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Selection for increasing 2n gamete production in red clover

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ABSTRACT - *Three selection cycles were performed in the red clover cultivars Quiñiqueli, Redland and Keenland to increase the 2n gamete production. Unreduced gamete production was estimated by the giant pollen grain (GP) production. Plants with more than 1% of GP were selected as progenitors from the original population; plants with at least 2% and 3% of GP, respectively, were selected and crossed for the second and third cycles. The GP production ranged from 1.4 to 7.5% in the first, 2.0 to 51.0% in the second, and 3.0 to 46.7% in the third cycle. Considering the three cultivars together, there was a significant increase in GP production from the first (4.0%) to the third (8.9%) cycles. 'Redland' presented higher GP percentages in each cycle and significant and steady GP production increases along the three cycles: 4.8, 13.4 and 19.7%, respectively, and should be included in further attempts to obtain sexual polyploids.*

Key words: meiotic chromosome doubling, sexual polyploidization, *Trifolium pratense*, unreduced gametes.

INTRODUCTION

Red clover (*Trifolium pratense* L.) is a typical forage legume of cultivated pastures in temperate regions (Fergus and Hollowell 1960, Taylor and Smith 1979, Zohary and Heller 1984, Taylor and Quesenberry 1996). It is diploid species with 2n=14 chromosomes, with no evidences that natural tetraploid cytotypes may occur (Parrott and Smith 1984). In some parts of Rio Grande do Sul, Southern Brazil (33º to 27º lat S), of temperate-like climate, red clover can be cultivated as a forage. However, the available commercial cultivars lack persistence, a problem that could be overcome by genetic breeding.

Polyploidy, the coexistence of three or more chromosome complements in the same nucleus has long been recognized as a main force in plant evolution (Ramsey and Schemske 1998). Polyploid organisms may arise by chromosome doubling of somatic cells (asexual polyploids), with the aid of substances such as colchicine, oryzaline and nitrous oxide (Elliot 1967, Taylor and Quesenberry 1996). The other means of polyploid formation is through functional unreduced gametes (sexual polyploids), acknowledged as the most common, if not exclusive, way of polyploidization in nature (Harlan and De Wet 1975, De Wet 1980, Ramsey and Schemske 1998). These gametes arise due to meiotic disturbances during micro or macrosporogenesis

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leading to chromosome non-reduction. The ability of a given individual to produce unreduced gametes (also called 2n gametes, meaning gametes with somatic chromosome number) is genetically controlled and generally highly inheritable (Hermsen 1984a, Veilleux 1985, Bretagnolle and Thompson 1995). Sexual polyploidization may be unisexual, when one unreduced gamete fertilizes a normal, reduced one, or bisexual, when two unreduced gametes fuse to form the zygote. Production of unreduced gametes has been reported in several economic important plants such as alfalfa (Vorsa and Bingham 1979, McCoy and Smith 1983, Mariani et al. 1992), ryegrass (Wagenvoort and Den Nijs 1992), potato (Peloquin et al. 1992), sweet potato (Lopez-Lavalle and Orjeda 2002), white clover (Hussain and Williams 1997), red clover (Parrott et al. 1985, Mousset-Déclas et al. 1992), among many others. In plant breeding, one of the main advantages of sexual polyploidization over somatic polyploidization is the maintenance of high heterozygosity levels. The first polyploid produced by human interference in 1928, *Raphanobrassica*, was probably formed by the union of unreduced gametes (Griffiths et al. 2000). Presently, sexual polyploidization is increasingly gaining importance in plant breeding. For an updated and comprehensive review on the subject, see Ramanna and Jacobsen (2003).

Induction of polyploidy in a plant breeding project may introduce new genetic combinations, therefore broadening the genetic basis from which the plant breeder selects. In red clover, artificial polyploidization, somatic and sexual, has been rather successful: in northern Europe; some chemically induced tetraploid cultivars performed better regarding forage yield, pest and disease resistance and persistence (Meglic and Smith 1992, Taylor and Quesenberry 1996). Several studies have pointed out the potential of sexual polyploidization in red clover breeding. The production of unreduced gametes is highly inheritable and may be increased in few generations (Parrott and Smith 1984, 1986, Parrott et al. 1985, Mousset-Déclas et al. 1992).

In this paper we report results of selection cycles aiming to increase the production of unreduced gametes in three cultivars of red clover. The study is part of a larger red clover breeding project, to select better adapted plants for Rio Grande do Sul´s local conditions, which includes the production of sexual polyploids.

MATERIAL AND METHODS

Plant material

Three populations of around 300 individuals each were established from seeds of three commercial red clover cultivars, Quiñiqueli, Redland and Keenland. The plants were kept in a greenhouse under a controlled photoperiod of 16 h light and watered whenever necessary. As commercial garden soil was employed, no further fertilization was applied during the one-year life cycle. Pests and diseases were controlled in the vegetative period when necessary using Malathiol (3 mL L^{-1}). No pesticides were employed after the first flower buds appeared.

Pollen analysis

Inflorescences from all plants that flowered were collected (fixed in 3:1 ethanol-acetic acid for 24 h) in order to estimate the percentage of unreduced gametes, assuming that "giant" pollen grains or pollen grains larger than normal grains are unreduced, as pollen grain size is considered to be a reliable method to estimate the percentage of unreduced gametes (Parrott and Smith 1984). Three flowers and a total of 1500 grains per plant were analysed. The anthers were squashed in propionic carmine and the pollen grains visually classified as normal (reduced) and "giant" (assumed to be unreduced, with the 2n chromosome number) pollen grains, henceforth referred to as GP. From each category, sub-samples of 15 and 10 grains, respectively, were measured (both axes). Pollen fertility was estimated by the staining reaction: full, stained grains were classified as fertile and not-stained or weakly stained grains were considered unfertile.

Crosses and selection cycles

Three cycles of phenotypic recurrent selection were performed involving three generations: P_1, F_1, and F_2. Reciprocal pollinations (crosses) were made with the aid of a small folded card among the selected plants within each cultivar and each selection cycle. From the first three original populations, those individuals producing more than 1% of GP were selected as progenitors for the next generation, according to Parrott and Smith (1984). For the second and third selection cycles, plants with at least 2% and 3% of GP production, respectively, were selected and crossed.

Each plant derived from each cultivar was designated with the cultivar's first letter (Q, R or K) and a number; for example, R14 indicated plant number 14 established from cv. Redland seed. After the crosses, seeds were collected from each plant individually and the plants grown from these seed were denominated with a letter and a number, the letter identifying the family and the number the individual within the family. For instance R14 D40 meant plant number 40 of family D originated from the R14 Redland plant.

Data analysis

In the first cycle, the treatments were the three cultivars, Quñiqueli, Keenland and Redland, and replicates were represented by the selected plants. In the second and third cycles,

each plant selected in the former cycle was a treatment and the respective progenies (families) were the replicates. Therefore, in all cycles, number of treatments and replicates were variable. Percentages of GP were compared among treatments by an F-test at 5% significance, and the averages by a Tukey test, using software Genes (Cruz 2001).

In order to verify if the visual selection for GP was efficient, that is if the supposedly unreduced pollen grains were really around 30% larger than reduced ones – which is considered as an indication of non-reduction in literature – the pollen grain area was calculated by the formula A=(*a* x *b*)/4, in which *a* is the larger axis and *b* the smaller one, and results compared by a t-Student test at 5% significance.

Selection gains and selection differentials for production of GP were calculated regarding each original cultivar and progenies along the three selection cycles and a final calculation compared all plants of all cultivars along the three cycles. Selection differentials (SD) were calculated as SD = average of the selected population - average of the original population; and selection gains (SG) as SD x 100/average of the original populations. This procedure allows an evaluation of the performance of each family in each selection cycle as well as the efficiency of selection.

The general ability of each family to produce GP, as well as its progress along the three cycles, was compared among the three cultivars by an ANOVA and a Tukey test at 5% significance. In this case, SD and SG compared the general averages of all three original population with the general averages of all selected populations in the second and third cycle.

RESULTS

First selection cycle

A total of 144 plants which flowered, derived from the original cultivars, were evaluated in the first cycle. From these, 53 (36.8%) produced at least 1% of GP, whose production varied from 1.4 to 7.5% among plants. Mean production of GP was not statistically different among cultivars (Table 1). All plants had pollen viability near or over 80%. GP grains were 39.6% larger than normal ones (standard deviation of 0.071).

From the 1421 flowers pollinated in this cycle, 67.4% produced seeds, resulting in 958 full F_1 seeds, which gave rise to the F_1 plants of the second cycle. Percentage of pollinated flowers that produced seeds varied from 16 to 100% among mother plants (data not shown).

Second selection cycle

A total of 106 adult F_1 plants belonging to 19 F_1 families were evaluated for the production of GP and 43 (40.6%) plants belonging to 12 F_1 families, which produced at least 2% of GP were selected. As these plants belonged to 12 different half-sib families, there were 12 different treatments at this stage. A high variation for GP production among plants was verified (2.0-51.0%) and statistical differences for GP production were detected among some families (Table 2). All plants had pollen viability near or over 80% and GP were 30.9% larger than normal ones (standard deviation 0.05).

A total of 975 F_2 seeds were recovered from 55.6% of the 1752 pollinated flowers. Percentage of pollinated flowers that produced seeds varied from 18-88% among plants (data not shown).

Third selection cycle

The 975 seeds from the second cycle originated 500 F_2 plants, 200 of which could be analysed regarding GP production. In this cycle, the 55 plants which produced at least 3% GP, and were therefore selected, belonged to 18 F_2 half-sib families. Consequently, there were 18 treatments. GP production among plants ranged from 3.0 up to 46.7% (Table 3). Pollen fertility for all plants was, as in the other selection cycles, near or over 80% and GP were 32% larger than normal ones (standard deviation of 0.07).

A total of 2046 flowers from 82 inflorescences from 28 of the selected plants were manually cross-pollinated and from these flowers 60.1% produced a total of 1230 F_3 seeds, that were stored for future use. Percentage of pollinated flowers that produced seeds ranged from 5-100% among plants (data not shown).

Table 1. Results from the first selection cycle for production of giant pollen grains (GP) in three red

Original population	Number of analyzed plants	Number of selected plants	Mean of GP %	Variation of GP production among the selected plants %
Quiñiqueli	78	20	3.57 a[1]	1.9 – 5.1
Redland	41	20	4.12 a	1.4 – 7.5
Keenland	25	13	3.89 a	2.3 – 7.2
Total/average	144	53	3.86	

[1]Means followed by same letter do not differ statistically by the Tukey test at 5% significance

Table 2. Results from the second selection cycle for production of giant pollen grains (GP) in three red clover cultivars

Original mother plant	Derived F_1 families, from which plants were selected	Number of analyzed F_1 plants	Number of selected F_1 plants	Mean of GP %	Variation of GP production among the selected plants %
R4	B	13	2	27.47 a[1]	24.1-30.8
Q178	Q	2	1	20.07 b	-
R27	G	14	4	15.45 bc	2.0-51.0
K78	H	4	2	8.09 cd	4.9-11.2
R1	A	3	1	7.60 d	-
K88	J	9	6	7.52 de	3.9-11.1
R14	D	17	9	5.71e	2.0-15.0
K52	O	1	1	4.47e	-
R59	E	8	7	4.55e	2.0-9.1
K86	I	7	4	4.20 ef	2.3-7.3
R60	F	9	2	3.30 f	2.9-3.7
R11	C	10	4	2.30 f	2.1-3.3
R49	-	1	0	-	-
K9	-	1	0	-	-
K49	-	2	0	-	-
Q25	-	1	0	-	-
Q54	-	2	0	-	-
Q159	-	1	0	-	-
Q274	-	1	0	-	-
Total	-	106	43	-	-

[1]Means followed by the same letter do not differ statistically by the Tukey test at 5% significance

Table 3. Results from the third selection cycle for production of giant pollen grains (GP) in three red clover cultivars

F_1 mother-plant	Number of analyzed F_2 plants	Number of selected F_2 plants	Mean of GP %	Variation of GP production among the selected plants %
G78	5	3	28.31 a[1]	3.3-46.7
J99	8	1	28.07 ab	-
D47	2	1	16.67 bc	-
H85	5	1	14.87 cde	-
G69	7	3	8.73 cde	6.1-12.8
E48	32	13	8.51 cde.	3.0-31.2
F61	9	2	8.50 def	3.0-14.0
J100	23	5	6.62 ef	3.1-11.3
D40	9	4	6.35 ef	4.07-9.0
J104	5	1	5.87 ef	-
Q119	20	7	5.76 ef	3.0-13.8
A1	6	1	4.87 efg	-
D22	8	2	4.60 efg	3.3-5.8
G76	3	1	3.90 fg	-
E50	10	3	3.44 fg	3.1-3.8
B6	13	1	3.40 fg	-
C20	4	1	3.40 fg	-
E55	12	5	2.52 g	3.1-5.7
C14	6	0	-	-
D24	5	0	-	-
H88	1	0	-	-
J102	7	0	-	-
Total	200	55		

[1]Means followed by the same letter do not differ statistically by the Tukey test at 5% significance

General analysis

Selection gains and selection differentials varied among cultivars (Table 4). Taking the three cultivars together it can be seen that there was a significant increase in the production of GP from the first (4.0%) to the third (8.9%) selection cycles (Table 5). However, analysing the performance of each cultivar in each cycle and in the three cycles together, differences among them were detected. Statistical differences for a higher total percentage of GP production were detected between Redland and the other two cultivars (Table 6). Quñiqueli and Redland presented statistically significant different productions of GP for two and for all the three cycles, respectively, while production in the three cycles was not statistically different for Keenland (Table 6). It should be stressed that for Quiñiqueli, in the second cycle, only one plant produced at least 2% GP, but it was a very high production (20.1%), which could have biased the results leading to the high SG for this cultivar in the second cycle (Table 4). Redland presented not only higher percentages of GP production in each cycle (4.8, 13.4 and 19.7%, respectively) but also a significant and steady increase in GP production along the three selection cycles (Table 6).

Table 4. Selection gains and selection differentials for the increase of giant pollen grain production in each cultivar and in each selection cycle

	Cycle I			Cycle II			Cycle III		
	Q	R	K	Q	R	K	Q	R	K
SG	264.28	103.96	91.58	485.13	103.96	63.16	141.0	179.33	401.96
SD	2.59	2.1	1.85	16.64	4.18	2.28	3.37	5.38	8.2
T	78	41	25	7	75	24	20	131	49

Q: cv Quiñiqueli; R: cv. Redland; K: cv. Keenland; SG: selection gain (%); SD: selection differential (%); T: total number of plants analyzed

Table 5. Mean frequency of giant pollen grains (GP) production in each cycle in the selected plants

Selection cycle	Averages of GP production%
Cycle I	4.00 c[1]
Cycle II	6.42 b
Cycle III	8.97 a

[1]Means followed by the same letter do not differ statistically by the Tukey test at 5% significance

Table 6. Performance of each original cultivar per cycle considering giant pollen grain (GP) production in the selected plants

Cycles	Averages of GP production %		
	Quñiqueli	Redland	Keenland
I	4.20 a[1]	4.84 c	2.97 a
II	1.18 b	13.41 b	4.68 a
III	2.37 b	19.72 a	4.82 a
Average	2.58 B	11.66 A	4.16 B

[1]Means followed by the same letter do not differ statistically by the Tukey test at 5% significance

DISCUSSION

The difference in size of about 30% between reduced and unreduced gametes we have found agrees with literature data reporting that 2n gametes are generally 30 to 40% greater than normal ones (Hermsen 1984b, Ramanna 1992). Therefore, our data confirm that phenotypic analysis based on pollen grain size is a reliable method to detect possibly unreduced gametes.

Results show that the selection procedure used in the present work was successful in increasing the frequency of GP production in the studied germplasm. Plants with exceptionally high GP production were detected in some selected families (Tables 2 and 3) and we managed to increase the average frequency of GP production up to 8.9% in three generations in the general population (Table 5) and up to 11.7% in one cultivar (Table 6). These values are smaller than those obtained by Parrott and Smith (1984), who evaluated six red clover cultivars and, after three cycles of recurrent phenotypic selection raised the 0.04% GP production in the original population to 47.4% at the end of the third cycle. However, it should be noted that they detected plants producing up to 84% GP already in the first cycle, whereas the highest individual GP production in our population in the first cycle was 7.5% (Table 1). Therefore, the amount of increase in GP production seems to depend on the genetic ability of GP production of the original population. Variations in the percentage of GP production among plants of a given family may be due to environmental effects or to different degrees of penetrancy and expressivity of the alleles responsible for this production (Carputo et al. 2003). This may explain the high variation in GP production among families and cycles in our work. Hermsen (1984a) commented that the great influence of micro and macro environmental conditions in the occurrence and frequency of 2n gametes fluctuation in most of the genotypes complicates genetic analysis. Other authors also report a wide

variation in the frequency of 2n gamete production, such as less than 1 up to 35% (Bretagnolle and Thompson 1995), or 1 up to 40% (Carputo et al. 2003). Parrott and Smith (1986) explained that the detected variation was also due to different sample sizes and maternal effect.

In conclusion, selection for increasing GP production in red clover was successful. Among the three cultivars we have tested, Redland is the best one not only for presenting the best performance for GP production but also for presenting a steady and increasing progress along the three cycles. This cultivar has a good potential as a source of plants producing unreduced gametes and should be included in further efforts to obtain sexual polyploids in red clover. As a sequence for the present work, further selection cycles, as well as a new experiment, with larger populations, are being planned.

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Seleção para incremento da produção de gametas 2n em trevo vermelho

RESUMO - *Três ciclos de seleção para incremento da produção de gametas 2n foram realizados nos cultivares Quiñiqueli, Redland e Keenland de trevo vermelho. A produção de gametas não-reduzidos foi estimada pela produção de grãos de pólen gigantes (GP). Na população original, plantas com mais de 1% de GP foram selecionadas como progenitores. Para o segundo e terceiro ciclos, plantas com no mínimo 2% e 3% de GP foram, respectivamente, selecionadas e cruzadas. A produção de GP variou de 4,0??? a 7,5% no primeiro, 2,0 a 51,0% no segundo e 3,0 a 46,7% no terceiro ciclo. Considerando os três cultivares em conjunto, houve um significativo aumento da produção de GP do primeiro (4,0%) ao terceiro (8,9%) ciclos. 'Redland' apresentou as maiores percentagens de GP em cada ciclo e um significante e constante aumento na produção de GP ao longo dos três ciclos: 4,8, 13,4 e 19,7%, respectivamente, devendo ser incluído em trabalhos futuros para obtenção de poliplóides sexuais.*

Palavras-chave: duplicação cromossômica meiótica, poliploidização sexual, *Trifolium pratense*, gametas não reduzidos.

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