.0 to 74.0. Having treated embryos with 0.1% of colchicine the number of embryos able to form embryogenic tissue and shoots declined by on average 5.4 times. The propagation coefficient was on average 1.2, and for different genotypes it varied from 0.0 to 44.0.

In the combination *T. ambiguum* x *T. hybridum* this combination of methods was less effective.

In the control treatment the embryos forming embryogenic tissue amounted to only 18.3%, and the embryogenic tissue regenerated shoots only at ½ rate almost twice as weakly. Multiplicitation coefficient reached on average 4.2, and for different genotypes varied from 0.0 to 9.0. Having treated the embryos with 0.1% colchicine, only 6 (13.6%) formed embryogenic tissue, which, unfortunately, did not form any regenerant.

The efficiency of polyploidization methods was determined by assessment of survival of explants after colchicine treatment, yield of fertile allotetraploids and formation of chimeras (Table 4). All efforts to restore fertility of *T. ambiguum* x *T. hybridum* hybrids did not give any positive results, whereas the fertility of hybrids between *T. pratense* and *T. diffusum* was successfully restored using different methods: embryo and meristem culture, colchicine treatment and microcloning.

Table 4. Comparison of the efficacy of different fertility restoration methods
4 lentelė. *Įvairių fertilumo atkūrimo būdų efektyvumo palyginimas*

Polyploidisation methods *Poliploidizavimo būdas*	Treated explants, number *Paveikta eksplantų vnt.*	Propagation coefficient *Pasidaugininimo koeficientas*	Survival after colchicine treatment % *Išgyveno po poveikio kolchicinu %*	Hybrid regenerants obtained, number *Gauta hibridų regenerantų vnt.*		Fertile allotetraploids % *Fertilių alotetraploidų išeiga %*	Sterile allodiploids % *Sterilių alodiploidų išeiga %*	Chimeras % *Chimerų išeiga %*
				In test tube *Mėgintuvėlyje*	In soil *Dirvoje*			
1	2	3	4	5	6	7	8	9
			T. pratense x *T. diffusum*					
Colchicine treatment of embryos *Gemalų kolchicinavimas*	119	–	23.5	28	22	59.1	22.7	18.2
Colchicine treatment and microcloning of embryos *Gemalų kolchicinavimas ir mikroklonavimas*	122	1.2	35.2	43	18	61.1	38.9	0

Table 4 continued
4 lentelės tęsinys

1	2	3	4	5	6	7	8	9
Colchicine treatment of meristems *Meristemų kolchicinavimas*	189	–	76.7	145	11	55.0	18.2	27.3
Colchicine treatment and microcloning of meristems *Meristemų kolchicinavimas ir mikroklonavimas*	111	1.4	72.0	148	76	48.7	48.7	2.6
			T. ambiguum x *T. hybridum*					
Colchicine treatment of embryos *Gemalų kolchicinavimas*	44	–	29.5	0	0	0	0	0
Colchicine treatment and microcloning of meristems *Meristemų kolchicinavimas ir mikroklonavimas*	44	1.3	27.3	12	8	0	100	0
Colchicine treatment and microcloning of embryogenic tissue *Embriogeninio audinio kolchicinavimas ir mikroklonavimas*	30	1.3	90.0	47	34	0	100	0
LSD_{05} / R_{05}			4.3			4.61	4.20	2.85

Embryos were found to be more sensitive to colchicine treatment. Averaged data suggest that 3.2 times more lateral meristems survived colchicine treatment compared with embryos. Especially sensitive to colchicine were the embryos of *T. ambiquum* x *T. hybridum*, all of which lost viability after treatment and ceased developing.

The application of microcloning enabled us to grow on average 1.6 times more regenerants. Propagation coefficient largely depended on the response of explants to colchicine treatment and in all treatments (when propagating both colchicine treated embryos and meristems) had a similar value 1.2–1.4.

The largest number of regenerants (76) was obtained in the treatment where colchicine treated *T. pratense* x *T. diffusum* meristems had been cloned. They were found to exhibit the least response to toxic effect of colchicine. As many as 53

meristems (47.7% from the total number of meristems treated and 66.3% from the number of meristems that survived after colchicine treatment) remained viable and did not lose the ability to form embryogenic tissue and regenerate shoots.

Expecting to transfer *T. ambiguum* x *T. hybridum* hybrids into tetraploid level and in this way restore their fertility, we tried another method of polyploidisation – 0.05% colchicine treatment and subsequent microcloning of embryogenic tissue obtained from allodiploid meristems. Out of the 30 explants treated, 27 formed shoots (90.0%). In total, 34 regenerants were grown (propagation coefficient 1.3). Like in previous treatment, polyploidisation did no give the expected result, the hybrids remained sterile allodiploids (2n = 16–32).

According to the yield of fertile allotetraploids, all fertility restoration methods in *T. pratense* x *T. diffusum* crossings were more or less equivalent, the yield varied from 48.7% to 61.1%. In total, 67 fertile allotetraploids (2n = 30) were grown. Only inappreciably more efficient (on average 8.5%) was embryo polyploidisation. It is likely that at colchicine treatment of explants at an earlier development stage the cells are more susceptible to polyploidogenic effect. In terms of fertile allotetraploids yield, polyploidisation of lateral meristems was less effective; however, this method is irreplaceable if it is necessary to transfer sterile adult hybrids into tetraploid level.

Another indicator of polyploidisation efficiency is the frequency of chimeric individuals formation. In our experiments chimeric individuals were not detected only in the treatments involving microcloning of colchicine-treated embryos. In the treatments involving microcloning of colchicine-treated meristems, the content of chimeras was small 2.6%. In other treatments the yield of chimeras was found to be 18.2% and 26.8%. In these cases regenerants are formed from the cell group, heterogeneous in terms of ploidy. Thus, having applied microcloning, chimeras were either not obtained at all or their number was 8 times lower than in the treatments not involving explant microcloning. Consequently, we can maintain that microcloning significantly reduces the chances of chimeric individuals formation. It is likely that under the effect of cloning the new plant starts forming from one cell, varied in terms of ploidy.

F_1 allotetraploids (2n = 30), developed using polyploidisation, formed full-fledged inflorescences. Averaged data suggest that the fertility of their pollen was as high as 93.2%, seed set by self-pollination amounted to 2.1% (0.0–12.2%) and when intercrossed 8.0% (0.4–20.5%). Although allogamy, inherited from *T. pratense,* was more markedly expressed than autogamy, inherited from *T. diffusum*, part of F_1 allodiploids inherited a clearly expressed self-pollination trait. For individual hybrids self-pollination rate was 5.4–12.2% and significantly surpassed the female form *T. pratense,* which is strictly cross-pollinating. The developed hybrids inherited the annuality trait, specific to the plants of male species *T. diffusum*. As a result, F_1 hybrids were later backcroseed with *T. pratense* (2n = 4x = 28), seeking to combine self-pollination and perenniality traits in one genome. Further success of the work was determined by the fact that part of the individuals of backcross lines did not possess annuallity specific to *T. diffusum* and inherited the self-pollination (1.5–2.0%) trait. The number of self-pollinating flowers for individual plants ranged from 0.0 to 69.4%, therefore we think

that identification of hybrids, characterised by self-pollination, will enable us to develop a qualitatively new hybrid population.

Discussion

Seeking to develop fertile *T. pratense* x *T. diffusum* hybrids many researchers polyploidised parental forms and crossed them. This tetraploid development method is insufficient. Many chimeric individuals develop from colchicine-treated seed or seedlings. A lot of time is required to select 'pure' allotetraploids from heterogenous population that could be suitable for interspecific crossings /Taylor et al., 1963; Рубцов, Комкова, 1983; Taylor, Gillet, 1988/.

Fertility can be restored by crossing diploid plants and by polyploidisation of the resulting sterile hybrids. Firsova with co-workers /Фирсова и др., 1980/ has reported about the attempts to treat apical points of the hybrids between *T. ambiguum* and *T. hybridum* with colchicine solution (dripping method); however, the attempts were unsuccessful. Anderson et al. (1990) polyploidised lateral meristems of allodiploids *T. ambiguum* x *T. repens* by culturing them on the medium supplemented with 0.1% colchicine. As a result, as much as 44% of shoot cells had a doubled number of chromosomes. Fertility of the hybrids between *T. repens* and *T. nigrescens* was restored in a similar way. Pollen fertility in the hexaploids produced by colchicine treatment of lateral meristems increased form 9.9% to 89.2% /Hussain et al., 1997/.

Another method used for the production of fertile interspecific hybrids, described in literature, is when unreduced gametes take part in fertilisation. No fertility restoration procedures are required in such cases, since chromosome balance is restored in hybrids immediately. While backcrossing F_1 (*T. pratense* x *T. diffusum*) with *T. pratense*, after fusion of diploid gametes we were able to obtain a few progeny, which were partly fertile /Schwer, Cleveland, 1972/. In this way, in recent years using backcross, fertile backcross lines in the combinations *T. repens* x *T. ambiguum* /Meredith et al., 1995/ and *T. repens* x *T. nigrescens* /Hussain, Williams, 1997 b; Abberton et al., 2003/ were developed.

In our laboratory we found out that it is expedient to carry out polyploidisation at young development stages – zygote, embryo, young inflorescence. Excellent results were obtained while developing ryegrass (*Lolium perenne* L.) polyploids by in vitro methods and while restoring the fertility of F_1 hybrids between ryegrass and fescue (*Lolium perenne* L. x *Festuca arundinacea* L.). Having treated embryos or young inflorescences with 0.3% colchicine, tetraploid yield amounted to 65.8–78.1% /Pašakinskienė, 2000/. This method was more than six times more effective than conventional colchicine treatment of emerged seed. Having treated *T. pratense* zygotes with nitrous suboxide gas and germinated applied embryoculture, the yield of autotetraploids reached 45.7%, and no chimeras were formed. The population obtained from colchicine treated *T. pratense* embryos contained 53.8% of individuals, which were 'pure' autotetraploids, and the number of chimeric clover was 2.6 times lower than in the populations, obtained by conventional colchicine treatment of germinated seeds /Basiulienė, Dabkevičienė, 1996; Dabkevičienė, 1999/. Polyploidisation of zygotes was also successfully used for fertility restoration of *T. pratense* x *T. diffusum* hybrids. Having treated allodiploid zygotes during the first division stage with nitrogen suboxide gas, the number of

chromosomes was doubled efficiently. As a result, 61.0% of survived individuals were fertile allotetraploids /Basiulienė, 1996/.

Belarussian researchers achieved good results in clover polyploidy by combining colchicine treatment with microcloning of lateral meristems applying BAP. Among the regenerants produced, autotetraploid percentage was 80.0–90.0%, and chimeras were not formed at all /Близнюк и др., 1994/. Microcloning using BAP allows to speed up the process, since regenerants are formed directly from embryoidogenic formations, skipping the calus stage /Близнюк, 1994; Чжун и др., 2001/. Regenerants developed in this way are genetically stable /Stanys, 1997/.

In our experiments, seeking to optimise the development process of fertile interspecific clover hybrids, we combined different methods: embryo and meristem culture, colchicine treatment, and microcloning. The use of microcloning while polyploidising *T. pratense* x *T. diffusum* hybrids reduced the chances of chimeric individuals formation. Having applied microcloning of colchicine-treated embryos or meristems, chimeras were either not obtained at all, or their number was 8.8 times lower than in the treatments without involving microcloning. It is likely, that during microcloning a chance increases for the regenerants to be formed from one cell. This is a great advantage of the use of microcloning.

Thus, in the crossing combination *T. pratense* x *T. diffusum* all methods gave positive results: the yield of allelotetraploids (2n = 4x = 30) reached 48.7–61.1%. However, all attempts to develop fertile allotetraploids in the combination *T. ambiguum* x *T. hybridum* were without success, they remained sterile allodiploids.

Conclusions

1. It was found expedient to use embryo culture for the production of interspecific hybrids (*T. pratense* x *T. diffusum* and *T. ambiguum* x *T. hybridum*). The yield of F_1 hybrids reached 15.7 and 9.6%, respectively.

2. Microcloning allowed us to keep and propagate the genotypes of interspecific hybrids: propagation coefficient was 4.2–8.2. A combination of microcloning with colchicine treatment resulted in the production of on average 1.6 times more of interspecific hybrid regenerants.

3. Fertility of interspecific hybrids *T. pratense* x *T. diffusum* was restored by a combination of different *in vitro* methods (embryo and meristem culture and microcloning) with colchicine treatment. However, all our attempts to restore fertility of *T. ambiguum* x *T. hybridum* allodiploids did not give any positive results.

4. According to the yield of fertile allotetraploids, all fertility restoration methods were more or less equivalent (48.7–61.1%). A slightly more efficient (on average by 8.3%) was polyploidisation of embryos.

5. Microcloning combined with polyploidisation reduced the chances of chimeric individuals formation by 8.8 times: when colchicine-treated embryos and meristems were microcloned, the yield of chimeras amounted to 0.0–2.6%, while in the treatments without microcloning the yield of chimeras was 18.2–26.8%.

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IN VITRO METODAI TAIKANT DOBILŲ ALOPOLIPLOIDIJĄ

G. Dabkevičienė

Santrauka

Dobilų selekcijoje, be vegetatyvinės masės derliaus padidinimo, svarbu sukurti ir atsparesnes ligoms veisles. Tam gali būti panaudotos laukinių dobilų rūšys. Sukurtų tarprūšinių hibridų fertilumui atkurti buvo taikyta poliploidija, ją derinant su *in vitro* metodais: gemalų bei meristemų kultūra ir mikroklonavimu.

Nustatyta, kad tarprūšiniams hibridams (*T. pratense* x *T. diffusum* ir *T. ambiguum* x *T. hybridum*) gauti tikslinga naudoti gemalų kultūrą. F_1 išeiga siekia atitinkamai 15,7 ir 9,6 %.

Tarprūšinių hibridų *T. pratense* x *T. diffusum* fertilumas atkurtas, suderinus įvairius *in vitro* metodus (gemalų bei meristemų kultūrą ir mikroklonavimą) ir kolchicinavimą. Fertilių alotetraploidų išeiga buvo 48,7–61,1 %. Nežymiai (vidutiniškai 8,3 %) efektyvesnis buvo gemalų poliploidizavimas, o visi bandymai atkurti *T. ambiguum* x *T. hybridum* alodiploidų fertilumą nedavė teigiamų rezultatų.

Mikroklonavimas sudaro galimybę išsaugoti ir padauginti tarprūšinių hibridų genotipus: padauginimo koeficientas siekia 4,2–8,2. Sujungus mikroklonavimą ir kolchicinavimą, vidutiniais duomenimis, pavyko išauginti 1,6 karto daugiau tarprūšinių hibridų regenerantų, be to, 8,8 karto sumažėjo chimerinių individų susidarymas.

Reikšminiai žodžiai: *Trifolium* spp., tarprūšiniai hibridai, gemalų ir meristemų kultūra, mikroklonavimas, kolchicinavimas.