biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2024) 68: 70-76 DOI: 10.32615/bp.2024.003

Special Issue "EUCARPIA2023"

BRIEF COMMUNICATION

Optimized tetraploidization strategies in tissue culture for *Lolium*, *Festuca*, and *Festulolium*

Marlies K.R. PEETERS[1,*], Isabelle MARYNS[1], An GHESQUIERE[2], Katrijn VAN LAERE[2], Yves VAN DE PEER[1], Tom RUTTINK[1,2], and Leen LEUS[2,*]

[1] *Ghent University, Department of Plant Biotechnology and Bioinformatics and Center for Plant Systems Biology (VIB), Gent, 9052, Belgium*
[2] *Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Plant Sciences Unit, Melle, 9090, Belgium*

[*]Corresponding authors: E-mails: marlies.peeters@ugent.be, leen.leus@ilvo.vlaanderen.be

Abstract

In plant breeding, polyploidization is an established technique to obtain superior phenotypic characteristics. In seed propagated agricultural crops, seed treatments with antimitotic agents are often used to obtain chromosome doubling. Here, we developed a method to induce polyploidization in clonally propagated fodder grasses *Lolium*, *Festuca*, and the intergeneric hybrid *Festulolium*. The aim was to obtain specific genotypes at both the diploid and tetraploid levels. We evaluated different types of plant explants, and the effects of the type, concentration, and application mode of three antimitotic agents (oryzalin, colchicine, and trifluralin) on the survival rate and the polyploidization efficiency. The treatment of greenhouse-grown tillers with antimitotics only resulted in mixoploids, while tissue culture propagated plants were successfully polyploidized. A shock pretreatment, using a high concentration of an antimitotic agent during a short period, successfully induced tetraploids in all three genera. Additionally, supplementing the tissue culture medium with a lower dosage of an antimitotic agent during minimal four weeks after the shock pretreatment further promoted polyploidization. By our methods, we were able to generate diploid and tetraploid plants with an identical genomic constitution but different ploidy allowing investigation of the effects of polyploidization on plant physiology and gene regulatory networks.

Keywords: artificial polyploidization, chromosome doubling, colchicine, fodder grass, *in vitro* culture.

Introduction

Polyploidy, the possession of more than two sets of chromosomes per nucleus, is a ubiquitous phenomenon in plants in nature. Also in agriculture, a wide range of important crops are polyploid, demonstrating that polyploidy comes with various genetic and phenotypic alterations (Renny-Byfield and Wendel 2014). In nature, polyploidization or whole-genome duplication arises mostly by gametes that have a complete set of duplicate chromosomes through nondisjunction during meiosis. These unreduced gametes increase genome complexity and may result in hybrid vigor or heterosis, and may serve to expand phenotypic plasticity and adaptability to new environments (Soltis and Soltis 2000, Sattler *et al*. 2016, Ebadi *et al.* 2023). Furthermore, polyploids

Received 1 December 2023, *last revision* 7 February 2024, *accepted* 6 March 2024.
Abbreviations: APM - amiprophos-methyl; BAP - 6-benzylaminopurine; FCM - flow cytometry; *Fp* - *Festuca pratensis*; *Lm* - *Lolium multiflorum*; *Lm* × *Fp* - *Festulolium*; *Lp* - *Lolium perenne*; NAA - naphthaleneacetic acid.
Acknowledgements: This work was supported by Ghent University (Bijzonder Onderzoeksfond UGent, Methusalem funding, BOF.MET.2021.0005.01).
Conflict of interest: The authors declare that they have no conflict of interest.

can function as a genetic bridge, allowing for gene flow of beneficial alleles between species and across ploidy levels (Mallet 2007, Cao *et al.* 2023). Given these features linked with genome doubling, artificial polyploidization can be seen as a powerful breeding tool to facilitate breeding in agronomically important crops for improved climate resilience and productivity. In plant breeding, polyploids are mostly created by mitotic polyploidization. Genomic changes in those mitotic polyploids are limited to additional copies of existing genes and chromosomes resulting in phenotypic changes. Typical characteristics aimed for in ploidy breeding are bigger or more compact plant and/or organ sizes, increased yield, restoration of fertility or induction of sterility, and an impact on other characteristics like stress resistance.

Ryegrasses, *Lolium* spp., are among the most prominent fodder grass species in European grasslands and are well appreciated for their high yield, productivity, and digestibility (Chaves *et al.* 2009). However, their sensitivity to drought, which is accompanied by yield losses, is challenging the usability of current *Lolium* cultivars, given that the frequency of drought periods is expected to increase due to climate change (Lee *et al*. 2019, Frisk *et al.* 2022). *Festuca* spp. cultivars, which occur in different ploidy levels ranging from di- to dodecaploidy (Martínez-Sagarra *et al.* 2021), can serve as a drought tolerant alternative forage crop, but provide lower yield and digestibility (Baert and van Waes 2014, Cougnon *et al.* 2017). Efforts to develop alternative climate-resilient cultivars stimulated the generation of interspecific *Lolium* and intergeneric *Festulolium* hybrids, combining the agronomically important traits with robustness in new environments (Baert *et al*. 2020). Next to hybridization, polyploidization can help to maximize the agronomic potential of fodder grass varieties (Lee *et al*. 2019) and it is a common technique used in grass breeding. In 2023, of all the perennial ryegrass (*Lolium perenne*) cultivars on the Belgian descriptive and recommended cultivar list, up to 70% were tetraploid. Tetraploids are characterized by an increased nutrients content, like sugars and proteins, and larger cells and therefore relatively more cell content compared to cell walls. This ensures better digestion and higher absorption of the nutrients in the grass and makes dairy cows produce more milk from the same amount of dry matter in tetraploid grass compared with diploid grass. Tetraploid cultivars also have larger seeds, thicker stems and broader leaves with a darker color. In tetraploids, the yield of fresh mass per hectare is higher, but due to the lower dry matter content (1 to 1.5% lower), the dry matter yield does not differ much when compared to diploids (Meehan and Gilliland 2019). Additionally, tetraploids have a lower susceptibility to rust (Fois *et al.* 2021). Because of the aforementioned agronomical benefits, artificial polyploidization is regularly used in grass breeding. The most common approach for polyploidization in grasses is the treatment of germinating seeds of a candidate cultivar with an antimitotic agent. As *Lolium* and *Festuca* are highly heterozygous outbreeding species, each seed is genetically unique. While seed-based polyploidization efficiently creates tetraploid individuals, there is no possibility to retain the original seed's genotype in its diploid state. Therefore, we produced both diploid and tetraploid plants with the same genome sequence but with a different number of chromosomes. For this, we developed a method to induce polyploidization using treatments with antimitotic agents on vegetative tissues of diploid *Lolium multiflorum* (Italian or annual ryegrass) (*Lm*), *Lolium perenne* (English or perennial ryegrass) (*Lp*), *Festuca pratensis* (meadow fescue) (*Fp*), and hybrids between *Festuca* and *Lolium*, so called *Festulolium* genotypes (*Lm* × *Fp*), to create their corresponding tetraploids.

Materials and methods

Plants and cultivation: Three different procedures (one in the greenhouse and two in tissue culture) to induce synthetic polyploids were applied on different diploid genotypes of *Festuca pratensis* Huds., *Lolium perenne* L., and *Lolium multiflorum* Lam. (ILVO breeding materials). Three antimitotic agents: colchicine, oryzalin, and trifluralin, were evaluated at varying exposure times and concentrations on two explant types. The same clonally propagated *Lm* and *Fp* genotypes were hybridized to create the respective intergeneric *Festulolium* hybrids *Lm* × *Fp* at the diploid level, of which eight hybrid genotypes were also included in the polyploidization experiment.

Method 1: For the first method (Method 1: "rooted tiller dipping in greenhouse"), three genotypes of *Lm* growing in substrate in pots in the greenhouse were separated into individual rooted tillers. Numbers of explants used are given in Table 1 Suppl. The tillers were cut above and below the apical meristem to a size of 1 to 2 cm and shaken for four hours in an antimitotic agent at different concentrations (Fig. 1, Table 1 Suppl.). Because of the difference in affinity for the plant tubulins, higher concentrations were tested for colchicine (0.75, 1.5, 2, and 3 g L^{-1}) compared to oryzalin and trifluralin (0.25, 0.5, and 1 g L^{-1}) (Eeckhaut *et al.* 2004, Dhooghe *et al.* 2011). A conversion table to different notations of the antimitotic concentrations (expressed in molar and volume %) can be found in Table 2 Suppl. After the pretreatment, the cut rooted tillers were directly planted in trays filled with potting soil for recovery. After four weeks, survival of the explants was checked, and the ploidy status was evaluated with flow cytometry (FCM) according to Leus *et al.* (2009). The same procedure was repeated on six genotypes of *Lm* for the treatment with colchicine but including lower concentrations (0.075, 0.15, 0.375, 0.75, and 1.5 g L^{-1}) and limiting the duration of the pretreatment to two hours.

Method 2: During the second method (Method 2: "antimitotic agent supplemented to *in vitro* culture medium"), sterile *in vitro* propagated explants of four genotypes of *Lp*, *Lm*, *Fp* and eight genotypes of the intergeneric hybrid *Lm* × *Fp* were used. Small explants

were generated by splitting tissue culture plants in individual tillers and excising explants of approximately 0.5 cm including the meristem using a binocular. The small explants (for numbers *see* Table 1 Suppl.) were grown for minimal four weeks on *in vitro* multiplication medium (4.4 g L^{-1} Murashige and Skoog medium including vitamins (*Duchefa*), 0.2 mg L^{-1} kinetine, 5 g L^{-1} plant tissue culture agar, 30 g L^{-1} sucrose, at pH 5.6) supplemented with different concentrations of colchicine (10, 30, 100, and 200 µM), oryzalin (1, 3, and 10 µM), or trifluralin (1, 3, and 10 µM) (Table 2 Suppl.). Similarly to method 1, the survival rate and the ploidy status was evaluated by FCM after four weeks of growth.

Method 3: Tissue culture plant explants, similiar to the plant explant type used in method 2, were treated in method 3 (Method 3: "shock pretreatment with antimitotic agent"), in which an additional pretreatment step was incorporated. The shock pretreatment was applied by incubating the explants for 24 h in sterile water with varying colchicine concentrations (500, 1 000, and 2 000 µM) before cultivation on culture medium without (0 µM) or with (30 or 100 µM) colchicine for minimal four weeks (cfr. method 2). For plant numbers *see* Table 1 Suppl. In line with method 1 and 2, the survival rate and the ploidy status was evaluated by FCM after four weeks of growth.

Stabilization of tetraploids: To generate stable tetraploids, plants identified as tetraploids during a first analysis by FCM were split into individual sprouts and cultured *in vitro* under similar conditions on the *in vitro* multiplication medium without antimitotics. Grown sprouts were subsequently subjected to FCM after four weeks of growth to confirm their tetraploid status. At least two iterative rounds of tetraploid identification, tiller splitting, cultivation, and FCM measurements were performed on seven *Lp* plants, eight *Lm* plants, and one *Fp* plant defined as tetraploid based on initial FCM measurements to generate stable tetraploids. Once confirmed by repeated measurements, tetraploid sprouts were transferred to the *in vitro* rooting medium, the basic medium as described above supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.05 g L^{-1} naphthaleneacetic acid (NAA) to stimulate root formation and later acclimated to greenhouse conditions.

Results

Whole genome doubling can be artificially induced by applying antimitotics, such as colchicine, amiprophos-methyl (APM), oryzalin, or trifluralin (Dhooghe *et al.* 2009). These bind to tubulin and interfere with chromosomal sorting during the mitotic cell division (Rauf *et al.* 2021). The polyploidization success of such treatments relies on *1*) type of antimitotic agent, concentration, and exposure time, *2*) the plant material, with variation between species, genotypes, and explant type, and *3*) the application method, *e.g.*, dripping of the antimitotics on the meristem, soaking of seeds or plantlets, application of antimitotics in tissue culture media, *etc*. (Dhooghe *et al.* 2009, 2011; Rauf *et al.* 2021). Here, we present an experimental set-up comparing three application methods: *1*) submerging of greenhouse cultivated rooted tillers, *2*) stem fragments cultivated in tissue culture on media supplemented with antimitotic agents, and *3*) like the second method but preceded by a 24-h shock pretreatment whereby the explants were shaken in liquid medium supplemented with a high concentration of antimitotic agent (Fig. 1). The succes of the method was evaluated by the overall plant survival rate and the polyplodization efficiency, calculated based on the estimation of the ploidy level per individual plant by FCM accoridng to Leus *et al*. (2009).

In method 1, "rooted tiller dripping in greenhouse", excised rooted tillers were subjected to colchicine, oryzalin, or trifluralin treatment, but no plants survived after four weeks (Fig. 2, Table 1 Suppl.). Repeating method 1 for five different concentrations of colchicine but reducing the duration of the treatment to only two hours resulted in a survival rate of 23, 3, and 5% for 0.15 g L^{-1} (376 µM), 0.75 g L^{-1} (1 878 µM), and 1.5 g L^{-1} (3 755 µM) colchicine, respectively (Fig. 2, Table 2 Suppl.). Only the highest two dosages were sufficient to induce mixoploidy, leading to one (0.75 g L^{-1}) and two (1.5 g L^{-1}) mixoploids (Table 1 Suppl.). Taken together, none of the set-ups of method 1, in which rooted tillers were dipped in the antimitotic agents, yielded fully tetraploid plants, despite testing variation in type, concentration, and duration of the antimitotic treatments.

The second method (method 2, "antimitotic agent supplemented to *in vitro* culture medium") used *in vitro* propagated plants as a source of small explants to be treated with antimitotic agents. This method tested whether the small explant size helps to improve penetration of antimitotics into cells of the meristem, which is smaller compared to the meristem of the greenhouse-grown tillers. Treatment of *Lp* plant explants with trifluraline illustrated similar survival rates for the two lowest concentrations (1 µM and 3 µM), while the survival rate decreased with an increasing oryzalin concentration. For both antimitotic treatments, the highest concentration of antimitotic agent (10 µM) resulted in the lowest survival rate. However, none of these treatments resulted in tetraploids (Fig. 2). The antimitotic treatment with colchicine was applied to four different species and our results illustrate the species-specific responses to *in vitro* propagated plant material. Survival rates in the control condition (0 µM) ranged from 14% (*Fp*) to 67% (*Lm*) while 30 µM colchicine led to survival rates varying from 61% of the *Fp* explants to 93% of the *Lm* explants (Fig. 2). Increasing the concentration to 100 µM colchicine affected the species differently, leading to the minimal survival rate of 19% for the *Festulolium* hybrids and the maximum survival rate of 66% for *Fp*. Finally, only one condition successfully generated tetraploids in method 2. Adding 100 µM colchicine to the culture medium resulted in two *Lp*, one *Lm*, and two *Festulolium* tetraploids. No plantlets of *Lp* and *Lm* survived on culture medium with 200 µM of colchicine. Overall, growing smaller explants on culture medium supplemented with 100 µM colchicine yielded tetraploids

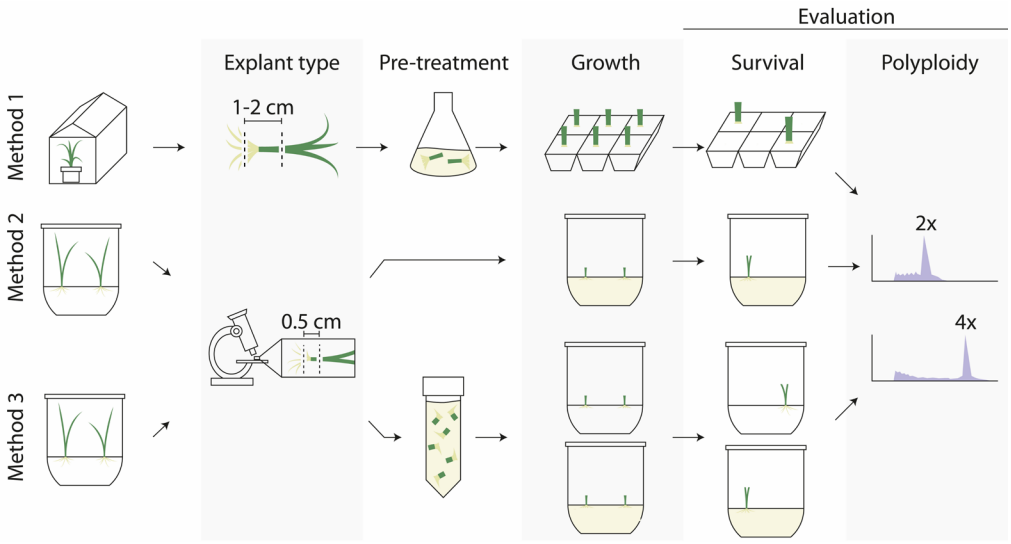

Fig. 1. Schematic overview of the experimental set-up of the study. The survival rate and polyploidization success were evaluated for three different experimental methods with varying explant type, pretreatment, and growth conditions. Method 1 started from grass tillers originating from potted plants in the greenhouse, which were cut to explants of 1 to 2 cm. After a pretreatment of 24 h in antimitotic agent, the explants were planted in trays. Method 2 and 3 used explants originating from *in vitro* grown grass tillers cut to 0.5 cm using a binocular. During method 2, these explants were grown on culture medium supplemented with an antimitotic agent, while in method 3, they were first subjected to a pretreatment of a high concentration of an antimitotic agent before growing on culture medium with or without antimitotic agent. After four weeks, the surviving plants were analyzed by FCM to determine their ploidy status. Yellow colored media represent the addition of an antimitotic agent, while the white media represent the conventional growth media/potting soil without antimitotic agent.

in three species, despite the lower survival rates due to higher antimitotic concentration.

The third method (method 3, "shock pretreatment with antimitotic agent") tested whether a shock pretreatment of small explants in a high concentration of an antimitotic agent in liquid medium could improve the polyploidization efficiency. By applying a shock pretreatment, the survival rates decreased for all species compared to the survival rates obtained in method 2, where no shock pretreatment was performed. For example, the pretreatment reduced the survival rate from 50 to 8% for *Lp*, from 38 to 12% for *Lm*, from 66 to 12% for *Fp* and from 19 to 9% for *Festulolium* explants in the treatment with 100 µM colchicine in the culture medium (Fig. 2). Additionally, as can be expected, increasing the concentration of the antimitotic agent in the culture medium further affected the survival rates, as already shown by the results obtained with method 2. Indeed, adding 30 µM colchicine to the culture medium after a colchicine pretreatment had limited effects on the survival rates of all species compared to the explants grown on culture medium without colchicine after the colchicine pretreatment, but a notable reduction in survival rate was observed when adding 100 µM to the culture medium posterior to the colchicine pretreatment.(Fig. 2, Table 1 Suppl.). Thus, the shock pretreatment had a negative effect on the survival rate, as well as an increased concentration of 100 µM of colchicine in the culture medium.

Subsequent evaluation of the polyploidization efficiency across the three tested methods revealed a positive effect of the shock pretreatment on the polyploidization efficiency, despite its negative effect on the survival rate. When we further increased the concentration of colchicine in the pretreatment to 2 000 µM for 24 h, a polyploidization efficiency of 5% (16 tetraploids) was observed, compared with 1% (5 tetraploids) for small explants without shock pretreatment, and only grown on culture medium supplemented with colchicine. This effect was observed for all the species. Moreover, the shock pretreatment of 2 000 µM colchicine allowed to yield tetraploids for all species. In addition to the shock pretreatment, a concentration of 100 µM instead of 30 µM colchicine in the culture medium further promotes polyploidization efficiency. The polyploidization efficiency increased from 10 to 20% for *Lp* and from 5 to 18% for *Lm* after increasing the colchicine concentration to 100 µM, but this effect was not observed in *Fp* and the *Festulolium* hybrids. However, when the survival rate is also taken into account, 30 µM colchicine in the culture medium led to a higher absolute number of tetraploids compared to 100 µM colchicine in the culture medium, respectively eight tetraploids (three *Lp*, four *Lm*, one *Fp*) *versus* three tetraploids (one *Lp*, two *Lm*). The species-specific response also indicates that the slightly higher polyploidization efficiency of *Lp* was compromised by the lower survival rate, finally resulting in a similar absolute number of tetraploids as in *Lm*, which had an overall higher survival rate. To summarize, a shock pretreatment of 2 000 µM colchicine prior to cultivation on culture medium supplemented with 30 µM colchicine balanced the survival rates and polyploidization efficiency, and was therefore the optimal set-up to induce polyploidization in *in vitro* propagated fodder grasses.

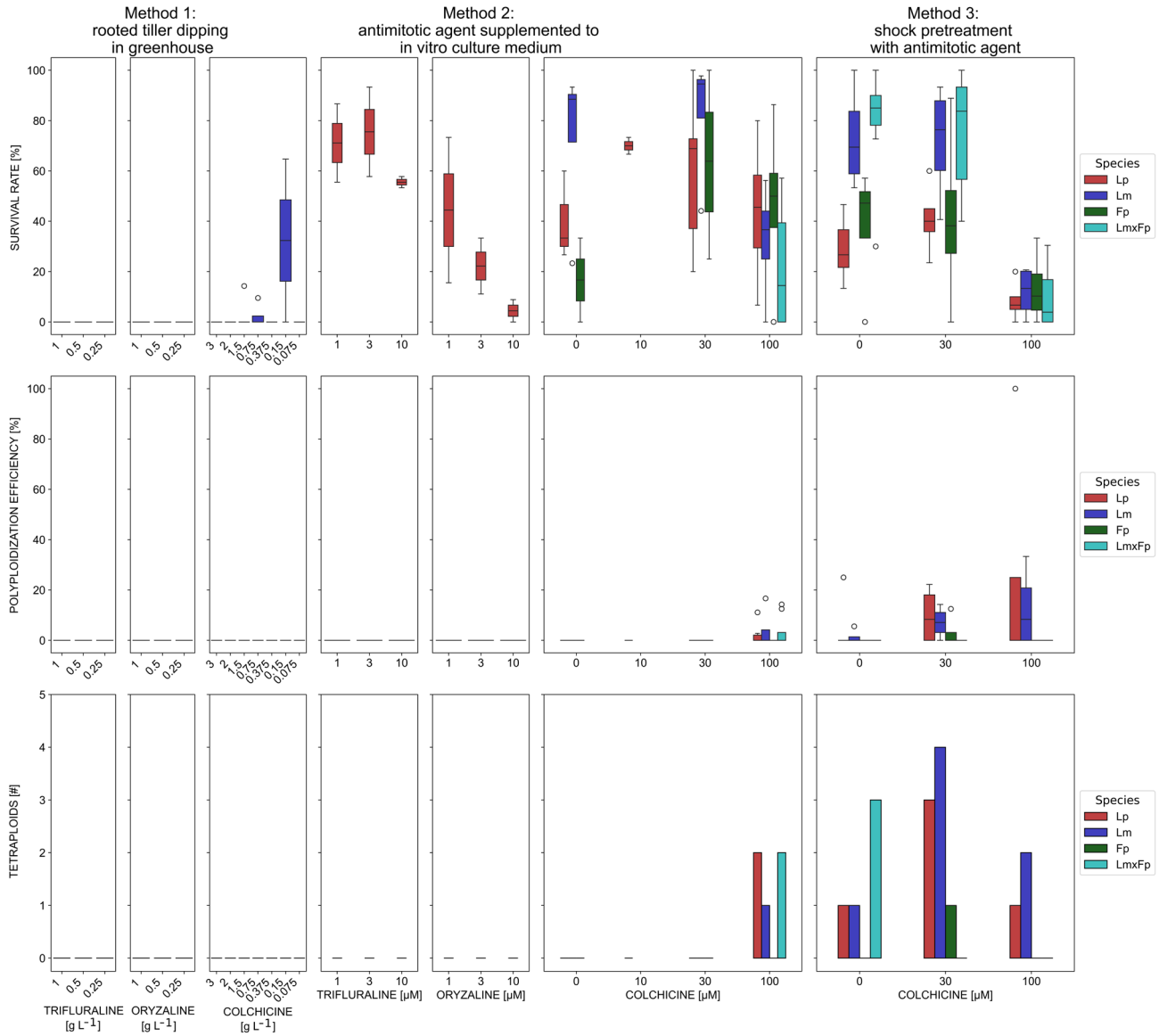

Fig. 2. Overview of survival rate, polyploidization efficiency, and number of tetraploids generated by the three different methods. For method 1, "rooted tiller dipping in greenhouse", only limited plant explants survived, but no tetraploids were obtained. Method 2, "antimitotic agent supplemented to *in vitro* culture medium", illustrates a species-specific survival response to the different procedures. Tetraploids were only succesfully generated at the highest colchicine concentration (100 µM) supplemented to the culture medium.The results of method 3, "shock pretreatment with antimitotic agent", demonstrate a negative effect of the pretreatment on the survival rate, but a positive effect on the tetraploidization success, since it induced tetraploidization in all species. A shock treatment is further sufficient to induce tetraploidization, shown by the obtained tetraploids after a shock treatment grown under normal conditions (0 µM). The 'survival rate' is defined as the number of explants that survived the treatment after four weeks and were sufficiently grown to be measured by FCM, divided by the total number of explants treated in that set-up. The 'polyploidization efficiency' represents the percentage of tetraploids calculated as the number of tetraploids divided by the number of surviving plantlets in that set-up, and 'tetraploids' means the absolute number of tetraploids plants identified by FCM.

Discussion

Our goal of artificial genome doubling *via* clonal propagation is to obtain diploid and tetraploid *Lolium* spp., *Festuca pratensis* and *Festulolium* genotypes with an identical genome constitution, but with difference in chromosome dosage per nucleus. Genome doubling in vegetatively propagated materials with multicellular meristems may result in mosaic tissues, so called mixoploids, as some sectors of the meristem of the explant contain the original diploid cells, and other sectors tetraploidized cells. In other cases, ploidy chimaera can arise as sectoral differences in ploidy levels or with different ploidy levels associated with different cell layers. It is especially important to repeat ploidy analysis on propagated materials to avoid misinterpretations of ploidy

measurements: *1*) mixoploidy could be overlooked in case of a low fraction of diploid cells (often the diploid cells outgrow the tetraploid cells after some time, thus repetition of the analysis after a few months is necessary); *2*) the formation of tillers from other (sub)meristems could result in shoots with different ploidy levels. Therefore, the ploidy level of the tillers should be confirmed after subculturing the individual tillers. So, several iterative rounds of tetraploid identification, tiller splitting, cultivation, and FCM measurements were performed on seven *Lp*, eight *Lm* plants, and one *Fp* plant defined as tetraploid based on initial FCM measurements. For example, the seven *Lp* plants resulted in a total of 34 individual tillers of which 10 were confirmed as tetraploids. In two additional iterations of the same procedure, eight were identified again as tetraploid, in a third round all 28 subcultured tillers were again confirmed as tetraploids. Altogether, these results stress the need for careful confirmation of the ploidy status by multiple measurements.

Polyploidization is a powerful tool in plant breeding but genome doubling methods require precise optimization of several parameters, such as the type of explant, type and concentration of antimitotic agent, should be tailored towards species and application (Dhooghe *et al.* 2011, Sattler *et al*. 2016, Pavlíková *et al.* 2017). In our set-up, two types of explants were considered in distinct growth conditions: *1*) rooted tillers from plants grown on substrate in the greenhouse cut to a size of 1 - 2 cm including the meristem, and *2*) small tissue culture explants excised to 0.5 cm. Treating individual rooted tillers failed to induce polyploidization, and induced only limited mixoploids, despite a variety of antimitotic agents, concentrations, and genotypes tested, while treating the small explants *in vitro* led to stable tetraploids. Thus, for fodder grasses, the treatment of small explants with small meristems in tissue culture improved the polyploidization efficiency compared to greenhouse-grown explants with larger meristems. These findings confirm previous reports stating that the small size of the explants can improve the penetration of colchicine into the cells to reach the growing meristems and more efficiently produce polyploids (Allum *et al*. 2007, Eng and Ho 2019). Secondly, the three antimitotic agents, oryzalin, trifluralin, and colchicine, were evaluated in different concentrations considering both survival rate and polyploidization efficiency. Of the three antimitotic agents used in this study, we observed that trifluralin could replace colchicine as antimitotic agent, but due to the dissolvability of colchicine in water, permitting proper sterilization for handling tissue culture, experiments were conducted with colchicine. In conclusion, we have shown that the combination of a short (24 h) shock pretreatment with a high concentration of colchicine in combination with cultivation of the explants on tissue culture media supplemented with antimitotic agents during minimal four weeks enhances the tetraploidization efficiency. We observed differences in the survival rate and polyploidization efficiency between individual genotypes of *Lp*, *Lm*, *Fp*, and *Festulolium* but managed to obtain diploid and tetraploid plants of all four species with an identical genomic constitution at the diploid and tetraploid level. These plants will allow to evaluate the effect of polyploidization on phenotypic differences, genomic constitution, and the processes driving genome dominance.

References

Allum J.F., Bringloe D.H., Roberts A.V.: Chromosome doubling in a *Rosa rugosa* Thunb. hybrid by exposure of *in vitro* nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. - Plant Cell Rep. **26**: 1977-1984, 2007.

Baert J., van Laere K., van Waes C. *et al.*: Breeding and genetics of two new amphiploid *Festulolium* synthetics with improved yield and digestibility. - Biol. Plantarum **64**: 789-797, 2020.

Baert J., van Waes C.: Improvement of the digestibility of tall fescue (*Festuca arundinacea* Schreb.) inspired by perennial ryegrass (*Lolium perenne* L.). - In: Hopkins A., Collins R., Fraser M. *et al.* (eds.): Grassland Science in Europe: EGF at 50: The Future of European Grasslands. Vol. 19. Pp. 172-174. IBERS/Aberystwyth University, Aberystwyth 2014.

Cao Y., Zhao K., Xu J. *et al.*: Genome balance and dosage effect drive allopolyploid formation in *Brassica*. - PNAS **120**: e2217672120, 2023.

Chaves B., De Vliegher A., Van Waes J. *et al.*: Change in agronomic performance of *Lolium perenne* and *Lolium multiflorum* varieties in the past 40 years based on data from Belgian VCU trials. - Plant Breeding **128**: 680-690, 2009.

Cougnon M., De Swaef T., Lootens P. *et al.*: In situ quantification of forage grass root biomass, distribution and diameter classes under two N fertilisation rates. - Plant Soil **411**: 409-422, 2017.

Dhooghe E., Grunewald W., Leus L., Van Labeke M.-C.: *In vitro* polyploidisation of *Helleborus* species. - Euphytica **165**: 89-95, 2009.

Dhooghe E., Van Laere K., Eeckhaut T. *et al.*: Mitotic chromosome doubling of plant tissues *in vitro*. - Plant Cell Tiss. Org. Cult. **104**: 359-373, 2011.

Ebadi M., Bafort Q., Mizrachi E. *et al.*: The duplication of genomes and genetic networks and its potential for evolutionary adaptation and survival during environmental turmoil. - PNAS **120**: e2307289120, 2023.

Eeckhaut T.G.R., Werbrouck S.P.O., Leus L.W.H. *et al.*: Chemically induced polyploidization in *Spathiphyllum wallisii* Regel through somatic embryogenesis. - Plant Cell Tiss. Org. Cult. **78**: 241-246, 2004.

Eng W.-H., Ho W.-S.: Polyploidization using colchicine in horticultural plants: A review. - Sci. Hortic.-Amsterdam **246**: 604-617, 2019.

Fois M., Malinowska M., Schubiger F.X., Asp T.: Genomic prediction and genotype-by-environment interaction analysis of crown and stem rust in ryegrasses in European multi-site trials. - Agronomy **11**: 1119, 2021.

Frisk C.A., Xistris-Songpanya G., Osborne M. *et al.*: Phenotypic variation from waterlogging in multiple perennial ryegrass varieties under climate change conditions. - Front. Plant Sci. **13**: 954478, 2022.

Lee M.A., Howard-Andrews V., Chester M.: Resistance of multiple diploid and tetraploid perennial ryegrass (*Lolium perenne* L.) varieties to three projected drought scenarios for the UK in 2080. - Agronomy **9**: 159, 2019.

Leus L., Van Laere K., Dewitte A., Van Huylenbroeck J.: Flow cytometry for plant breeding. - Acta Hortic. **836**: 221-226,

2009.
Mallet J.: Hybrid speciation. - Nature **446**: 279-283, 2007.
Martínez-Sagarra G., Castro S., Mota L. *et al.*: Genome size, chromosome number and morphological data reveal unexpected infraspecific variability in *Festuca* (Poaceae). - Genes **12**: 906, 2021.
Meehan E.J., Gilliland T.J.: Differences in dry matter content between forage varieties of *Lolium perenne* L. - Biol. Environ. **199B**: 123-137, 2019.
Pavlíková Z., Paštová L., Münzbergová Z.: Synthetic polyploids in *Vicia cracca*: methodology, effects on plant performance and aneuploidy. - Plant Syst. Evol. **303**: 827-839, 2017.
Rauf S., Ortiz R., Malinowski D.P. *et al.*: Induced polyploidy: a tool for forage species improvement. - Agriculture **11**: 210, 2021.
Renny-Byfield S., Wendel J.F.: Doubling down on genomes: Polyploidy and crop plants. - Am. J. Bot. **101**: 1711-1725, 2014.
Sattler M.C., Carvalho C.R., Clarindo W.R.: The polyploidy and its key role in plant breeding. - Planta **243**: 281-296, 2016.
Soltis P.S., Soltis D.E.: The role of genetic and genomic attributes in the success of polyploids. - PNAS **97**: 7051-7057, 2000.