



Autopolyploids in fodder grass breeding: induction and field performance

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Abstract

Doubling of chromosome set directly affects plant performance through increase of organ size, higher feeding value and increased resistance to adverse environmental factors. Therefore efficient methods of polyploid induction are needed in order to develop new varieties of naturally diploid fodder grass species. The efficiency of antimetabolic agents as colchicine, amiprofos-methyl, trifluralin and oryzalin was compared in a series of tetraploid induction experiments in *Lolium multiflorum*, *L. perenne* and *Festuca pratensis*, while newly developed tetraploid plants were compared to standard tetraploid varieties in the field trials. Colchicine treatment proved to be the most efficient method for *in vitro* cultured embryos in comparison with the other agents. Induced tetraploids of *F. pratensis* produced higher dry matter and seed yield and could be used for the development of new varieties. Induced tetraploid plants of *Lolium* spp. were equal to the standard varieties in field trials, therefore they could be used as parental genotypes in crosses. Induced tetraploids of *F. pratensis* produced higher dry matter and seed yield and could be used for development of new variety.

Additional keywords: embryo culture; meadow fescue; mitotic inhibitors; polyploidization; ryegrass.

Abbreviations used: APM (amiprofos-methyl).

Authors' contributions: Conceived and designed the experiments, and analysed the data: GD and VK. Performed the experiments and wrote the paper: GD, VK and GS. Coordinated the research project: NL and GB.

Citation: Dabkevičienė, G.; Kemešytė, V.; Statkevičiūtė, G.; Lemežienė, N.; Brazauskas, G. (2017). Autopolyploids in fodder grass breeding: induction and field performance. Spanish Journal of Agricultural Research, Volume 15, Issue 4, e0706. <https://doi.org/10.5424/sjar/2017154-11357>.

Received: 08 Mar 2017 **Accepted:** 15 Dec 2017.

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Funding: Research Council of Lithuania (grant No. MIP-064/2015, ADAPTGENAS).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Polyploidy is defined as presence of three or more complete chromosome sets per cell nucleus. It is a common phenomenon in plant kingdom and is thought to be major driving force behind plant evolution (Soltis *et al.*, 2009). Ancient events of polyploidization led to emergence of many cultural plant species. Polyploidization often seems to be associated with increased plant vigour, higher adaptability to environmental conditions, increased cell size and, consequently, increased organs although the latter one is not always the case as the number of cell division can be reduced. Other benefits of being polyploid include better salinity, drought or extreme temperature stress tolerance (reviewed in Sattler *et al.*, 2016). Gene duplication in autopolyploids can cause transcriptomic changes leading to higher adaptability compared to diploids (del Pozo & Ramirez-Parra, 2015), therefore if genotype undergoing whole genome duplication possesses allelic variants of

genes conferring increased resistance to freezing temperatures (Aleliūnas *et al.*, 2015) or genes involved in regulation of plant architecture traits (Statkevičiūtė *et al.*, 2015), highly beneficial effect in stress response and growth habit of induced tetraploid can be expected. All these characteristics are highly desirable in cultivated crop varieties, with the exception of crops cultivated for seeds as polyploids tend to have lower seed yield compared to their diploid counterparts. Therefore breeders have been searching for most efficient methods of artificial autopolyploid induction in economically important yet naturally diploid plant species. Polyploids can be induced by doubling of the chromosome number in somatic tissues (mitotic polyploidization) or by generating unreduced gametes (meiotic polyploidization) (Younis *et al.*, 2014). First attempts at inducing agricultural polyploids were reported nearly eight decades ago (Blakeslee & Avery, 1937). Colchicine was used in pioneering experiments, and it remained as a very popular antimetabolic agent up to this day. Various tech-

niques were developed to induce mitotic chromosome doubling in different plant species, including treatment of shoots, leaves, buds, node sections, embryos and cell suspension cultures (Dhooghe *et al.*, 2011). Colchicine was proven to be very useful in most of these methods as well as in many plant species, however many researchers postulated the need for new antimitotic drugs as colchicine application is both hazardous due to its' toxicity, and costly, because high concentrations of colchicine are needed. Attention was turned towards antimicrotubular herbicides due to their ability to disrupt cell mitotic division. Herbicides oryzalin, trifluralin and amiprofosmethyl (APM) were proven to be just as effective or even more so – and in much lower concentrations – compared to colchicine (Yemets & Blume, 2008). Despite tremendous progress has been made since first attempts, methods of autopolyploidy induction in vitro are still being modified as the whole procedure has to be adapted for each plants species individually, and even after elaboration of efficient technique high variability of polyploid yield remains due to differences in genotype response.

Ryegrass is one of most important grass for forage and lawn in the world. Two subspecies of *Lolium multiflorum* Lam.: *italicum* (Italian ryegrass) and *multiflorum* (Westerwolths ryegrass) are used for fodder and erosion control. Perennial ryegrass (*Lolium perenne* L.) is sown for both fodder and lawn purposes (Humphreys *et al.*, 2010). Meadow fescue (*Festuca pratensis* Huds.) is another important forage species in Northern countries, widely used in the mixtures (Fjellheim *et al.*, 2007). Ryegrass species and meadow fescue are often used for development of hybrid festulolium. This hybrid is characterized by better stress tolerance (cold and drought) than ryegrass and improved fodder value compared to the fescue (Humphreys *et al.*, 2004). Both ryegrass species and meadow fescue exist as diploid ($2n = 2x = 14$) in nature. Therefore mitotic polyploidization is used not only for development of higher yielding fodder cultivars but also for restoration of fertility in interspecific hybrids.

The aim of this study was to compare effectiveness of different in vitro mitotic polyploid induction methods in *Lolium perenne*, *Lolium multiflorum* and *Festuca*

pratensis, and evaluate field performance of newly created tetraploids.

Material and methods

Research was carried out at the Institute of Agriculture, Lithuanian Research centre for Agriculture and Forestry.

Plant material

The germplasm consisted of 16 ryegrass and 7 fescue diploid genotypes (Table 1). All of the breeding lines used in the experiment were developed locally.

Induction of autotetraploids

Three treatment methods and four antimitotic agents were used in the polyploidization experiments: colchicine (10 mM), oryzalin (50 μ M), trifluralin (50 μ M) and APM (100 μ M). All solutions were prepared in sterile dd H₂O.

Embryos of Westerwolths, Italian and perennial ryegrass and meadow fescue were excised from mature seeds following surface sterilization with 50% sulfuric acid for 15 min, 70% ethanol for 2 min. and 50% commercial bleach containing up to 5 % of sodium hypochlorite for 7 min. Thereafter embryos were cultivated in 10 mL test tubes on Murashige-Skoog basal medium (Sigma, 2/3 of salts) containing 0.2 mg of indoleacetic acid (IAA), 0.25 mg kinetin, 1 mg thiamin, 0.1 g pyridoxine, 30 g sucrose and 7 g agar per liter of medium. Germinated embryos (8-14 mm long) were treated with antimitotic agents for 4 hours, then rinsed with sterile distilled water and placed on fresh medium. The experiment was carried out in three replicates, 734 germinated embryos of Westerwolths ryegrass, 420 embryos of Italian ryegrass, 525 embryos of perennial ryegrass and 730 embryos of meadow fescue were treated with each antimitotic agent in total, 100-105 per each genotype. Embryos were kept in test tubes (24°C, 16 h light) for 45-60 days, then transferred to peat substrate. Chromosomes were counted in root

Table 1. Plant material used in the polyploid induction and the field experiment.

| Species, subspecies | Type | Name |
|---|---------------|--|
| Westerwolths ryegrass (<i>Lolium multiflorum</i> subsp. <i>multiflorum</i>) | cultivar | Varpė, Vitesse, Gulf, Shoot, Lirasand, Druva, Weldra |
| Italian ryegrass (<i>L. multiflorum</i> subsp. <i>italicum</i>) | cultivar | Aber Mario, Aber Epic, Fastyl, Montfort |
| Perennial ryegrass (<i>L. perenne</i>) | breeding line | LZI-3548, LZI-3532, LZI-3722, LZI-3569, LZI-3534 |
| Meadow fescue (<i>Festuca pratensis</i>) | cultivar | Sigita, Kaita DS |
| | breeding line | LZI-5048, LZI-4998, LZI-4832, LZI-5234, LZI-5241 |

tip meristem cells, aceto-carmin solution (3%) was used as staining agent. Five or more root tips were sampled from each plant, chromosomes were counted in at least 5 clear metaphase plates in each root tip. The plants with less than five diploid metaphase plates in total were classified as tetraploid.

Field experiment

Tetraploid genotypes of studied ryegrass and meadow fescue genotypes (Table 1) were set up in the trial nurseries (separate for herbage and for seed yield evaluations) in Dotnuva (55°23'N, 23°57'E) during 2012, 2013 and 2014 for Westerwolths ryegrass and during 2013, 2014 and 2015 for meadow fescue, Italian and perennial ryegrass. Eighteen plants were planted per plot (9 plants in each of 2 rows), at a distance of 50 × 50 cm, using a randomized design with 3 replications. Standard variety of each species was included: Westerwolths ryegrass, Elunaria (Germany); Italian ryegrass, Ugnė (Lithuania); perennial ryegrass, Sodrė (Lithuania); and meadow fescue, Raskila (Lithuania).

The soil of the experimental fields was Endocalcari – Epihypogleyic Cambisols (CMg-p-w-can), characterised by a homogeneous texture, pH_{KCl} 7.3-7.0, humus content 1.9-2.2%, available P₂O₅ 206–270 mg/kg and K₂O 101-154 mg/kg. In the autumn of each year of use phosphorus and potassium fertilisers (P₆₀ K₉₀) were applied. Nitrogenous fertilisers (N₁₅₀) were applied each year of herbage use in several applications: in spring N₆₀ and N₄₅ after cuts.

Winter survival (WS, %), heading date (HD), plant height (PH, cm), flag leaf length (FLL, cm), flag leaf width (FLW, cm), inflorescence length (IL, cm), dry matter yield (DMY, g/plant), seed yield (SY, g/plant) and re-growth after cuts (RGR, score) were assessed.

Statistical analyses

Statistical reliability of research data was assessed with the lowest significant difference (*LSD*₀₅, *LSD*₀₁).

Computer software STATISTICA 7 (StatSoft Inc., USA) was used for the statistical analysis.

Results

Post-treatment survival and tetraploid induction efficiency of different mitotic inhibitors

In this experiment, concentration of oryzalin used proved to be the most toxic, the lethal effect of both colchicine and APM treatment was similar while trifluralin treatment was the least toxic (Table 2). The response of different species was also diverse with Westerwolths ryegrass being the most recalcitrant for polyploidy induction. The proportions of induced tetraploid plants after treatment with different mitotic inhibitors in mature embryo culture was analysed in a separate experiment. Again, oryzalin was the most efficient in tetraploid induction (Table 3). However extremely low survival of plants regardless of species indicates that application of this mitotic inhibitor needs adjustments. Trifluralin application induced lowest mean proportion of tetraploid plants, only treatment of perennial ryegrass embryos yielded high induced tetraploid proportion (81.3%) when this antimicrotubular chemical was used (Table 3). The mean efficiency of colchicine was the highest (58.2%), it proved to be the most suitable agent for polyploidization of both ryegrasses and meadow fescue if we take into account the surviving plants. The variation in the tetraploid induction efficiency (cv%) between the treated populations in each species ranged from 17.6 to 91.3 (Table 4). Again the mean variation of colchicine efficiency (21.6-53.3%) was lower compared to other antimitotic agents.

Field experiment

Seeds of *Lolium* spp and *F. pratensis* tetraploid plants were collected and sown in the field trials. The average results of each species and the results of the best

Table 2. Survival of *Lolium multiflorum*, *L. perenne* and *Festuca pratensis* plants (%) after treatment with mitotic inhibitors.

| Species | Colchicine | APM | Trifluralin | Oryzalin |
|---|------------|------|-------------|----------|
| <i>L. multiflorum</i> subsp. <i>multiflorum</i> n = 734 | 51.3 | 54.8 | 78.9 | 22.3 |
| <i>L. multiflorum</i> subsp. <i>italicum</i> n = 420 | 28.6 | 41.3 | 93.9 | n.d. |
| <i>L. perenne</i> n = 525 | 33.0 | 27.8 | 60.3 | 1.7 |
| <i>F. pratensis</i> n = 730 | 38.6 | 21.8 | 66.0 | 0.6 |
| Mean | 36.7 | 36.4 | 72.3 | 8.2 |
| LSD ₀₅ | 5.95 | 5.19 | 5.38 | 2.48 |

APM: amiprofos-methyl; LSD: least significant difference, *p* < 0.05; n.d.: no data available.

Table 3. Tetraploid induction rate (%) after treatment with different mitotic inhibitors in culture of mature embryos of *Lolium multiflorum*, *L. perenne* and *Festuca pratensis*.

| Species | Colchicine | APM | Trifluralin | Oryzalin |
|---|-------------|-------------|-------------|-------------|
| <i>L. multiflorum</i> subsp. <i>multiflorum</i> n = 734 | 70.1 (36.2) | 58.1 (30.6) | 17.0 (13.5) | 67.8 (15.1) |
| <i>L. multiflorum</i> subsp. <i>italicum</i> n = 420 | 54.2 (15.5) | 39.5 (16.3) | 1.0 (0.8) | n.d. |
| <i>L. perenne</i> n = 525 | 78.3 (25.9) | 76.3 (21.2) | 81.3 (49.0) | 100 (1.7) |
| <i>F. pratensis</i> n = 730 | 35.9 (15.2) | 19.7 (4.8) | 15.2 (9.0) | 100 (0.5) |
| Mean | 58.2 (21.9) | 48.4 (18.2) | 22.9 (14.5) | 89.2 (5.8) |
| LSD ₀₅ | 5.89 (5.08) | 6.35 (5.25) | 3.68 (3.78) | 4.37 (3.62) |

Tetraploid plant proportion calculated from the number of surviving plants, proportion calculated from the number of plants in pre-treatment group shown in parenthesis; APM: amiprofos-methyl; LSD: least significant difference, $p < 0.05$; n.d.: no data available.

Table 4. Tetraploid induction variation in *Lolium multiflorum* subsp. *multiflorum* (7 cultivars), *L. multiflorum* subsp. *italicum* (4 cultivars), *L. perenne* (5 breeding lines) and *Festuca pratensis* (2 cultivars, 5 breeding lines) after treatment with different mitotic inhibitors in culture of mature embryos.

| Species | Colchicine | | APM | | Trifluralin | | Oryzalin | |
|---|------------|------|-----------|------|-------------|------|-----------|------|
| | Min-Max | cv% | Min-Max | cv% | Min-Max | cv% | Min-Max | cv% |
| <i>L. multiflorum</i> subsp. <i>multiflorum</i> | 55.9-100 | 21.6 | 31.3-66.7 | 26.7 | 0-33.3 | 88.4 | 44.0-80.0 | 25.1 |
| <i>L. multiflorum</i> subsp. <i>italicum</i> | 35.7-80.0 | 32.3 | 3.3-50.0 | 17.6 | 0-3.4 | 90.3 | n.d. | n.d. |
| <i>L. perenne</i> | 51.1-95.5 | 21.0 | 50.0-100 | 23.7 | 51.1-95.5 | 22.3 | 0-100 | 91.3 |
| <i>F. pratensis</i> | 9.1-85.7 | 53.3 | 0-42.9 | 69.1 | 0-46.7 | 89.5 | n.d. | n.d. |

Calculations based on tetraploid proportion (%) in the group of surviving plants; APM: amiprofos-methyl; cv%: coefficient of variation; n.d.: no data available.

tetraploid genotype were compared with the standard tetraploid variety. The percent of survival after winters of Italian and perennial ryegrass were significantly ($p < 0.05$) lower for all tetraploids compared with the standard varieties (Table 5). Winter damage of meadow fescue was very low regardless ploidy level of the plant. Significantly highest seed yield per plant was assessed for *L. perenne* (6.45 g, $p < 0.05$) and meadow fescue (28.58 g, $p < 0.01$) compared with the standard (5.53 and 17.27 g respectively) (Table 6).

Average data of dry matter yield and other agrobiological traits of meadow fescue tetraploids showed the most statistically significant differences compared with standard, especially for plant height (Table 5 and Table 6). The results in other species were similar to standards varieties.

Discussion

The success of autopolyploid induction in any plant species depends not only on the choice of mitotic inhibitor and application method, but also on responsiveness of particular genotype. High coefficients of variation were obtained in each grass species in this experiment, affirming that any method might yield rather different results when applied to distinct populations/cultivars.

There is no consensus on the best method for tetraploid induction in fodder grasses. The popular method for ryegrass polyploidization is colchicine treatment of germinated seeds or young plants, with surviving success rate up to 40% of induced tetraploids plants, depending on colchicine concentration and treatment duration (Joshi & Verma, 2004; Nair, 2004; Pereira *et al.*, 2014). In some studies treatment of dry seeds rather than germinated proved to be more efficient, where tetraploid induction rate in surviving perennial ryegrass plants was 64% (Dapkienė *et al.*, 1999), yet the survival rate itself was low making this method applicable only when initial material is ample. Successful polyploidization results were obtained when germinated seeds of *Dactylis polygama* were treated with colchicine, while treatment of dry seeds was completely inefficient (Dabkevičienė *et al.*, 2013). The data on application of in vitro embryo culture for plant polyploidy induction is scarce. Embryo culture was tested in cotton, however the authors found seed treatment to be more reliable due to rather complicated procedure of embryo in vitro cultivation in cotton (Omran & Mohammad, 2008). Treatment of germinated embryos with 7.5 mM colchicine in combination with low cultivation temperature and increased sucrose concentration in germination medium was the most efficient tetraploid induction method in perennial ryegrass (Pašakinskienė, 2000).

Table 5. Dry matter yield (DMY) and yield-related traits (WS: winter survival; PH: plant height; RGR: re-growth after cuts) of tetraploid genotypes of *Lolium multiflorum*, *L. perenne* and *Festuca pratensis*.

| Species | DMY, g/plant | WS, % | PH, cm | RGR, score |
|---|--------------|--------|----------|------------|
| <i>L. multiflorum</i> subsp. <i>multiflorum</i> | | | | |
| Standard | 22.10 | n.d. | 70.67 | 7.00 |
| Mean | 17.97 | n.d. | 64.24 | 5.89 |
| Druva [#] | 18.30 | n.d. | 76.40 | 7.00 |
| <i>L. multiflorum</i> subsp. <i>italicum</i> | | | | |
| Standard | 547.13 | 68.33 | 75.00 | 8.33 |
| Mean | 258.80 | 51.78* | 85.50 | 5.00 |
| Fastyl [#] | 298.47 | 55.56* | 88.50 | 7.00 |
| <i>L. perenne</i> | | | | |
| Standard | 436.67 | 66.11 | 63.00 | 7.50 |
| Mean | 416.75 | 44.78* | 53.50 | 8.13 |
| LZI-3722 [#] | 419.50 | 42.86* | 55.50 | 8.50 |
| <i>F. pratensis</i> | | | | |
| Standard | 139.67 | 91.67 | 55.50 | 8.83 |
| Mean | 140.13 | 99.78 | 91.92** | 7.33 |
| LZI-4832 [#] | 175.90* | 100.00 | 107.33** | 8.00 |

*: $p < 0.05$; **: $p < 0.01$ significant differences compared with standard variety; # : best performing genotype of the tetraploid group; n.d.: no data available. n=18 plants per replicate, 54 plants per each genotype in total.

Table 6. Seed yield per plant (SY) and seed yield-related traits (HD: heading date; FLL: flag leaf length; FLW: flag leaf width; IL: inflorescence length) of tetraploid genotypes of *Lolium multiflorum*, *L. perenne* and *Festuca pratensis*.

| Species | SY, g | HD | FLL, cm | FLW, cm | IL, cm |
|---|---------|------|---------|---------|--------|
| <i>L. multiflorum</i> subsp. <i>multiflorum</i> | | | | | |
| Standard | 2.27 | 191 | 19.20 | 0.83 | 26.83 |
| Mean | 1.92 | 190 | 16.75 | 0.85 | 22.85 |
| Vitesse [#] | 2.50 | 190 | 31.00** | 1.10* | 28.50 |
| <i>L. multiflorum</i> subsp. <i>italicum</i> | | | | | |
| Standard | 21.53 | 145 | 22.07 | 0.90 | 30.73 |
| Mean | 21.67 | 145 | 23.23 | 0.93 | 33.87 |
| Fastyl [#] | 27.00* | 145 | 29.10 | 0.90 | 36.20 |
| <i>L. perenne</i> | | | | | |
| Standard | 5.53 | 143 | 18.40 | 0.66 | 21.67 |
| Mean | 6.45* | 150* | 15.67 | 0.61 | 20.43 |
| LZI-3532 [#] | 10.33** | 150* | 19.03 | 0.70 | 23.00 |
| <i>F. pratensis</i> | | | | | |
| Standard | 17.27 | 139 | 10.89 | 0.75 | 30.40 |
| Mean | 28.58** | 142* | 13.27 | 0.92* | 31.87 |
| LZI-4832 [#] | 35.43** | 140 | 11.90 | 0.80 | 34.83 |

*: $p < 0.05$; **: $p < 0.01$ significant differences compared with standard variety; # : best performing genotype of the tetraploid group. n=18 plants per replicate, 54 plants per each genotype in total.

Since 1990s when the attention was turned towards alternatives to colchicine, dinitroanilines (oryzalin and trifluralin) and phosphoroamidates (APM) have been tested in various plant species. Some researchers claim trifluralin and oryzalin are less lethal to treated plants than colchicine (Hansen & Andersen, 1996; Cheng *et al.*, 2012). Oryzalin was the most effective antimetabolic agent in cork oak double haploid production (Pintos & Manzanera, 2007) and fertility restoration of interspecific currant hybrids (Stanys *et al.*, 2004), trifluralin application was the most efficient in *Rosa chinensis minima* polyploidization (Zlesak *et al.*, 2005), APM was the best chromosome doubling agent for *Allium cepa* (Grzebelus & Adamus, 2004). Trifluralin was the least toxic agent for *Lolium* spp and *Festuca pratensis* alike in this experiment, yet, at our experiment conditions, it was also the least effective, while oryzalin killed almost all plants. Only APM efficiency was similar to colchicine, making it second-best choice in *Lolium* and *Festuca* polyploidization experiments.

Good overwintering is important for grasses, especially for ryegrass, in the Nemoral environmental zone which covers Southern Scandinavia and Baltic states. There is no consensus data in the literature for ploidy level influence on plant over-wintering. Humphreys (1991) indicated that tetraploids often are more tolerant to abiotic and biotic stresses in comparison to diploids. Whereas Sugiyama (2006) proposed that tetraploid genotypes of Italian and perennial ryegrass tended to show lower survival under freezing stress conditions than diploid as related to large shoot mass and high tissue water content of tetraploids. *Lolium* tetraploids suffered higher winter damage than diploids in this experiment, whereas winter survival rate of meadow fescues was close to 100%, which is the reason this species used for crossing with ryegrass to develop festulolium (Fjellheim *et al.*, 2007).

The seed yield of grasses is a complex trait (Elgersma, 1990) and often negatively correlates with the dry matter yield (Bugge, 1987). It is generally stated, that herbage yield of tetraploid plants is higher compared to diploids (Burns *et al.*, 2013; Kemesyte *et al.*, 2017). Standards varieties of this study were tetraploid, therefore we cannot confirm or disprove these propositions. However all tetraploids populations in this study produced no significant difference of dry matter yield compared with registered varieties, and tetraploid meadow fescue line produced higher dry matter yield compared to the standard.

To conclude, considering all factors that define the efficiency of polyploid induction method (plant survival, tetraploid induction rate and variation between genotypes), colchicine in the present conditions, is proved to be the most effective antimetabolic agent for

Lolium multiflorum, *L. perenne* and *Festuca pratensis*. New tetraploid lines, developed during the experiment, were equal or superior to the registered cultivars.

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