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Plant regeneration from immature inflorescence-derived callus of Italian ryegrass (*Lolium multiflorum* Lam.): chromosome number and fertility of regenerated plants

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Abstract. Twenty field-grown genotypes of diploid Italian ryegrass (*Lolium multiflorum* Lam., 2n = 2x = 14) were tested for their ability to induce callus and regenerate plants. Callus cultures were initiated from segments of immature inflorescences cultured on the MS medium supplemented with 4.0 mg L^{-1} 2,4-D. The calluses were subcultured first on the maintaining medium (MS medium with 2.0 mg L^{-1} 2,4-D) and later on the rooting medium (MS medium with 0.2 mg L^{-1} 2,4-D). The frequency of callus induction varied depending on the source of explant and the initial genotype. A total of 473 green plantlets were regenerated, of which 420 were established in the soil. All these plants had the morphological characteristics of Italian ryegrass. Among 372 regenerants analysed cytologically, 302 (81.2%) had the expected diploid chromosome number (2n = 2x = 14), 65 (17.5%) were tetraploid (2n = 4x = 28); several aneuploids and mixoploids were also observed. All diploid and tetraploid regenerants were male and female fertile. However, a great variation of female fertility within and between both groups of regenerants was observed.

Key words: callus culture, chromosome doubling, immature inflorescence, *Lolium multiflorum*, plant regeneration.

Introduction

Regeneration of plants from tissue cultures provides an important method to produce new types of plants, as an alternative to conventional plant breeding

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methods. In the family Poaceae, different types of explants, i.e., mature and immature embryos, basal leaf sections, nodes, internodes and immature inflorescences, were used for callus induction and plant regeneration. Immature inflorescences appear to be the most suitable source of explants for callus tissue cultures in the cross-pollinating species of the *Lolium-Festuca* complex, e.g., in Italian ryegrass (*L. multiflorum* Lam.) (DALE et al. 1981, DALE, DALTON 1983, CREEMERS-MOLENAAR et al. 1988, RAJOELINA et al. 1990), perennial ryegrass (*L. perenne* L.) (DALE, DALTON 1983, CREEMERS-MOLENAAR et al. 1988) and tall fescue (*F. arundinacea* Schreb.) (DALE, DALTON 1983, EIZENGA, DAHLEEN 1990).

Somaclonal variation among in vitro regenerated plants is a relatively common phenomenon. Cytological and morphological variation among plants regenerated from callus in the grasses of the *Lolium-Festuca* complex have been reported by several authors, e.g., KASPERBAUER et al. 1979, AHLOOWALIA 1983, RYBCZYŃSKI et al. 1983, ZWIERZYKOWSKI et al. 1985, JACKSON, DALE 1988, GARCIA et al. 1994. Variation involved chromosome number, chromosome structure and meiotic behaviour.

Italian ryegrass (*Lolium multiflorum* Lam.) is one of the most important forage grass species. In natural conditions it is diploid (2n = 2x = 14), but in grass breeding both diploid and tetraploid forms are used. The tetraploids are usually produced by chromosome doubling of diploid plants using colchicine.

Fifteen years ago we initiated experiments to use tissue culture procedures for chromosome doubling in sterile *Lolium* × *Festuca* hybrids. The explants used for culture initiation were excised from nodes, internodes, peduncles, rachises and immature inflorescences, and cultured on MS medium supplemented with various concentrations of 2,4-D (RYBCZYŃSKI et al. 1983, ZWIERZYKOWSKI et al. 1985, ZWIERZYKOWSKI, RYBCZYŃSKI 1994).

This paper presents the results of plant regeneration of *Lolium multiflorum* genotypes from callus cultures derived from immature inflorescences. Chromosome numbers and fertility of the regenerated plants are also described.

Material and methods

Twenty field-grown genotypes of diploid (2n = 2x = 14) Italian ryegrass (*Lolium multiflorum* Lam.) cv. Tur were used in the experiment. Tillers with unemerged inflorescences collected at random from different plants, were surface sterilized in 70% ethanol for 30 s, followed by 5% (w/v) calcium hypochlorite for 10 min. and rinsed five times in sterile distilled water. The im-

mature inflorescences of 1-12 cm in length (entire inflorescences of 1-6 cm in length and for the inflorescences over 6 cm in length only their 6-cm basal parts) were aseptically excised and cut into 0.5-cm segments. The inflorescences used for experiments were divided into four groups according to their length: 1-3 cm, 4-6 cm, 7-9 cm and 10-12 cm, and the callus formation response of explants of each group was pooled. The inflorescence segments were plated onto the MS medium (MURASHIGE, SKOOG 1962) supplemented with 4.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (initiation medium) in 8-12 cm Petri dishes. Callus cultures were initiated during first 2-4 weeks in the darkness and then exposed to light (4000 lx) with a 16/8 h photoperiod at 25°C. After 4-5 weeks of culture, calluses were excised from the explants and subcultured on the MS medium supplemented with 2.0 mg L^{-1} 2,4-D. On this medium calluses were maintained for 8-12 weeks. Every 3-4 weeks of subculture, regenerated shoots were selected and transferred into the rooting medium (MS medium supplemented with 0.2 mg L^{-1} 2,4-D). All media contained 30 g L^{-1} sucrose and were solidified with 6-8 g L^{-1} agar (Difco Bacto Agar); they were adjusted to pH 5.8 before autoclaving at 121°C for 20 min. Regenerated plantlets at the 3-4 leaf stage were transferred to pots in a greenhouse where all the well-established plants were vernalized (during winter season at 1-5°C) and grown up to maturity.

Chromosomes were counted in root tips. The roots collected in ice water were refrigerated overnight, fixed in a mixture of absolute ethanol and glacial acetic acid (3 : 1) and stored in refrigerator until used. Root-tips from individual plants were hydrolysed in 1N HCl at 60°C for 5-6 min., and stained in a drop of 2% acetoorcein directly on a slide and squashed.

Male fertility (pollen stainability) was determined by staining pollen grains in a mixture of acetocarmine and glycerine (1 : 1). Female fertility was determined on the basis of seed set under open pollination of the regenerated plants.

Results

Callus induction and plant regeneration

Immature inflorescences of all the twenty used genotypes of diploid *Lolium multiflorum* cv. Tur produced callus (Table 1). Callus initiation was observed during the first 2-4 weeks of culture. Usually, the calluses developed at both ends or on the entire surface of rachis of the explants, sometimes on glumes enclosing the floral primordia. Two types of callus were observed: (1) regener-

able (white, compact and nodular), and (2) non-regenerable (yellow-greenish, soft and watery).

The callus formation response of explants originating from inflorescences 1-3 cm and 4-6 cm in length was 73.3% and 74.6%, respectively, and in the case of inflorescences 7-9 cm and 10-12 cm in length was significantly lower, 46.7% and 43.0%, respectively.

Table 1. Callus induction and plant regeneration from immature inflorescences of selected *Lolium multiflorum* genotypes

Initial genotypes	Inflorescence segments cultured no.	Explants forming callus		Plants regenerated	
		no.	%	no.	%[1]
I/1-1	26	16	61.5	10	38.5
I/2-2	37	19	51.4	0	0
I/3-3	25	24	96.0	63	252.0
I/4-4	41	25	61.0	30	73.2
I/5-5	50	36	72.0	4	8.0
I/6-6	26	13	50.0	14	53.8
I/7-7	23	5	21.7	11	47.8
I/8-9	11	10	91.0	16	145.5
I/9-10	46	13	28.3	0	0
I/10-11	17	11	64.7	6	35.3
II/1-1	123	30	24.4	8	6.5
II/2-4	155	62	40.0	46	29.7
II/3-5	108	67	62.0	76	70.4
II/4-8	93	43	46.2	20	21.5
II/5-9	105	33	31.4	34	32.4
II/6-10	48	22	45.8	15	31.3
II/7-13	108	64	59.3	30	27.8
II/8-16	77	77	100	23	29.9
II/9-18	175	131	74.9	45	25.7
II/10-21	164	93	56.7	22	13.4
Total	1458	794	54.8	473	32.4

[1]Plants/100 explants

Considerable variation in the ability of different genotypes to initiate callus was observed. Although calluses were obtained from each of the 20 genotypes tested, the frequencies of callus induction ranged from 21.7% (genotype No. I/7-7) up to 100% (genotype No. II/8-16). Of the 1458 explants cultured, 794 (54.8%) formed callus. A total of 473 green plantlets from 18 genotypes were regenerated. The most responsive genotypes, Nos. I/3-3 and II/3-5, produced 63 and 76 plants, respectively. A great majority of the regenerated plants were green; only several albino plantlets were observed during the whole experiment.

Of the 473 plantlets regenerated, 420 were established in the soil and grown in a greenhouse till maturity. All these plants had the morphological characteristics of Italian ryegrass; however, some variation in plant height, leaf and inflorescence shape and size was observed. Additionally, tetraploid regenerants were more vigorous and had markedly broader leaves than the diploid regenerants.

Table 2. Chromosome numbers of plants regenerated from the inflorescence-derived callus of diploid *Lolium multiflorum* (2n=2x=14) genotypes

Initial genotypes	Regenerants studied	No. of regenerants with the chromosome number		
		diploid (2n=2x=14)	tetraploid (2n=4x=28)	aneuploid
I/1-1	10	10		
I/3-3	63	37	26[a]	
I/4-4	30	30		
I/5-5	4		4	
I/6-6	14	12	2	
I/7-7	11	11		
I/8-9	16	4	12	
I/10-11	6	6		
II/1-1	4	4		
II/2-4	20	14	5[b]	1[c]
II/3-5	68	59	7	2[c]
II/4-8	17	17		
II/5-9	30	27	3	
II/6-10	13	13		
II/7-13	29	23	6	
II/8-16	12	12		
II/9-18	15	14		1[d]
II/10/21	10	9		1[e]
Total	372 (100%)	302 (81.2%)	65 (17.5%)	5 (1.3%)

[a]mixoploid plant (2n=28/29), [b]mixoploid plant (2n=27/28), [c]plants with 2n=27, [d]plant with 2n=15, [e]plant with 2n=16

Chromosome number of regenerated plants

Of the 420 regenerants successfully established in the soil, 372 were analysed for somatic chromosome number (Table 2). Among them 302 plants (81.2%) had the unchanged diploid chromosome number (2n = 14) and 65 plants (17.5%) were tetraploid (2n = 28). Among tetraploids, two plants were mixoploids, with 2n = 27/28 and 28/29 chromosomes. Additionally, five aneuploid plants with 15, 16 and 27 chromosomes were observed. Regenerants with a doubled chromosome number were obtained in 8 of the 20 initial genotypes used in the experiment. In the initial genotype No. I/5-5 all the re-

generants had a tetraploid chromosome number, and in two other genotypes, Nos. I/8-9 and I/3-3, 75.0% and 41.3% of regenerants, respectively, were tetraploids. Spontaneous chromosome doubling was more common among plants originated from callus cultured on the MS medium with 2.0 mg L^{-1} 2,4-D for 8-12 weeks, than plants originated from 4-8 weeks of culture.

Pollen stainability and seed set of regenerated plants

All 420 regenerants had dehiscent anthers and were male fertile. Pollen stainability, which was analysed in 94 diploid and 35 tetraploid regenerants, ranged from 70.0% to 100% and from 83.5% to 98.5%, respectively (Table 3).

Table 3. Pollen stainability and seed set of diploid and tetraploid regenerants obtained from inflorescence-derived callus of diploid *Lolium multiflorum* genotypes

Ploidy of regenerants	Pollen stainability (%)					Seed set (%)				
	plants studied no.	min.	max.	mean	CV	plants studied no.	min.	max.	mean	CV
2x	94	70.0	100	94.1	4.66	96	28.1	87.4	62.0	21.27
4x	35	83.5	98.5	92.2	9.79	35	2.2	32.5	10.7	84.61

CV – coefficient of variation

Female fertility measured as seed set under open pollination, was checked in 96 diploid and 35 tetraploid regenerants. Considerable variation was present in both groups; the seed set ranged from 28.1% to 87.4% in diploids and from 2.2% to 32.5% in tetraploids. Low seed set of the tetraploids was probably a result of insufficient pollination under the greenhouse conditions. In two test crosses using a standard tetraploid *L. multiflorum* cultivar as a source of pollen, seed set reached 60.7% and 66.7% which was comparable to that observed in the standard cultivar itself. The results of the test crosses suggest that the regenerated tetraploids did not suffer any reduction in female fertility. Seed germination rate was similar in the diploids and the tetraploids and ranged from 57.1% to 98.2%.

Discussion

In the present study twenty genotypes of diploid Italian ryegrass were tested for their ability to induce callus and regenerate plants. Immature inflorescences of 1-12 cm in length were used as a source of explants. DALE et al. (1981) reported that 40% of the explants from young inflorescences of *L. multiflorum*

(up to 5 cm long) produced compact callus. CREEMERS-MOLENAAR et al. (1988) obtained an even higher response, with up to 100% compact callus formation in *L. multiflorum* and *L. perenne*, but they used very young, 4-7-mm-long inflorescences. In this study, considerable variation in callus induction rate was also observed depending on the length of inflorescences used. For the shorter inflorescenses, up to 6 cm in length, about 74% callus induction rate was observed, as compared to about 45% callus induction rate for the longer (up to 12 cm) inflorescences. However, only a portion of the formed callus was recognized as compact and capable to regenerate organs for both the above mentioned groups.

Variation among genotypes in their capacity to initiate callus and regenerate plants has been observed in many species, including the *Lolium-Festuca* complex (CREEMERS-MOLENAAR et al. 1988, JACKSON, DALE 1988, EIZENGA, DAHLEEN 1990). In this study, considerable variation in the ability of different genotypes to initiate callus was also noted. Callus was obtained from all of the twenty initial genotypes tested. However, the frequency of callus induction ranged from 21.0% to 100% (54.8% on average). The number of regenerated plants per studied genotype ranged from 0 to 76.

A majority (81.2%) of plants regenerated from callus cultures of diploid *L. multiflorum* explants had the unchanged diploid chromosome number (2n = 2x = 14). Most of these plants were regenerated after a shorter period of culture (from culture initiation to regeneration) – about 4-8 weeks. On the other hand, almost all regenerants with doubled chromosome numbers were obtained after a longer period of culture – about 8-16 weeks. Although no detailed observations were made on the type of the regeneration process involved, we suggest that in most cases the plant regeneration might have been from callus through organogenesis.

Induction of chromosome doubling in cultures was described in several studies involving the *Lolium-Festuca* complex, e.g., KASPERBAUER et al. 1979, AHLOOWALIA 1983, JACKSON, DALE 1988, but the frequencies were always low. The percentage of plants with a doubled chromosome number in this study was 17.5, i.e. comparable to that observed earlier in the *Lolium* × *Festuca* hybrids, for example 19.1% regenerants with a doubled chromosome number in the diploid *L. multiflorum* × *F. pratensis* hybrids (ZWIERZYKOWSKI et al. 1985).

In conclusion, the study suggests that the genotype of the explant source is an important factor in the success of callus formation and plant regeneration. It would seem reasonable to believe that with the variation observed here, "super-responsive" genotypes could be selected. The procedure of plant regeneration used here was efficient not only for regeneration of large numbers of plants but also for producing tetraploid plants from diploid *L. multiflorum*.

It seems that in certain situations, the tissue culture method could be used as an alternative to the colchicine method of chromosome doubling in diploid species of the *Lolium-Festuca* complex. In this case the mutagenic effect and environmental hazards of colchicine can be avoided.

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