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S. W. Hussain · W. M. Williams
C. F. Mercer · D. W. R. White

Transfer of clover cyst nematode resistance from *Trifolium nigrescens* Viv. to *T. repens* L. by interspecific hybridisation

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Abstract Resistance to clover cyst nematode (*Heterodera trifolii*) has been successfully transferred from *Trifolium nigrescens* to *T. repens* by interspecific hybridisation. A sterile triploid hybrid (H-6909-5, $2n = 3x = 24$) was initially produced with the aid of embryo culture. The hybrid was chromosome-doubled from axillary meristems by an in vitro colchicine method. Three chromosome-doubled plants were obtained, and these showed a marked increase in pollen stainability from 10% in 3*x* H-6909-5 to an average of 89% (range 88–91%) in 6*x* H-6909-5. *T. nigrescens* was a source of clover cyst nematode resistance. A mean of 23 (range 0–150) cysts per plant was recorded for *T. nigrescens* in comparison to a mean of 150 (range 50–240) cysts per plant for *T. repens*. The 3*x* and 6*x* interspecific hybrids were shown to be as resistant as the most resistant. *T. nigrescens* genotype and were significantly lower in cyst number per gram of root dry weight than the susceptible *T. nigrescens* and *T. repens* genotypes.

Key words *Trifolium* spp · Interspecific hybrids · Nematode · Chromosome doubling · *Heterodera trifolii*

Introduction

Clover cyst nematode (*Heterodera trifolii* Goffart) is a serious pest of white clover (Cook and Yeates 1993; Mercer and Watson 1996) which depresses both yield and the rate of nitrogen fixation (Yeates 1977; Watson et al. 1994). While control measures other than crop rotation and host resistance seem impracticable (Skipp and Gaynor 1987), crop rotation is rarely an option for clover-based pastures, and resistant cultivars are not yet available. Although some resistance to clover cyst nematode has been found in *T. repens* (van den Bosch and Mercer 1997) its nature and durability are unknown. Consideration has therefore been given to sources of resistance from other species of *Trifolium* (Litz 1986; Boerma and Hussey 1992).

T. nigrescens Viv., is an annual, diploid ($2n = 2x = 16$), non-stoloniferous and free-seeding species occurring in natural pastures of the Mediterranean area (Gillett 1985; Britten 1963; Pritchard 1969). Although it has not yet been used as a source of germplasm for white clover improvement, this species has been shown to have resistance to clover cyst nematode (Mercer 1988) and could be used in hybridisation with white clover for incorporating clover cyst nematode resistance into white clover germplasm.

T. repens and *T. nigrescens* cross with some difficulty, although certain combinations of plants produce large number of hybrids (Williams et al. 1978; Marshall et al. 1995). The cross can be more successful when *T. repens* is used as the female parent (Kazimierski and Kazimierska 1970; Hovin 1962). Hybrids between *T. repens* and *T. nigrescens* have also been obtained by Keim (1953a,b), Evans (1962) and Trimble and Hovin (1960). These authors have reported that hybrid plants were intermediate in morphology between the parent species and showed low fertility. Hybrids were weakly perennial, rooted infrequently at the nodes and may have been more susceptible to viruses than *T. repens* (Gibson et al. 1971). Studies of chromosome pairing at meiosis in *T. repens* × *T. nigrescens* hybrids indicate some homology between the chromosomes of the two species (Hovin 1962; Chen and Gibson 1970; Brewbaker and Keim 1953; Hussain et al. 1997).

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S. W. Hussain · W. M. Williams (✉)
C. F. Mercer · D. W. R. White
AgResearch Grasslands, Private Bag 11008, Palmerston North, New Zealand
Fax: + 64 6 351 8047
E-mail: williamsw@agresearch.cri.nz

There are no published reports on the evaluation of *Trifolium* interspecific hybrids for resistance to *H. trifolii*. Pederson and Windham (1989), however, evaluated seven interspecific *Trifolium* hybrids for resistance to the southern root-knot nematode (*Meloidogyne incognita*). Hybrids with mean gall indices greater than 2 were (*T. repens* × *T. nigrescens*) × *T. repens*, *T. isthmocarpum* × *T. repens* and *T. repens* × *T. uniflorum*. Because of its cross-fertility and high level of resistance, Pederson and Windham (1989) found that *T. nigrescens* may be a valuable source of resistance to *M. incognita* for white clover improvement.

Interspecific *Trifolium* hybrids range in fertility from highly sterile to partially fertile. Wide variation is observed not only for different interspecific combinations but also for different F_1 derivatives and backcrosses of the same combination (Hovin 1962; Williams and Verry 1981; Pandey et al. 1987). Gametic sterility in hybrids may be chromosomal and/or genetic (Williams 1987). Chromosomal sterility is associated with gross meiotic abnormality resulting from the failure of distantly related genomes to pair or from the presence of an uneven number of genomes of a different basic number. Alternatively, sterility may occur even in the presence of effective chromosome pairing as a result of physiological or genetic disturbances during gametogenesis. When sterile intergeneric or interspecific hybrids are obtained, chromosome doubling is often attempted to restore the fertility of the hybrids (Hovin 1963; Williams et al. 1982). Chromosome doubling does not necessarily restore full fertility immediately if genomic incompatibility (genetic sterility) is also present. For example, colchicine doubling of the chromosomes of a highly sterile diploid hybrid of *Ornithopus pinnatus* × *O. sativus* restored only 9% pollen fertility, which increased to 18% at the F_4 generation (Williams and DeLautour 1981).

Chromosome doubling of clovers can be readily achieved with aqueous colchicine by the immersion of germinating seeds (Evans 1955; Berthaut 1965) or of the shoot apex of germinating seedlings (Brewbaker 1952). However, chromosome doubling at the mature vegetative stage is more difficult. Apical and axillary meristems are protected by sheathing-leaf bases, so that the immersion of cuttings in a colchicine solution has had only limited success (Brewbaker 1952; Evans 1955; Berthaut 1965). Treatment of the mature flowering plant with nitrous oxide under pressure has given higher percentages of chromosome doubling (Berthaut 1968; Taylor et al. 1976) as has spontaneous chromosomal non-reduction during meiosis (Taylor and Giri 1983; Parrott and Smith 1984), but these techniques are limited to genotypes with the ability to produce seeds. Anderson et al. (1991) applied an in vitro chromosome doubling method to clover species and their hybrids. This technique combined colchicine treatment with shoot proliferation to enhance the frequency of meristematic target cells.

The objectives of the research presented here were to transfer clover cyst nematode resistance from *T. nigrescens* to *T. repens* through interspecific hybridisation, to double the chromosome number of the triploid hybrid by in vitro colchicine treatment and to test the resulting plants for fertility and clover cyst nematode resistance.

Materials and methods

Chromosome-doubling method

An individual interspecific hybrid was obtained following the pollination of *T. repens* ($2n = 4x = 32$; plant 96EXO, cv 'El Lucero') with *T. nigrescens* ($2n = 2x = 16$; plant 6893/3-1, selected from Az 2225 for clover cyst nematode resistance, method given later). Fourteen days after pollination, 12 embryos were rescued from 13 pods and placed on EG medium (Przywara et al. 1989). Six plantlets grew to maturity, of which only 1 ($3x$ H-6909-5) survived, and this plant was resistant to clover cyst nematode.

A modification of the in vitro technique reported by Anderson et al. (1991) was used to double the chromosome number of $3x$ H-6909-5. When the vegetatively propagated plants of $3x$ H-6909-5 had developed several stolons, stem segments with nodes were removed from the plants, trimmed to remove most of the leaf material, cut into about 3-cm segments and washed with tap water in a beaker. The stem segments were then trimmed above and below each axillary meristem to a total length of approximately 5 mm. Segments were twice surface-sterilised by immersion in 70% ethanol for 1 min followed by rinsing with sterile water for 1 min. Segments were then further surface-sterilised in 5% sodium hypochlorite (10% available chlorine) for 4 min, followed by five rinses of 1 min each in sterile water. Subsequent manipulations were carried out under aseptic conditions in a laminar flow hood. The stem segments were further trimmed on a presterilised filter paper to leave the axillary meristem and a minimal amount of subtending tissues (about 0.5–1 mm total length).

WCSP (white clover shoot proliferation) medium was used for in vitro culture of meristems. This medium is a modification of B5 (Gamborg et al. 1968) and was prepared by adding 0.15 mg of 6-(γ-γ dimethylallylamino)-purine (2iP) and 0.15 mg of indole-3-acetic acid (IAA) to 1 l of medium. The final pH was adjusted to 5.5 with 0.2 *N* KOH or 0.2 *N* HCl and the medium solidified with 0.8% agar.

Trimmed, surface-sterilised meristems were precultured, 10–15 meristems per petri dish, on 25 ml of medium without colchicine in 22 × 85-mm presterilised plastic petri dishes for 7 days. Dishes were incubated at 23°–25°C under cool white fluorescent tubes (approx. $10\ \mu Em^{-2}s^{-1}$) except during the colchicine treatment. During the preculture period, meristems with bacterial and fungal contaminations were discarded, and the clean meristems were frequently transferred to fresh medium.

Five treatments, comparing different concentrations and durations of colchicine application, were applied to the precultured meristems (Table 1). The WCSP media containing 0.1% and 0.05% colchicine were prepared by mixing 50 ml and 25 ml, respectively, of filter-sterilised 2% aqueous colchicine solution in 950 ml and 975 ml of the medium shortly after autoclaving. All meristems which had received colchicine treatments and the untreated control were incubated in the dark at 4°C for the respective time period. Following this, meristems were placed on fresh WCSP medium, and the dishes were incubated at 23°–25°C under cool white light (approx. $10\ \mu Em^{-2}S^{-1}$) for the remaining period of culture. The meristems were periodically transferred to fresh medium.

Meristems remained in the medium for 5–7 weeks during which time they developed 3–5 roots and one or two shoots. They were then transferred to presterilised moist sand in 6.5-cm diameter

Table 1 Responses of the *T. repens* × *T. nigrescens* (3*x* H-6909-5) F_1 hybrid to protocols for in vitro chromosome doubling[a]

Treatment[b]	Number meristems cultured	Meristems bleached		Meristems contaminated[c]		Meristems surviving		Meristems with doubled chromosomes[d]	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
C_1 0.1%, 48 h	152	68	(45)	27	(18)	0	(0)	0	(0)
C_2 0.1%, 72 h	170	43	(25)	48	(28)	0	(0)	0	(0)
C_3 0.05%, 48 h	68	29	(43)	19	(28)	14	(21)	1	(1.5)
C_4 0.05%, 60 h	70	33	(47)	15	(21)	18	(26)	2	(3)
C_5 0.05%, 72 h	133	56	(42)	23	(17)	0	(0)	0	(0)
C_6 0.0%, –	57	21	(37)	13	(23)	22	(39)	0	(0)
Mean		40		23					

[a] Colchicine was applied at 0.1% or 0.05% in solidified WCSP medium
[b] Concentration and duration of colchicine application
[c] Bacterial and fungal
[d] Based on meristems that survived from initial culturing until transfer to pots in the glasshouse

plastic pots. The pots were covered with a plastic bag and kept in a growth chamber at 16°C with a 16-h photoperiod for 4 weeks. The young plants were supplied with half-strength (0.9 g/l) "Thrive" (N : P : K 27 : 5.5 : 9) every 14 days. After 4 weeks in the growth chamber, plants were transferred to 10-cm diameter plastic pots containing potting mix and were kept in the glasshouse.

Chromosome-doubled plants were identified by chromosome counts in root-tip meristems and pollen mother cells (PMCs) as well as by pollen-fertility estimates and morphology of dry pollen grains using procedures described by Hussain et al. (1997).

Screening for clover cyst nematode resistance

Seeds of *T. nigrescens* (Az 2225) and *T. repens* cv 'Grasslands Huia' (C6484) were obtained from the Margot Forde Forage Germplasm Centre, AgResearch Grasslands, Palmerston North, New Zealand. *T. nigrescens* is a diploid ($2n = 2x = 16$) accession of ssp *nigrescens* obtained from Perugia, Italy, as exchange germplasm. 'Grasslands Huia' is a commercial white clover cultivar ($2n = 4x = 32$) and a known host to clover cyst nematode (Mercer 1988).

Glasshouse cultures of clover cyst nematodes were initiated from material collected from pastures at Fitzherbert West, Palmerston North, New Zealand, and were maintained on white clover. Inoculum was prepared from these cultures using the method described by Mercer (1990).

Seedlings of *T. nigrescens* and *T. repens* were initially screened for clover cyst nematode resistance/susceptibility to identify resistant and susceptible genotypes in both species for comparison with 3*x* and 6*x* H-6909-5.

Fifty germinating seeds of *T. nigrescens* and 20 germinating seeds of *T. repens* were transferred to a pasteurised sand/soil mix in 6.5-cm diameter plastic pots, with 1 germinating seed per pot. The pots were randomly arranged in 40 × 80-cm steel trays with 35 pots per tray, and the trays were kept in a glasshouse at a soil temperature of 18°–25°C and natural daylight for the whole growing period. Water was added to the trays as required so that pots were moistened by capillary action. Nutrients were applied every 14 days using a half-strength solution of "Thrive". The trays were drained using capillary wicks.

Seedlings were inoculated by syringing a 3-ml suspension of about 2000 eggs into a hole angled under a 2-week-old seedling. During inoculation the egg suspension was continuously stirred to get reasonably uniform numbers of eggs for each pot inoculated. The inoculated plants remained in the glasshouse at a soil temperature of 18°–25°C and in natural daylight.

Eight weeks after inoculation, roots of inoculated plants were washed free of soil in an elutriation apparatus (Wood and Foot 1977) and the cysts collected in a clean 180-μm sieve. Cysts were transferred to a Doncaster dish, and the number of cysts obtained from each plant was counted with the aid of a light microscope. Data were recorded as numbers of cysts per plant.

For rescreening, 10–15 stem cuttings of 2–4 cm in length from each of 16 surviving *T. nigrescens* and 5 *T. repens* genotypes (included as a test of the inoculum) were planted in plastic trays containing potting mix. The trays were kept in the glasshouse. Three weeks after planting, at least 5 cuttings (where available) of each plant with 5–10 roots were uprooted from the plastic trays, and the roots thoroughly washed free of potting mix and trimmed to an approximately uniform size of 2–3 cm. The plants were then planted in 6.5-cm diameter plastic pots containing a pasteurised soil/sand mix. Pots were randomly placed in steel trays with a maximum of 35 pots per tray and inoculated. The growing conditions and inoculation procedures were the same as for the initial screening. Eight weeks after inoculation, the numbers of cysts per plant were counted by the method described for the initial screening.

Based on the comparison of cyst numbers for each of the 16 plants of *T. nigrescens* and 5 plants of *T. repens* in the two screenings (Table 3), 2 plants of *T. nigrescens* (1 resistant and 1 susceptible) and 1 plant of *T. repens* (susceptible) were selected for comparison with 3*x* and 6*x* H-6909-5 in the next screening. As *T. nigrescens* is an annual species, the 2 plants were kept alive by frequent vegetative propagation from stem cuttings in a temperature-controlled glasshouse.

Ten stolon cuttings of 3*x* H-6909-5 and 20 cuttings (10 each from 2 plants) of colchicine-doubled 6*x* H-6909-5 were included for clover cyst counts using the procedure described for rescreening *T. nigrescens* and *T. repens*. Twelve stem cuttings from 2 previously screened *T. nigrescens* genotypes (1 with a low and the other with a high number of cysts) and 6 stolen cuttings from one *T. repens* genotype (with a high number of cysts) were used as controls and tests of inoculum, respectively. The experiment was completely randomised. After washing out the cysts the roots were detached from the shoots, oven-dried at 80°C for 24 h and weighed. Data were first recorded as numbers of cysts per plant and were then calculated as cysts per gram root dry weight.

Data for cyst number per plant after the rescreening of *T. nigrescens* and *T. repens* genotypes were analysed according to a completely randomised design with equal replication by the use of analysis of variance. Genotype means were compared using Fisher's LSD test at the 5% level of probability.

Data for numbers of cysts per gram root dry weight were analysed according to a completely randomised design with unequal

replication by the use of analysis of variance. Genotype comparisons were performed using Duncan's multiple range test at the 5% probability level.

Results

Chromosome doubling

Responses of the detached meristems of the triploid *T. repens* × *T. nigrescens* interspecific F_1 hybrid (3*x* H-6909-5) to different colchicine treatments for in vitro chromosome doubling are presented in Table 1.

Treatments C_1, C_2 and C_5 killed all meristems, whereas treatments C_3 and C_4 gave very similar results for meristem survival. Apart from bacterial and fungal contamination, which was assumed to be random, death of the meristems was due partly to the bleaching effect of surface sterilisation and partly to the toxic effect of the colchicine treatment. The bleaching effect was evident during the preculturing period prior to colchicine application, during which time between 25% and 47% (average 40%) of the meristems died. The chromosome-doubling frequency of meristems was 1.5% (7% of the surviving meristems) for treatment C_3 and 3% (11% of the surviving meristems) for treatment C_4. In total only 3 plants, 1 from treatment C_3 and 2 from treatment C_4, out of 32 (approx. 10%), showed doubled chromosomes. No chromosome-doubled plants were obtained from the control treatment C_6.

Identification of chromosome-doubled plants

The doubling of chromosomes in the 3 plants (designated as CT-1, CT-14 and CT-28) obtained after colchicine treatment was confirmed in at least ten cells from 5–10 root tips for each plant by the presence of $2n = 6x = 48$ chromosomes (Fig. 1B) in contrast to the triploid hybrid (3*x* H-6909-5) which had a 2*n* chromosome number of 24 (Fig. 1A). Chromosome doubling in the shoot was confirmed by the presence of 48 chromosomes in the PMCs of all 3 colchicine-induced autoallohexaploids (6*x* H-6909-5). Somatic chromosomes from root-tip squashes were also counted for 3 other colchicine-derived plants showing low pollen stainability (average 8.9%) and a cylindrical or oval dry pollen shape. These plants showed an undoubled number of $2n = 24$.

Examination of dry pollen shape was found to be a rapid method for identifying chromosome-doubled shoots of colchicine-derived plants. The triploid *T. repens* × *T. nigrescens* hybrid (3*x* H-6909-5) with $2n = 3x = 24$, produced cylindrical to oval pollen grains (Fig. 1C). Pollen grains from the autoallohexaploid 6*x* H-6909-5 were found to be tetrahedral (Fig. 1D).

Pollen stainability was markedly increased in the colchicine-induced autoallohexaploid of H-6909-5. The 3 colchicine-induced hexaploid (6*x*) plants had an average of 89.2% pollen stainability in contrast to only 9.9% for the triploid hybrid (3*x* H-6909-5) (Fig. 1E, F).

Description of 3*x* and 6*x* H-6909-5

Trifolium repens × *T. nigrescens* H-6909-5 showed similar morphological characters at both the triploid and hexaploid levels except for leaflet and inflorescence sizes which were comparatively larger for 6*x* H-6909-5 than 3*x* H-6909-5. The inflorescence size of 3*x* H-6909-5 was intermediate between that of *T. repens* and *T. nigrescens* while that of the 6*x* H-6909-5 was similar to that of *T. repens*. However, both ploidy levels of H-6909-5 exhibited the stoloniferous growth habit of *T. repens* with long internodes, but with infrequent root primordia and nodal roots only at the basal two to three nodes. The 3*x* and 6*x* plants of H-6909-5 flowered profusely like the *T. nigrescens* parent, and flowering continued through the year. Self-pollination of 50 inflorerscences of 3*x* H-6909-5 (more than 1200 flowers) and 20 inflorescences (more than 500 flowers) each from the 3 colchicine-induced hexaploid plants of H-6909-5 produced no seed, showing that 3*x* H-6909-5 and colchicine-derived 6*x* H-6909-5 plants were self-incompatible.

Clover cyst nematode resistance and susceptibility

Initial counts of *H. trifolii* cysts on *T. nigrescens* and *T. repens* (Table 2) confirmed the susceptibility of *T. repens* to clover cyst nematode and the effectiveness of the inoculum. Of the 50 initially screened *T. nigrescens* plants 27 (54%) had 0–10 cysts per plant, while 80% of *T. repens* plants had more than 100 (a range of 104–239) cysts per plant. The remaining 20% of *T. repens* plants ranged from 49 to 69 cysts per plant.

Mean numbers of cysts recorded for 16 *T. nigrescens* genotypes and 5 *T. repens* genotypes after rescreening (Table 3) were reasonably consistent with the initial screening. Significant differences in cyst number occurred among the 16 *T. nigrescens* genotypes. Six *T. nigrescens* genotypes had significantly fewer cysts than the most resistant *T. repens* genotype, while 4 of the 5 *T. repens* genotypes had significantly more cysts per plant than all 16 *T. nigrescens* genotypes. Based on the results of both screenings (Table 3), 2 genotypes of *T. nigrescens*, Tn-14 and Tn-30, with high and low numbers of cysts, respectively, and 1 genotype of *T. repens*, Tr-10, with a high number of cysts, were selected and included for comparison in the screening of triploid and hexaploid *T. repens* × *T. nigrescens* F_1 hybrids (H-6909-5).

The 3*x* and 2 6*x* H-6909-5 plants (CT-1 and CT-14) were as resistant as the resistant *T. nigrescens* (Tn-30)

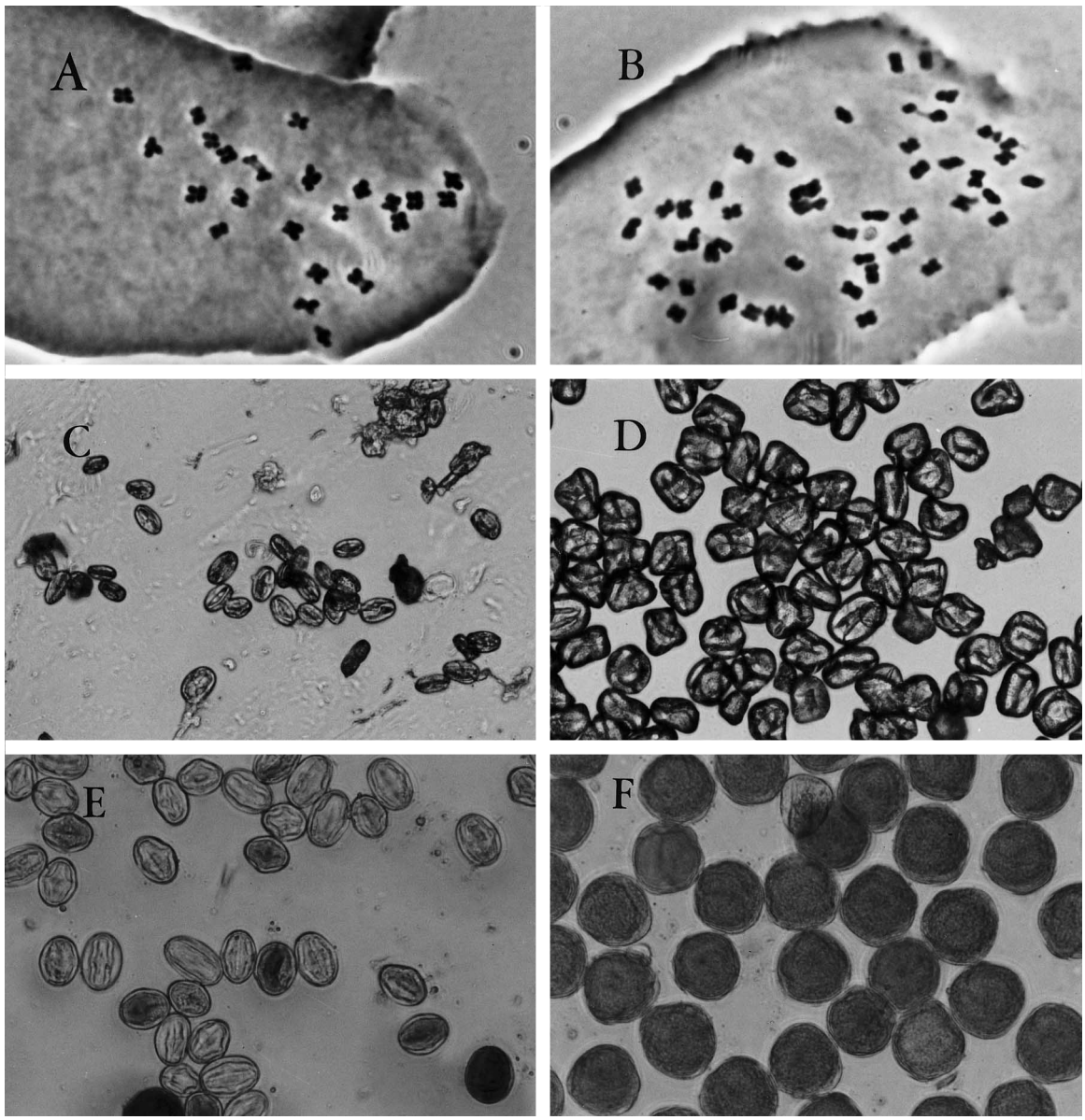

Fig. 1A–F Somatic chromosome numbers, dry pollen shapes and pollen stainabilities of 3*x* H-6909-5 (*left column*, **A, C, E**) and 6*x* H-6909-5 (*right column*, **B, D, F**). **A, B** × 1600, **C–F** × 300

genotype and were significantly lower in cyst number per gram of root dry weight than the susceptible *T. nigrescens* (Tn-14) and *T. repens* (Tr-10) genotypes (Table 4). The susceptible *T. nigrescens* genotype was significantly lower in cyst number per gram than the susceptible *T. repens* genotype.

Discussion

Chromosome doubling

The production of chromosome-doubled plants in the present study indicates the effectiveness of in vitro colchicine application for chromosome doubling. However, the chromosome doubling rates of 7% and 11% of the surviving meristems for treatments C_3 and C_4, respectively, were lower than the 44% obtained by Anderson et al. (1991) for a *Trifolium ambiguum* × *T. repens* hybrid. This difference could have been due to the lower colchicine concentration (0.05% as against 0.1% used by Anderson et al. 1991) used for treatments C_3 and C_4. However, increasing the concentration and/or time of application of colchicine had a severe toxic effect on the growth of *T. nigrescens*-based hybrid meristems in culture, as was evident from treatments C_1, C_2 and C_5. These results are in contrast to those obtained by Anderson et al. (1991) who did not observe significant differences for two durations of 48 and 72 h with 0.1% in vitro colchicine application to *Trifolium* interspecific hybrids. The present results (Table 1)

Table 2 Mean number and range of *H. trifolii* cysts produced on two species of *Trifolium* after initial screening

Species	Accession no.	Number of plants tested	*H. trifolii* cyst number per plant	
			Mean	Range
T. nigrescens	Az 2225	50	23	0–150
T. repens cv Grasslands Huia	C 6484	20	150	49–239

Table 3 Comparison of the number of *H. trifolii* cysts per plant after initial and rescreening of 16 *T. nigrescens* and 5 *T. repens* genotypes

Species	Plant no.	Number of cysts after	
		Initial screening[a]	Rescreening[b]
T. nigrescens[c] (Az 2225)	Tn-4	2	9
	Tn-8	38	31
	Tn-9	1	3
	Tn-14	140	89
	Tn-17	2	20
	Tn-20	1	28
	Tn-26	0	8
	Tn-27	150	60
	Tn-29	44	33
	Tn-30	0	1
	Tn-32	0	23
	Tn-36	0	4
	Tn-39	9	51
	Tn-42	12	31
	Tn-46	10	48
	Tn-48	3	9
T. repens (C 6484)	Tr-5	54	49
	Tr-10	211	199
	Tr-12	49	117
	Tr-14	236	165
	Tr-19	69	126
LSD ($P < 0.05$)			31

[a] Number of cysts per plant
[b] Mean of five cuttings for each genotype
[c] Plants which survived after the initial screening

Table 4 Number of *H. trifolii* cysts produced on *T. nigrescens*, *T. repens* and 3*x* and 6*x* H-6909-5

Species/hybrid	Plant no.	Number of plants tested	Mean number of cysts/g of RDW[a]
T. repens (C 6484)	Tr-10	6	522.8 a[b]
T. nigrescens (Az 2225)	Tn-14	6	340.3 b
T. nigrescens (Az 2225)	Tn-30	6	9.6 c
3*x* H-6909-5	–	10	6.1 c
6*x* H-6909-5	CT-1	10	3.2 c
6*x* H-6909-5	CT-14	10	2.6 c

[a] RDW, Root dry weight
[b] Means with a common letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test

suggest that *T. nigrescens*-based hybrids may be more sensitive to colchicine, both with respect to the concentration and duration of application, than the *T. ambiguum* hybrids used by Anderson et al. (1991). This emphasises the need to determine specific culture conditions for each new set of material before in vitro meristem culture for colchicine-mediated chromosome doubling is carried out.

The high meristem mortality rates (25–47%) during the preculture period in all six treatments were in large part due to the bleaching effect of the method used for surface sterilisation. However, reducing the concentration of sodium hypochlorite from 5% to 4% resulted in almost 100% microbial contamination. Some of the bacterial and fungal contaminations observed in the present investigation may have been internal in origin and presumably could have been reduced by the addition of antibiotics such as sodium cefotaxime (Anderson et al. 1991). Although the bleaching effect of surface sterilisation on most of the meristems in culture was quite obvious after 10–12 h of preculturing, it was uncertain whether the meristem mortality rate of between 25% and 47% in all the six treatments during preculture was due only to the bleaching effect, or to a combination of bleaching and sensitivity of the meristems to preculturing conditions. Anderson et al. (1991) also observed a significant reduction in meristem survival during preculturing. It is likely that the surface-sterilisation technique and the preculturing periods could be further refined to increase the meristem survival rate, which should eventually result in a higher frequency of chromosome-doubled plants. This requires further investigation.

Identification of chromosome-doubled plants

The chromosome-doubled plants did not exhibit marked morphological differences from 3*x* H-6909-5, with the exception of leaflet and inflorescence size. Alteration in leaflet size and shape as a result of polyploidy were also observed by Anderson et al. (1991) for *T. ambiguum* × *T. repens* hybrids, and by DeRoo (1975) for red clover, where colchicine-doubled plants in both cases had larger and more rounded leaflets than the undoubled plants.

Dry pollen shape was found to be a rapid method for identifying the chromosome-doubled plants. Taylor

et al. (1976) in red clover and Anderson et al. (1991) in *T. ambiguum* × *T. repens* hybrids also observed altered pollen shape and size as a result of chromosome doubling. The present results are consistent with these reports.

The chromosome-doubled plants of H-6909-5 in the present study showed marked improvement in fertility, as estimated by pollen stainability. These results are consistent with those reported by Brewbaker and Keim (1953), who recorded 90% pollen stainability for a hexploid *T. nigrescens* × *T. repens* hybrid obtained after doubling the chromosomes of the parental species before hybridisation, and Hovin (1963), who reported greater than 90% pollen stainability for a colchicine-doubled hexaploid *T. repens* × *T. nigrescens* hybrid as against less than 20% for the triploid hybrids.

These results suggest that alteration in dry pollen shape and increased pollen stainability are two rapid methods for assessing chromosome doubling after colchicine treatment. Such methods should be supported, however, by chromosome counts because aneuploidy is a common phenomenon after chemical treatment for chromosome doubling (Taylor et al. 1976; Anderson et al. 1991). However, no aneuploidy was found in the present study, and so it is uncertain whether aneuploids would show altered pollen shape and stainability in this material.

Clover cyst nematode resistance/susceptibility

The present study reports for the first time the screening of interspecific *T. repens* × *T. nigrescens* hybrids for resistance to clover cyst nematode. Results of both the initial and rescreening tests of *T. nigrescens* and *T. repens* showed that *T. nigrescens* was more resistant to clover cyst nematode than *T. repens*. These results are consistent with those reported by Mercer (1988) who found a mean number of 1.6 (range 0–5) cysts per plant on *T. nigrescens* in contrast to a mean number of 500 cysts per plant for white clover. In the present experiment we considered plants with 0–5 cysts per plant as resistant. Consequently, 34% and 19% of the *T. nigrescens* plants were resistant in the initial and rescreenings tests, respectively. No resistant plant was observed for *T. repens* in either screening. The rescreening results were reasonably consistent with the initial screening, indicating the repeatability of the screening technique.

Both the 3*x* and 6*x* H-6909-5 showed the same degree of resistance to clover cyst nematode and were as resistant as the selected resistant *T. nigrescens* genotype. This high transmission of resistance from parent to hybrid in one generation was consistent with control by one or a small number of dominant genes and contrasted with white clover where resistance appears to be controlled by more than one gene (Dijkstra 1971).

The H-6909-5 hybrids were not evaluated for agronomic traits, but at both ploidy levels they appeared to be markedly inferior to white clover in growth, perenniality and fertility. Nevertheless the 6*x* H-6909-5 hybrid has provided potentially useful genetic material for backcrossing to *T. repens*. Fertile backcross progenies have been produced between 6*x* H-6909-5 and both of its parental species (Hussain et al. 1997).

Although Gibson et al. (1971) concluded that interspecific *T. repens* × *T. nigrescens* hybrids were not useful for the improvement of *T. repens* due to poor agronomic performance and lack of virus resistance, the present results suggest that *T. nigrescens* is a valuable species to hybridise with *T. repens* in order to improve the resistance of the latter to clover cyst nematode. The species was also reported to be resistant to *Meloidogyne incognita* (Pederson and Windham 1989). The advantages of using *T. nigrescens* in interspecific hybridisation with *T. repens* are the ease of producing interspecific hybrids (relative to other interspecific *Trifolium* hybrids), fertility of the backcrosses and the level of resistance to clover cyst nematode in *T. nigrescens* and H-6909-5. It is emphasised at this point that further investigations are needed to determine the genetic basis of resistance in *T. nigrescens* and the hybrid plants.

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