Reproductive characters of interspecific hybrids between *Trifolium repens* L. and *T. nigrescens* Viv.

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The amphidiploid *Trifolium repens* L. ($2n = 4x = 32$) is one of the most agronomically important of the 250–300 species in the genus *Trifolium*. Seed production is, however, variable and often poor, because of a number of interacting reproductive and climatic factors. Improvement to those characters influencing seed production may be achieved by interspecific hybridization using a closely related species within the genus. One species which has been demonstrated to have a close affinity with *T. repens* is the annual *T. nigrescens* ($2n = 2x = 16$), a prolific flower producer with a less intermittent pattern of flowering than *T. repens*. Specific cross-combinations of *T. repens* and *T. nigrescens* readily produced hybrids without the need of embryo culture. Some F_1 hybrids produced large numbers of seed when backcrossed to *T. repens*. Chromosome counts, isoenzymes and leaf markers confirmed that these were true hybrids. The F_1 s and BC_1 s were vigorous plants demonstrating intermediate reproductive characters inherited from the two parental species. This novel approach may be a means of extending the range of heritable variation useful in the breeding and selection of *T. repens*.

Keywords: interspecific hybrids, reproductive growth, *Trifolium nigrescens*, *Trifolium repens*.

Introduction

Trifolium repens L. (white clover), a self-incompatible perennial species ($2n = 4x = 32$), is the most important forage legume in temperate northern Europe. In a mixture with grasses it provides herbage of a high nutritive value and contributes a considerable quantity of nitrogen to the sward (Erith, 1924). The main objective of *T. repens* breeding programmes has been to improve important agronomic characteristics such as dry matter production and persistency. However, the commercial success of a variety also depends upon its ability to produce reasonable quantities of seed. Unfortunately, within the U.K. seed yields of *T. repens* are variable and often low (Marshall, 1994).

Several factors contribute to the low seed yields of *T. repens*. Climatic conditions, and in particular excessive rainfall during pollination and at harvest, have a major detrimental effect (Marshall, 1994). The growth of the plant during the reproductive phase is also important, as the indeterminate flowering and intermittent pattern of flowering along the stolon influence the number of ripe inflorescences at harvest and can cause variation in seed yield.

Inflorescences, the main seed yield component (Marshall, 1994), are produced from axillary buds in the axils of leaf primordia on stolons (Thomas, 1981). A stolon node may produce an inflorescence, may be vegetative and produce a secondary or tertiary stolon or remain dormant. Inflorescences may occur at one or two nodes along a stolon, with a gap of several nodes followed by more reproductive nodes (Thomas, 1981). Consequently, inflorescences are produced over a prolonged period and only a proportion of them will contain mature seed at any one time. These characteristics make it difficult for seed growers to optimize the harvest date, as only a proportion of the mature inflorescences is harvested (Marshall *et al.*, 1989).

Improvements to the characters that influence seed production may be achieved by selecting genotypes with the desired floral characteristics from within *T. repens* (Hill *et al.*, 1989). An alternative approach is to use interspecific hybridization. This is a means of increasing the genetic variability available for selection beyond the normal range of a single species and of combining useful traits from different species. Within the *Trifolium* genus, there are more than 250 species and a number of these, including *T. occidentale*, *T. uniflorum* and *T. ambiguum*, can produce occasional hybrids with *T. repens* (Gibson *et al.*, 1971; Williams,

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1978). *Trifolium nigrescens* Viv. (Ball clover), a self-incompatible annual ($2n=2x=16$) which has a number of useful reproductive characters, has also been shown to be cross-compatible with *T. repens* (Trimble, 1951; Keim, 1953; Hovin, 1962). It is a prolific producer of inflorescences and appears to have a less intermittent pattern of flowering than *T. repens*. Observations suggest it may also be particularly attractive to insect pollinators.

The present paper describes the development of hybrids between *T. repens* and *T. nigrescens* and compares the reproductive characters of the two species and their related hybrids. Particular attention is given to flowering characteristics and the relative influence of the *T. nigrescens* and *T. repens* genomes on these and on other traits associated with seed production.

Materials and methods

Production of hybrids

In summer 1990, individuals from three populations of *T. nigrescens* were crossed to a large number of *T. repens* genotypes from widely differing populations. With *T. nigrescens* as the pollen parent, most crosses produced some seed. No seed was produced in any of the reciprocal crosses. Embryo rescue techniques were used for the first few crosses, but this proved unnecessary, as healthy seed developed *in situ*. There was considerable variation in cross-compatibility, but two interspecific combinations of accessions gave consistently high seed set, averaging 1.6 seeds per pod.

Material for further work was derived from one of these two relatively successful combinations. Five hand-crosses were produced between Ac3715, a prolific inflorescence producer and high seed yielding population of *T. repens* collected from Southern Ireland and Ah1524, a population of *T. nigrescens* from Portugal. Table 1 shows that the seed set in each of the crosses was similar to the normal seed set found in *T.*

repens (Marshall, 1994). As *T. repens* is self-incompatible (Williams, 1925), all crosses were carried out without emasculation.

In April 1991, 80 F_1 hybrid seeds from the above hand-crosses were sown into a John Innes No. 2 loam-based compost in a glasshouse. The seed germinated readily, producing 50 healthy seedlings, and cytological examination confirmed that they were triploid ($3x=24$). When these hybrid plants began flowering they were placed outside the glasshouse and surrounded with a wide range of *T. repens* genotypes. Some of these white clover plants contained homozygous leaf markers (red leaf, red midrib and inverted V). Each of these distinctive leaf markers is controlled by a single dominant gene and was used as a genetic marker (Corkill, 1971). During the summer the plants were pollinated by bumble bees and honey bees. Ripe inflorescences were harvested from each of the 50 hybrid plants. Of these, 23 plants produced no seed, 12 plants produced 2–3 seeds, nine plants produced 30–40 seeds and six plants produced 100–200 seeds. Chromosome counts, isoenzyme analysis and leaf marker genes (see later) indicated that seed was produced as a result of backcrossing between the F_1 hybrids and *T. repens* rather than intercrossing among the F_1 s. Backcross seed (BC_1) from individual F_1 plants was bulked.

Isoenzyme analysis

Thirty seeds of each parent species and derived F_1 and BC_1 hybrids were scarified and sown in shallow boxes ($51 \times 38 \times 8$ cm) containing John Innes loam-based compost in a glasshouse at 15°C . After 10 weeks, a young leaf was collected from 20 plants within each of the four groups. Each leaf was crushed in a buffer consisting of two drops of 0.05 M tris/mercaptoethanol at pH 7.2. The crude extract from each sample was then absorbed onto 9×2 mm paper wicks. Starch gel electrophoresis was used to separate the isoenzymes as described by Michaelson-Yeates (1986).

Table 1 Mean number of seeds/floret from hand-crosses between *Trifolium repens* and *T. nigrescens*

Genotype cross	Inflorescence 1		Inflorescence 2		Mean seeds/floret
	Florets	Seed	Florets	Seed	
Ac3715 (2) $\times$ Ah1524 (1)	38	55	54	99	1.67
Ac3715 (4) $\times$ Ah1524 (2)	29	66	25	31	1.80
Ac3715 (6) $\times$ Ah1524 (5)	46	74	30	17	1.20
Ac3715 (9) $\times$ Ah1524 (7)	36	89	40	76	2.17
Ac3715 (10) $\times$ Ah1524 (8)	40	21	43	78	1.19

The three isoenzymes investigated were phosphoglucoisomerase (PGI) which catalyses the reversible isomerization of glucose 6-phosphate and fructose 6-phosphate, leucine aminopeptidase (LAP) which is an exopeptidase that hydrolyses the n-terminal residues of peptides, and amylase, an enzyme that hydrolyses starch. These were assayed in the dark at room temperature. After an hour, zones of enzyme activity (PGI and LAP) appeared on the gels as blue and red bands respectively. For the amylase enzyme the gel was left in ethanol overnight, and banding patterns due to the hydrolysis of the starch were visible after 12 h.

Experimental plant material

On the 6th January 1992, 50 seeds of each of *T. repens*, *T. nigrescens*, F_1 and BC_1 hybrids were spread on wet germination pads and placed in a germination tank maintained at a temperature of 20°C. Most seeds had germinated after three days, when they were transplanted into Jiffy pot trays containing a John Innes loam-based compost. The trays were kept in a glasshouse maintained at a day/night temperature of 15/5°C. On the 9th March, they were repotted, singly, into 9 cm diameter pots. Chromosome counts were made on the F_1 and BC_1 hybrids by pretreating root tips for 5 h in H_2O at 0–2°C, fixing in acetic acid:ethanol (1:3) and then staining in Feulgen (Meredith, pers. comm.). All F_1 plants that were used in the following experiments were triploid ($3x=24$). Chromosome counts in the BC_1 plants showed numbers between 28 and 30, and 15 per cent of them contained one of the distinguishing leaf markers from the pollen parent, *T. repens*. Plants of the parental species and derived F_1 and BC_1 hybrids are shown in Fig. 1.

Experiment 1

On the 23rd April, eight plants of each of the two species and the F_1 and BC_1 hybrids were repotted into 12.5 cm diameter pots. The pots were placed in a controlled environment in which the day/night temperatures were maintained at 20/15°C. The pots were placed on saucers for watering and arranged in a fully randomized design. The light regime of 16 h was provided by an irradiance of $530 \mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by a bank of warm white fluorescent tubes. A vapour pressure deficit of 6×10^{-4} MPa was maintained.

Growth and development

Though the lateral stems of *T. nigrescens* are not true stolons, 'stolon' has been used to describe their lateral stems and the similar structures of the hybrids.

On each pot, two primary stolons were marked with a ring of coloured wire behind the first trifoliate leaf. At weekly intervals throughout the experiment (13 weeks) the following parameters were recorded on each labelled stolon: (i) stolon length from the ring of coloured wire to the apical growing point; (ii) the total number of nodes; (iii) the number and position of secondary stolons arising from the labelled primary stolon; (iv) the total number and position of inflorescences on each stolon (node 1 being the first node after the ring and therefore the oldest). The number of inflorescences on each secondary stolon was also counted.

During the experiment, a total of four inflorescences in full bloom (inflorescences with all florets open) on unlabelled primary stolons were collected from each pot. All inflorescences were removed 2 h after the beginning of the light period. The florets were carefully removed and counted. One floret from the top, middle and bottom of each of two inflorescences was dissected and the length and width of each ovary was measured under the light microscope. Nectar volume and sugar concentration were measured on a subsample of 10 florets, by inserting a $10 \mu\text{L}$ capillary tube into the lip of the floret. One tube was used to draw off the nectar from all ten florets, the length of the column of nectar was then measured and the per cent sugar content determined with a refractometer.

Harvest measurements

After 13 weeks, the labelled stolons were removed and the total length of the primary stolon and number of nodes, secondary stolons and inflorescences on the primary and secondary stolons recorded. The position of the reproductive nodes on each primary stolon was noted using the system described by Thomas (1981). The first node after the coloured wire was called node 1 and nodes distal to this 2, 3, 4 and so on. The number of nodes that bore an inflorescence at each position was then expressed as a proportion of the total nodes. On the rest of the plant, the numbers of inflorescences, primary stolons and the numbers of reproductive (those bearing inflorescences) and nonreproductive (not bearing inflorescences) stolons were recorded.

Experiment 2

On the 24th May 1992, eight plants of each of the two species and the F_1 and BC_1 hybrids were repotted into deep boxes ($51 \times 38 \times 15$ cm) containing the soil mix described in experiment 1 and transferred to an unheated glasshouse. On each plant two stolons were marked as in experiment 1. At weekly intervals on each

marked stolon, stolon length and the number of open inflorescences were recorded as well as the number of inflorescences on the whole plant. After eight weeks of measurement, all above ground material of each plant was harvested. The plants were separated into inflorescences and vegetative material (stolons and leaves). The numbers of fully opened and unopened inflorescences were counted, and inflorescences and vegetative material dried in an oven at 80°C for 12 h and weighed.

Results

Isoenzyme analysis

A previous extensive survey at the *PGI/2* locus of *T. repens* (Michaelson-Yeates, 1986) identified five alleles (*a, b, c, d, f*) controlled by a single codominant gene that

behaved in a normal Mendelian manner. These alleles gave rise to ten different banding patterns and because of gene dosage, genotypes containing different proportions of the same allele could be distinguished. The survey also revealed that out of the five alleles, only *c* was homozygous. In this study (Fig. 2), the 20 *T. repens* plants produced four different isoenzyme banding patterns expressed by three alleles (*b, c, d*). *T. nigrescens* was more uniform as only two different types of single band were observed, one of which corresponded to the position of the *c* allele in *T. repens*. The same single band was the only one observed among the 20 F_1 hybrids while the BC_1 isoenzymes consisted of five different banding patterns. These were controlled by four alleles which were common to those that exist in *T. repens*.

The genetic control of LAP and amylase in *T. repens* is unknown. LAP isoenzyme banding patterns for both species and their hybrids are shown in Fig. 3, each

Fig. 1 Plants of *Trifolium repens* (bottom left), *T. nigrescens* (bottom right), F_1 (three plants, middle row) and BC_1 (top) hybrids at the onset of flowering.

group being represented by three genotypes. The LAP survey showed clear differences in banding positions between *T. repens* and *T. nigrescens*. A distinct lower band was observed in all of the *T. nigrescens* plants but was absent from *T. repens*. The F_1 s and BC_1 s showed hybrid bands between the two parental species, although the BC_1 s often had a banding pattern similar to *T. repens*. A less concentrated lower band similar to *T. nigrescens* appeared in all the F_1 hybrids but was present in only 25 per cent of the BC_1 plants.

Amylase banding among the species and their hybrids is shown at the bottom of Fig. 3. A single band showing variation was observed in all the *T. repens* material, but this was only faintly expressed among 15 per cent of the *T. nigrescens* plants. The amylase banding was very apparent in the F_1 and BC_1 plants.

Experiment 1

The general pattern of node production on the primary stolon was similar for all species and hybrids although stolons of *T. repens* had more nodes than those of *T. nigrescens* and all hybrids were intermediate (Fig. 4a). There were, however, some differences at the beginning of stolon growth as stolon nodes were visible in week one for both parent species and the BC_1 but not until week two for the F_1 .

There were few differences in the numbers of axillary buds/primary stolons until week four when numbers on *T. nigrescens* reached a peak and remained constant to the end of the experiment whilst those on *T. repens* and the hybrids continued to increase (Fig. 4b). From six weeks until the end of the experiment, the F_1 s

Fig. 2 Observed isoenzyme banding patterns at the *PGI/2* locus among a sample of five plants from each *Trifolium* species and associated hybrids.

Fig. 3 LAP (top) and amylase (bottom) isoenzyme banding patterns of, from left to right, *Trifolium repens*, *T. nigrescens*, F_1 and BC_1 hybrids.

had more axillary buds than *T. repens* and the BC_1 's while *T. nigrescens* had fewer axillary buds.

Inflorescences were first visible in week three and from then *T. nigrescens* produced more inflorescences per primary stolon than *T. repens* and all hybrids (Fig. 4c). *T. repens* produced inflorescences later and also produced fewer inflorescences than the hybrids, which were intermediate between the parent species. *T. nigrescens* produced more inflorescences on secondary stolons than *T. repens* with the hybrids being intermediate (Fig. 4d). There were also differences in the date of appearance of inflorescences. Inflorescences were visible in week four for *T. nigrescens*, in week five for the hybrids but not until week seven for *T. repens*. The F_1 s produced more inflorescences than *T. repens* and although the BC_1 s also produced more inflorescences than *T. repens* these differences were not always significant.

At harvest, *T. repens* had more primary stolons/plant than *T. nigrescens* and both hybrids, and *T. nigrescens* had fewer than all plants except the F_1 s (Table 2). *T. repens* also had more vegetative stolons

and a higher proportion of vegetative stolons than *T. nigrescens* and the F_1 hybrids; the difference between *T. nigrescens* and the F_1 , however, was not significant. *T. nigrescens* had fewest reproductive stolons but the highest per cent reproductive stolons whilst *T. repens* had a lower proportion of reproductive stolons than *T. nigrescens* and all hybrids. Overall, *T. nigrescens* produced more inflorescences per plant than the hybrids, which were found to be similar, while *T. repens* produced fewest.

The pattern of flowering on the primary stolons is shown in Fig. 5. *T. repens* did not produce inflorescences until node nine and thereafter no more than 31 per cent of nodes at any position produced a reproductive bud. Few reproductive buds were observed beyond node 19. *T. nigrescens*, however, produced an inflorescence at node six and all nodes from eight to 12 were reproductive, after which there was then a gradual decline in the percentage of nodes forming reproductive buds until node 19. Generally, all the hybrids were intermediate between *T. nigrescens* and *T. repens*, having more inflorescences than *T. repens* but not as

Fig. 4 The number of (a) nodes/primary stolon, (b) axillary buds/primary stolon, (c) inflorescences/primary and (d) inflorescences secondary stolon of *Trifolium repens* (—□—), *T. nigrescens* (—○—) and the F_1 (—△—) and BC_1 (---○---) hybrids. Vertical bars represent L.S.D. at $P=0.05$.

many as *T. nigrescens*. The F_1 s produced a reproductive bud at node seven, reaching a maximum of 75 per cent at node 12 before declining until node 24. The BC_1 s, however, produced the first reproductive bud at node five, reached a maximum of 50 per cent at nodes 13 and 14 before gradually declining until there were no reproductive nodes at node 23. The number of florets per inflorescence was highest in *T. repens* and lowest in *T. nigrescens* with the hybrids intermediate (Table 3). Ovary length but not width (data not shown) also differed as *T. repens* had longer ovaries than *T. nigrescens* but not longer than the hybrids which were not significantly different from each other. *T. repens* also had more ovules/ovary than the F_1 s and both had more than the BC_1 s and *T. nigrescens*, which were not significantly different from each other. Nectar production also differed as florets of *T. repens* and all hybrids produced more nectar than *T. nigrescens*, which had a significantly higher nectar sugar concentration than *T. repens* and the BC_1 s but not the F_1 s.

Experiment 2

The numbers of harvested inflorescences of *T. repens*, *T. nigrescens* and the hybrids are shown in Table 4. *T. nigrescens* had many more inflorescences than *T. repens* and both hybrids, whilst *T. repens* had fewest inflorescences but only significantly fewer than the F_1 s. There was no significant difference between the hybrids. This was reflected in the dry weight of the reproductive and vegetative components. The vegetative dry weight of *T. repens* was higher than *T. nigrescens* and all hybrids, whilst *T. nigrescens* was lower than the BC_1 s but not the F_1 s. The per cent vegetative growth was lower in *T. nigrescens* than *T. repens* and all hybrids. *T. nigrescens* and the BC_1 s had a higher inflorescence dry weight than *T. repens* but not the F_1 s and there was no difference between *T. repens*

and the F_1 s. *T. nigrescens* had the highest per cent inflorescence dry weight and *T. repens* the lowest, with both hybrids intermediate.

Discussion

Species hybridization is a means of extending the range of heritable variation, which can be exploited by the plant breeder. Brewbaker & Keim (1953) were among the first to suggest interspecific hybridization of *Trifolium* spp. as a means of improving *T. repens*. They identified potential benefits in disease and pest resistance, persistency, root development and cold and drought tolerance. The objective of the present study was to determine the potential for transferring reproductive characters from *T. nigrescens* to *T. repens* by interspecific hybridization.

Data presented in Table 1 confirmed results from other studies (Hovin, 1962), which demonstrated that by using *T. repens* as the female parent, specific genotype cross-combinations with *T. nigrescens* can produce large numbers of hybrids. In this paper we also describe the characteristics of a backcross generation to *T. repens*. Although the majority of BC_1 plants were expected to be true backcrosses, it is possible that some intercrossing did occur among the F_1 s. Chromosome counts, isoenzyme analysis and leaf markers confirmed, however, the true origin of the F_1 s and BC_1 s used in this study. Among the F_1 hybrids, six plants produced as many as 200 seeds each, which is considerably more than the only other backcross hybrids thus far reported (Hovin, 1962). This is the first time that large numbers of hybrids have been produced by interspecific hybridization between these species and this enables an investigation of the morphological characteristics of the F_1 s and the BC_1 s.

In perennial forage species such as *T. repens*, the correct balance between vegetative and reproductive

Table 2 Vegetative and reproductive stolons and inflorescences per plant of *Trifolium* species and hybrids

Species/hybrid	Primary stolons/ plant	Vegetative stolons/plant		Reproductive stolons/plant		Inflorescences/ plant
		No.	% of total	No.	% of total	
<i>T. repens</i>	18.5	9.4	50.8	9.1	49.2	26.5
F_1 hybrid	10.5	2.5	23.8	8.0	76.2	83.9
BC_1 hybrid	14.4	5.6	38.9	8.8	61.1	80.9
<i>T. nigrescens</i>	7.5	0	0	7.5	100.0	265.4
LSD ($P=0.05$)	5.9	6.0	24.0	4.4	24.0	38.0

Fig. 5 The percentage of nodes producing a reproductive bud on the labelled primary stolon (node 1 = 1st node produced after the label and therefore the oldest) of (a) *Trifolium repens*, (b) F_1 hybrid, (c) BC_1 hybrid and (d) *T. nigrescens*.

growth is important to ensure good forage production and the production of acceptable quantities of seed. In *T. repens* the number of reproductive nodes per stolon and their pattern along the stolon determine the number of ripe inflorescences at harvest, the main seed yield component (Hollington *et al.*, 1989). To combine high seed yields, and good forage production and persistency, *T. repens* would ideally have stolons which change from vegetative to reproductive, producing a series of reproductive nodes, then revert to being vegetative. These vegetative nodes are important to ensure perennality and persistency in the sward from one season to the next.

In both controlled environment and glasshouse experiments *T. nigrescens* produced an inflorescence at almost every node and, once flowering began, no secondary stolons were produced. In contrast, *T. repens* produced fewer inflorescences and the pattern of flowering on the primary stolon was intermittent. The hybrids had reproductive characters inherited from both parental species. Their pattern of flowering was intermediate between the parental species, though the BC_1 s had a pattern more like that of *T. repens*. *T. nigrescens* had a more concentrated flowering pattern and therefore is likely to produce more ripe inflorescences at harvest. The F_1 hybrids were intermediate between the two species, and even the BC_1 s had more inflorescences per plant and per stolon than *T. repens*. In practice, seed yield is the product of several yield components (Marshall, 1994), of which inflorescence number is the most important. The F_1 s and BC_1 s had smaller inflorescences with fewer florets and fewer ovules per floret than *T. repens*; however, their reproductive potential, as a product of the individual components, was considerably higher than that of *T. repens*. The reproductive characters of the F_1 hybrids lie close to the mid-parent indicating little overall dominance, but as expected, the expression of characters in the BC_1 s was, in general, shifted towards *T. repens*.

It is important, however, to combine the desired reproductive characters with those vegetative characters that already exist in *T. repens*. The number

Table 3 Reproductive characters of *Trifolium* species and hybrids

Species/hybrid	Florets/ inflorescence	Ovules/ ovary	Nectar volume/ 10 florets (nL)	Nectar sugar concentration (%)
<i>T. repens</i>	63.8	5.7	1.5	42.7
F_1 hybrid	44.6	4.8	1.4	52.3
BC_1 hybrid	41.2	4.2	1.5	50.6
<i>T. nigrescens</i>	28.6	4.0	0.6	56.4
LSD ($P = 0.05$)	4.8	0.6	0.5	4.6

Table 4 Inflorescence production and vegetative and reproductive dry weight for *Trifolium* species and hybrids (g)

Species/hybrid	Inflorescences/plant	Vegetative		Inflorescence	
		Dry weight (g)	% of total	Dry weight (g)	% of total
<i>T. repens</i>	67.0	385.0	98.3	6.6	1.7
F ₁ hybrid	460.0	123.0	86.0	20.0	14.0
BC ₁ hybrid	283.0	205.0	89.0	25.3	11.0
<i>T. nigrescens</i>	868.0	30.0	59.1	20.8	40.9
LSD ($P = 0.05$)	240.2	104.2	9.8	NS	9.8

of nonreproductive nodes, vegetative stolons and nodal roots are all essential to ensure perenniality and persistency in the sward from one season to the next. Being an annual species, *T. nigrescens* does not require this characteristic and it is not surprising that no vegetative stolons and nodal roots were observed. The F₁s had more vegetative stolons than *T. nigrescens*, but no nodal roots. However, the BC₁s had more vegetative stolons than the F₁s whilst retaining the same number of reproductive stolons as *T. nigrescens*. Although the BC₁s had fewer vegetative stolons than *T. repens*, it should be possible to combine characteristics which will ensure persistency similar to *T. repens* whilst increasing reproductive growth. Though there were no nodal roots in the F₁s, they were apparent in some of the BC₁s, suggesting that the production of nodal roots may be controlled by recessive genes. It is clear that those characteristics which are associated with the annual species *T. nigrescens* (profuse flowering, absence of nodal roots) are dominant over the same characters found in *T. repens*. Further breeding work is necessary to determine the extent to which good vegetative characters may be maintained whilst seeking to improve reproductive growth. This will also necessitate an examination of hybrid-derived material under field conditions.

The characteristics which determine the attractiveness of an inflorescence to insect pollinators are largely unknown, but floral scent and nectar production are likely to be important. Previous observations suggest that *T. nigrescens* and the hybrids are particularly attractive to pollinators (Michaelson-Yeates, unpublished). In the present experiments, *T. nigrescens* produced relatively small amounts of nectar but of a higher sugar concentration than *T. repens*. There was no difference between the F₁s and the BC₁s and they both produced similar amounts to *T. repens* but of a higher sugar concentration. Other substances (volatiles) have been suggested as important in the

attraction of pollinators. Their importance in *T. nigrescens* and the derived hybrids merits investigation.

Disomic inheritance and the prevalence of regular pairing and disjunction of the chromosomes at meiosis have indicated that *T. repens* is an amphidiploid (Atwood and Hill, 1940). A number of research workers have suggested that *T. nigrescens* may be one of the possible ancestors of *T. repens* (Brewbaker & Keim, 1953; Hovin, 1962). A survey at the *PGI/2* locus of *T. repens* strongly suggested that *T. repens* behaves as an amphidiploid in which one of its genomes expresses a multiallelic series whilst the other is homozygous for the *c* allele (Michaelson-Yeates, 1986). The results of the *PGI/2* survey in this paper revealed that, out of the five alleles found in *T. repens*, the only one that could be identified with a band in *T. nigrescens* was the *c* allele. Although all chromosomes from *T. repens* and *T. nigrescens* are present in the F₁s, the expression of *PGI/2* alleles from *T. repens* seems to be suppressed except for the *c* allele. This was not evident in the BC₁ generation as four alleles common to those that exist in *T. repens* were observed. The banding patterns of the F₁s could also be explained if all five *T. repens* parents in the original hand-crosses were homozygous for the *c* allele. Although the banding patterns of the original parents are not known, the probability of all five plants of *T. repens* being homozygous for the *c* allele is extremely low. The results warrant further investigation of chromosomal behaviour in the hybrids at the *PGI/2* locus.

This study has further demonstrated that certain cross-combinations between the two species can produce large numbers of hybrids without resorting to artificial techniques, indicating a close relationship between *T. repens* and *T. nigrescens*. Useful genetic variation in the form of reproductive characters can therefore be exploited by the plant breeder as a possible means of improving the seed production of *T. repens*. These hybrids along with their parental species

may also provide information on the control of the various biochemical and developmental pathways associated with these reproductive characters.

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