SUBSPECIATION IN *TRIFOLIUM SUBTERRANEUM*

By F. H. W. Morley,* R. D. Brock,* and C. I. Davern*

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Summary

Subspeciation in *Trifolium subterraneum* was indicated by the presence of reproductive and vegetative abnormalities in crosses between some varieties.

In F_1's and F_2's there was pollen sterility, reduced seed set, and embryo abortion. Dwarf and semi-dwarf plants segregated in some F_2's. The occurrence and severity of different abnormalities were strongly correlated between F_2's. Within F_2's the correlation was slight or absent which suggested that these abnormalities were independently determined. Chromosomal rearrangement was indicated by failure of pairing, reduced chiasma frequency, and multivalent formation at meiosis of some F_1's. Less than 40 per cent. of observed pollen sterility could be explained by such structural differences. The main cause of sterility seems to be genic.

The presence of genic and structural sterility barriers suggests that many of the varieties were reproductively isolated long before they were introduced to Australia. Major varietal differences are unlikely to have arisen from selection of locally adapted ecotypes. There is thus ample justification for a programme of hybridization and selection to improve and to extend the range of the species. Such a programme would be impeded, but not prevented, by the presence of reduced fertility in some segregates.

I. Introduction

Subterranean clover (*Trifolium subterraneum* L.) consists of numerous self-pollinating varieties showing a wide morphological and physiological diversity. The natural habitat of the species is the Mediterranean region, the Iberian peninsula, France, and the British Isles. Strains from Portugal, Morocco, Malta, England, and commercial Australian varieties have $2n = 16$ chromosomes. A "strain" from Israel has $2n = 12$ chromosomes (Yates and Brittan 1952).

In Australia the species is distributed mainly in southern areas with 20 in. or more annual precipitation. Collections made in this area were described by Aitken and Drake (1941) who recorded over 40 distinct types. According to these authors, and to Donald and Smith (1937) and Frankel (1955) these were probably introduced independently, either deliberately or accidentally. Davies (1951) considered that some introductions came from England and not from the Mediterranean.

The existence of a cryptic species in Israel (Brock 1953), and the variable success of intervarietal crosses (Hutton and Peak 1954) suggested that differences of subspecific rank may distinguish some Australian varieties. Accordingly various characteristics of parent and hybrid plants were examined. The results support the view that some strains are differentiated by genic and chromosomal sterility barriers. Such differences indicate long term genetic separation and, we consider, constitute the process of subspeciation such as described in other genera by Stebbins (1950), Baker (1951), Clausen (1951), and others.

* Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T.

TABLE 1

DISTRIBUTION OF POLLEN STERILITY IN PARENTS AND CROSSES

Numbers of plants showing different pollen scores

Parents and Crosses	Plants	Total Plants	Mean Pollen Score										Mean
			5	15	25	35	45	55	65	75	85	95	
Parents	Tallarook	5	5										5
	458	35	31	2			1					1	6
	Clare	3	3										5
	Mulwala	5	4	1									7
	Gin Gin	4	4										5
	Red Leaf	4	4										5
	Mt. Barker	5	4				1						13
	Dalliak	4	3	1									7
	Yarloop	5	1	1	1	1		1					27
	Dwalganup	4	4										5
	N_1E	5	5										5
	Pink Flower	5	2	3									11
F_1's	Burnerang × Wenigup*	2									2		85
	Tallarook × Clare*	2								1	1		80
	,, × Wenigup*	2								1	1		80
	,, × 458	7							5	2			68
	,, × Dwalganup	19			4	5	8			2			41
	,, × Pink Flower	4				2	2						40
	,, × Red Leaf	3		2		1							22
	,, × Gin Gin	9	9										5
	,, × Mt. Barker	12	12										5
	Mt. Barker × Dwalganup	15		4	6	3	1			1			29
	Pink Flower × Red Leaf*	1		1									15
	Yarloop × Yabba North	2		2									15
F_2's	Tallarook × Clare	65	5	3	1		9	8	14	12	10	3	60
	,, × Yarloop	20	1	3		1	1	3	3	2	1	5†	60
	,, × 458	108	21	12	12	7	13	11	10	3	2	17†	43
	,, × Dwalganup	86	33	15	12	12	8	2	3	1			22
	,, × Pink Flower	39	17	12	2	3	1		1	2	1		20
	,, × Mulwala	24	11	7	1		3	1		1			20
	,, × N_1E	23	16	3		3				1			13
	,, × Cranmore	13	8	2	2	1							13
	,, × Dalliak	47	34	8	5								9
	,, × Red Leaf	5	5										5
	,, × Gin Gin	8	8										5
	,, × Mt. Barker	23	22						1				8
	Mt. Barker × Pink Flower	33	14	4	4	4	3	4					22
	,, × Dwalganup	56	24	16	5	4	1	2	2		1	1	19
	,, × N_2E	20	18		1	1							7
	N_1E × Pink Flower	22	11	3	3	1	1	2	1				20
	,, × N_2E	14	12	1			1						9
	,, × Dwalganup	23	19	4									7
	,, × Mt. Barker	23	21	2									6
	,, × Cranmore	10	9	1									6
	Clare × Pink Flower	24	9	8	1	1	2	1	1	1			21

* Plants grown in glasshouse, remainder in field.

† These plants classified "empty anthers".

TABLE 1 (*Continued*)

Parents and Crosses	Plants	Total Plants	Mean Pollen Score										Mean
			5	15	25	35	45	55	65	75	85	95	
Backcross to Tallarook of F_2's of:	Dwalganup*	2		2									15
	Yarloop*	1							1				65
	N_1E*	1	1										5
	Gin Gin*	3	3										5

* Plants grown in glasshouse, remainder in field.

II. METHODS AND MATERIAL

Most of the plants studied (see Table 1) were grown at Dickson Experiment Station, Canberra. Seedlings were grown in flats and transplanted to the field in May 1954, spaced 7 lk each way between plants. Spray irrigation was provided when necessary. The original crosses were made in connection with the programme of Dr. E. M. Hutton in his investigations of inheritance in this species. Some additional material was provided from crosses made by the senior author in the spring of 1954, and grown in the glasshouse during the summer 1954–1955.

(*a*) *Pollen Sterility*

Pollen sterility was assessed by the reaction of grains to staining with cotton blue in lactophenol after fixation in acetic alcohol (Darlington and La Cour 1947). During November 1954 two inflorescences, just prior to anthesis, were collected from five plants of each parent variety, from all F_1's, and from at least 25 plants of most F_2 lines.

Because of variability between anthers and flowers and the large amount of material involved, a rapid rating for pollen sterility was used. Slides were graded 0–10, 10–20, . . . , 90–100 estimated percentage of abnormal pollen, using 5, 15, . . . , 95 for calculating means. This scoring system was found to be satisfactory and it gave good agreement with actual counts.

(*b*) *Seed Set*

Seed set was examined in (1) a sample of 10 well-developed burrs taken during late November from each of four to five plants, and (2) whole runners collected during the first week in December. These were stored by refrigeration for examination during the subsequent 3 weeks. Seeds were classified as follows:

(i) *Normal.*—Seeds with fully distended seed coats, apparently viable (but not necessarily large).

(ii) *Shrivelled.*—Slightly to very shrivelled seeds, with incompletely developed embryos. These were considered to correspond to the aborted seeds noted in many interspecific crosses (Brink and Cooper 1947).

(iii) *Empty Flowers.*—No seed development, indicating failure of fertilization or very early embryo degeneration.

(c) *Meiosis*

Meiosis was studied in acetic orcein squashes of pollen mother cells after fixation in Carnoy fluid (6:3:1) (Darlington and La Cour 1947).

III. Results

(a) *Pollen Sterility*

Table 1 shows the pollen scores of parental, F_1, and F_2 lines in terms of number of plants in each class. An additional class of "empty anthers" gives the number of plants in which no organization of pollen grains was noted in all anthers examined (usually 8-10). Occasional loose pollen grains were seen in a few of these slides. These plants were allocated a value of 95 when compiling means.

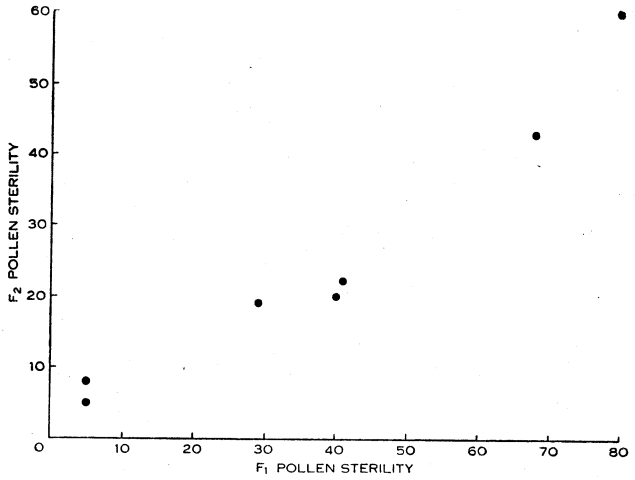


Fig. 1.—Correlation between pollen sterility in F_1 and F_2.

The data of Table 1 indicate that, with few exceptions, plants in the parental lines were highly fertile. There is relatively little variation in pollen score among plants in an F_1, but there is a wide range between different F_1's. There is a wide variation within and between F_2's, indicating segregation for this characteristic. F_1 lines of high pollen sterility segregated high and low sterility plants in F_2. However, fully fertile F_1's showed no segregation for pollen sterility in F_2.

The correlation between pollen score in F_1 and F_2 is shown in Figure 1. The correlation is high, especially in view of the small number of plants scored in F_1. In general, the F_2's were more fertile than F_1's, presumably because many unfavourable genotypes were eliminated in F_1.

Glasshouse material was collected from plants grown during January-March 1955 under a 16-hr day (part of period only) after 3 weeks vernalization. Pollen scores indicate a strong incompatibility between Tallarook and Wenigup, Burnerang and Wenigup, and Tallarook and Clare. The last observation is in accord with field observations, and the pollen scores of backcrosses suggest that environment conditions were not unfavourable to normal pollen formation.

The plants with empty anthers in the crosses Tallarook × 458 F_2 and Tallarook × Yarloop F_2 are of particular interest. The anthers were approximately normal in size, and appeared distended with cellular material, but no organization of pollen grains was apparent (Plate 1). It is tempting to suggest that this abnormality is determined by a recessive gene which is segregating in a 3:1 ratio in these F_2's. In view of the breeding habit of the parent varieties such an explanation is very unlikely, as one parent would be homozygous for the gene in question. A 13:3 ratio is consistent with the data. Nearly all these empty anther plants set a small quantity of seed, so that breeding tests will be possible. An occasional apparently normal pollen grain was noted in some preparations. In one plant which set no seeds, no pollen grains were found in 93 anthers examined.

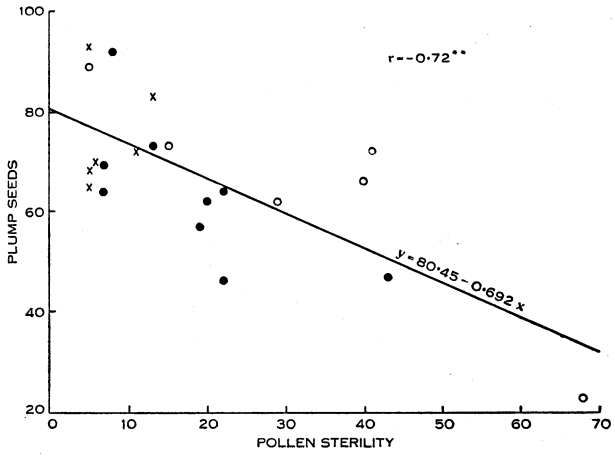


Fig. 2.—Relationship between pollen sterility (Table 1) and formation of plump seeds (Table 2). × Parents. ○ F_1's. ● F_2's.

In addition to the plants with empty anthers there were plants in which some sectors of some anthers examined contained little or no pollen. There were a few obvious variations between anthers of the same flower or different flowers on the same or different inflorescences. Such variation was not noted in any preparation from parent lines, except in the variety Yarloop. The significance of these variations will be discussed in Section IV of this paper.

(*b*) *Seed Set*

Seed set as indicated in Section II (*b*) was determined from burr samples and on whole runners. Whole runners give an indication of the ability to set seed under the particular set of external and internal environmental conditions over a period of time. These conditions will also vary throughout the field, so that comparisons between groups are not strictly valid, because there will be differences due to maturity, onset of senescence, and disease.

If p were the inherent fertility of the plant, then the probability of any of the four flowers which normally constitute an inflorescence failing to set and develop seed would be $(1-p)^4$. The sample of 10 burrs per plant would exclude the $(1-p)^4$

inflorescences which failed completely, but would include inflorescences in which one or more seeds developed, as burr formation is normal in these cases. However,

TABLE 2

PERCENTAGE OF THE DIFFERENT CATEGORIES OF SEEDS IN PARENTS AND HYBRIDS

Parents and Crosses	Plants	No. of Plants Sampled	Total Flowers	Plump Seeds (%)	Shrivelled Seeds (%)	Empty Flowers (%)	Seed Abortion (%)
Parents	Tallarook	5	328	93	1	6	1
	Tallarook*	5	196	98	—	2	0
	458	10	272	70	6	25	8
	458*	5	192	74	2	24	3
	Mt. Barker	6	219	83	4	13	5
	Mt. Barker*	5	200	87	1	12	1
	Dwalganup	8	223	68	5	27	7
	Dwalganup*	5	202	94	1	5	1
	N_1E	5	203	65	5	30	7
	Pink Flower	12	334	72	7	22	9
	Pink Flower*	4	160	88	5	7	5
F_1's	Tallarook × 458	5	314	23	25	51	52
	Tallarook × 458*	5	200	40	40	20	50
	Tallarook × Dwalganup*	5	199	72	17	11	19
	Tallarook × Pink Flower	4	116	66	7	27	10
	Tallarook × Pink Flower*	4	161	61	24	15	28
	Tallarook × Mt. Barker	3	88	89	3	8	3
	Tallarook × Mt. Barker*	4	158	91	2	7	2
	Mt. Barker × Dwalganup	6	234	62	15	23	20
	Mt. Barker × Dwalganup*	5	200	64	25	11	28
	Mt. Barker × Tallarook	5	135	92	—	8	0
	Mt. Barker × Tallarook*	5	200	93	3	4	3
	Yabba North × Yarloop*	4	117	73	1	26	1
F_2's	Tallarook × 458	19	391	47	17	35	27
	Tallarook × 458†	15	296	60	19	21	24
	Tallarook × Dwalganup	25	710	64	16	20	20
	Tallarook × Pink Flower	26	847	62	13	24	17
	Tallarook × N_1E	25	735	73	15	12	17
	Tallarook × Mt. Barker	25	849	92	3	5	3
	Mt. Barker × Pink Flower	26	993	46	22	32	32
	Mt. Barker × Dwalganup	21	569	57	23	20	29
	Mt. Barker × N_2E	27	585	64	19	17	23
	N_1E × Dwalganup	25	755	69	11	20	14

* Plants sampled by collection of 10 well-formed burrs. Remainder sampled by taking one runner.

† Excluding empty anthers.

the frequency of inflorescences failing to produce burrs was considerably higher than expected. This is not surprising since all flowers of an inflorescence would be influenced by the same environment, and would tend to succeed or fail together.

The per cent. seed set estimated from the 10 burr samples would therefore be, and generally was, higher than the average for the whole runner. Nevertheless, the burr-sample method is probably to be preferred since an estimate, although slightly biased, is thus obtained of potential fertility, and real differences are less likely to be obscured by gross environmental variations. Results are presented in Table 2.

The correlations among per cent. plump seeds, per cent. empty flowers, per cent. seed abortion, and pollen sterility, derived from "runner sample" data of Table 2, are shown in Table 3 and Figures 2 and 3.

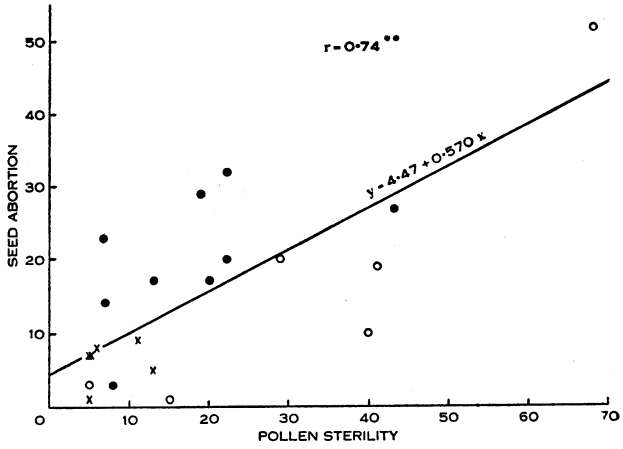


Fig. 3.—Relationship between pollen sterility (Table 1) and seed abortion (Table 2). × Parents. ○ F_1's. ● F_2's.

Sterility factors operating after fertilization are indicated by the presence of large numbers of shrivelled seeds in both F_1 and F_2 plants. Such embryo abortions are usually associated with endosperm failure (Brink and Cooper 1947). The aborted seeds are unlikely to represent all cases of embryo failure, for, as shown by Brock (1955), a large proportion of failure occurs shortly after fertilization. The numerous empty flowers in, for example, Tallarook × 458, may result from failure after fertilization rather than absence of fertilization. The correlation between seed set and pollen score (Fig. 2) may therefore reflect a common basis for pollen sterility and early embryo failure rather than pollen infertility *per se* especially as the number of pollen grains available is unlikely to limit fertilization.

The correlation between observed embryo abortion and pollen inviability (Fig. 3) may depend on the ratio of early to late abortions. Both pollen sterility and embryo abortion reflect the extent of incompatibility in these hybrids. In those hybrids in which sterility barriers are most severe only few balanced types would develop beyond early stages, and the ratio of "aborted" to "viable" seeds would probably fall, so that the correlation could become markedly curvilinear. The higher proportion of aborted seeds in the F_2 Mt. Barker × Pink Flower and Mt. Barker × Dwalganup relative to Tallarook × 458 and of F_2's relative to F_1's (Figs. 2 and 3) may be explained on this basis.

The existence of two ovules per ovary in this species (Aitken and Drake 1941) in addition to possible selection of balanced and viable female gametes (Renner effect) would further obscure relationships between male and female fertility. Normally

TABLE 3

CORRELATIONS BETWEEN PERCENTAGE SEED SET AND PERCENTAGE POLLEN STERILITY

Correlation coefficients—data from Table 2. All values significant at 1% level

Character	Plump Seeds	Empty Flowers	Seed Abortion	Pollen Sterility
Plump seeds	—	−0·90	−0·90	−0·72
Empty flowers	−0·90	—	0·65	0·61
Seed abortion	−0·90	0·65	—	0·74
Pollen sterility	−0·72	0·61	0·74	—

only one ovule develops, and in so doing suppresses the other. If p is the potential proportion of viable zygotes, then potentially 0, 1, or 2 viable seeds per flower would be produced in $(1-p)^2$, $2p\ (1-p)$, and p^2 of cases respectively. The proportion of

TABLE 4

DEVELOPMENT OF BOTH OVULES IN SHRIVELLED OVARIES OF F_1 TALLAROOK × 458

20 burrs of F_1 and parents examined on November 17, 1954

Ovary Development				No. of Ovaries with 0, 1, and 2 Ovules		
Parents or F_1	Plump	Shrivelled	Empty Flowers	0	1	2
Tallarook	80	—	—	—	80	—
458	65			—	65	—
		1		—	1	—
			4	4	—	—
F_1	27			—	27	—
		27		—	17	10
			26	26	—	—

flowers actually developing seed would then be $p\ (2-p)$. Thus if p were 0·5, the actual seed production would be 0·75 (assuming all fertilized and viable zygotes developed).

The presence of two ovules increases the possibility of selection operating both at fertilization, and during embryo development. This effect is demonstrated in the

F_1 of Tallarook $\times$458 (Table 4). Here all ovaries containing plump seeds had only one ovule developing. However, in 10 out of 27 ovaries containing shrivelled seeds two ovules were developing. Presumably these gametes were balanced and capable of being fertilized. However, sterility effects acting on both ovules after fertilization may have prevented full suppression of one by the other, so that both were represented as poorly-developed embryos.

The correlations between the pollen viability and the seed set among lines indicates that varieties, distinguishable by differences of subspecific rank in one character, tend to be distinguishable in other characters. But this finding does not indicate that the same factors operated to produce the different symptoms. This possibility can be tested by examining the segregation of different factors in the F_2 generation. Segregation for pollen sterility and seed setting in a number of F_2's is examined in Table 5.

TABLE 5

SEED SETTING IN F_2 PLANTS OF HIGH AND LOW POLLEN STERILITY

F_2	Pollen Sterility*	Plump Seeds (%)	Shrivelled Seeds (%)	Empty Flowers (%)	Seed Abortion (%)	No. of Flowers	No. of Plants
Tallarook × 458	Low	82	13	5	14	112	6
	High (49)	46	23	30	33	184	9
	Empty anthers	8	14	79	64	96	4
Tallarook × Dwalganup	Low	69	12	19	15	256	8
	High (29)	62	20	18	24	343	13
Tallarook × Pink Flower	Low	63	14	22	18	425	14
	High (24)	63	11	26	15	296	9
Mt. Barker × Dwalganup	Low	62	23	14	27	276	9
	High (32)	50	23	27	32	216	9
Mt. Barker × Pink Flower	Low	41	22	37	35	321	8
	High (23)	48	26	27	35	222	6

* Low, less than 10 %. High, greater than 10 %; averages in parenthesis.

In the F_2 of Tallarook $\times$458 there was a close relationship between the two types of abnormalities, which suggested a common cause, presumably genetic. However, in the other F_2's, this relationship was less evident or was absent, which suggested that the factors operating to produce pollen sterility and those influencing failure at or after fertilization were independent. Such contradictory results are difficult to reconcile, but may be considered in terms of interactions of possible processes, such as the "two-ovule effect", the Renner effect, linkage, segregation of univalents, and possible tapetum-pollen interactions.

The two-ovule effect and the Renner effect would diminish the correlation, but would not obscure it. If the production of normal gametes were determined by the balanced effects of many genes both on the same and on different chromosomes

of a homozygous line, and if the balance were achieved in a different way in different lines for a number of different characters, hybridization, and subsequent recombination among genes, would destroy the necessary balance for all these characters. The incidence or severity of abnormalities would therefore tend to be correlated, being high in generally unbalanced individuals, low in generally balanced. Probably the most balanced individuals would be most like one of the parents. The greater the number of possible combinations which would be unbalanced for two characters, the higher the F_2 correlation would be. We would thus expect a tendency for higher correlations between the more complex characters and in the hybrids from the more divergent parents. Abnormalities determined by relatively simple mechanisms would occur more or less independently of others. This theory suggests that hybridization, by destroying the gene balance (both inter- and intra-chromosomal) produces correlated unfavourable effects by a mechanism similar to that suggested by Mather and Harrison (1949) to accompany intense selection. These authors demonstrated that in *Drosophila* extreme selection for one character in either direction could be accompanied by similar unfavourable responses in other characters. If a finely-adjusted genic balance exists in a species such as *Drosophila* and can be disturbed by selection, it is certainly likely to exist in normally self-fertilizing species, and to be disturbed by hybridization.

Individual plants with good seed set and high pollen sterility and vice versa occurred in each of the 5 F_2's studied. Therefore the two types of abnormality were determined by different genotypes on some occasions. Correlation between abnormalities may therefore express complexity of determination rather than common causation. This seems to be a reasonable explanation for variations among within-F_2 correlations.

(c) *Vegetative Abnormalities*

In two F_2 lines, dwarf and semi-dwarf individuals occurred (Table 6). No such plants were found in any of the parent lines, F_1's, or other F_2's. It is interesting that these two F_2's are among those which had the greatest pollen sterility.

Plants were classified as dwarfs, semi-dwarfs, and normals on October 2, and in late November plant diameters were measured (Table 7). The plant sizes in the relative classes generally confirm the ratings. However, subsequent to the time of rating, which was taken at the main period of flowering, some of the semi-dwarfs, and one of the dwarfs, made considerable vegetative growth. Further investigations are being made of the inheritance of the dwarf conditions in these crosses.

Table 7 also illustrates the vegetative vigour of the F_1's and the normal F_2's. The average end-of-season diameter of both the F_1's and F_2's of Tallarook $\times$ 458 exceeded that of either parent. F_1 plants of Tallarook $\times$ Clare grown in the glasshouse appeared correspondingly vigorous.

The relationship within F_2's between vegetative abnormalities and pollen viability is shown in Table 8. Within F_2's the correlation between these two characters was small and non-significant. However, the fact that the F_2's showing vegetative

abnormalities were those showing greatest pollen sterility suggests that the former is a symptom of relatively extreme genetic divergence. The mechanism of dwarfing such as this is unknown, although dwarf and weak plants are frequently met in relatively wide crosses. If the genic balance and linkage theories described earlier

TABLE 6

PERCENTAGE OF ABNORMAL PLANTS IN THREE F_2'S AND PARENTS

Parents and F_2 Crosses	No. of Seedlings	Missing (%)	Dwarf (%)	Semi-dwarf (%)	Normal (%)
Tallarook	65	—	—	—	100
Clare	152	—	—	—	100
458	141	1	—	—	99
Tallarook × Clare	91	18	48	11	22
Tallarook × 458	122	6	21	—	73

are correct, the lack of correlation would indicate a relatively simple genetic determination of dwarfing. It is possible that these plants have small chromosomal deficiencies or duplications, but this possibility could not be investigated.

TABLE 7

AVERAGES AND RANGES OF PLANT DIAMETERS OF PARENTS AND CROSSES WHICH SHOWED SEGREGATION OF DWARFS

Genotype	Type	No. of Plants	Average Diameter (in.)	Range (in.)	
				Smallest	Largest
458	Normal	45	37·5	26·0	48·0
Tallarook	Normal	43	40·5	28·5	52·5
Clare	Normal	62	37·9	26·5	50·5
Tallarook × 458 F_1	Normal	8	51·2	43·0	57·0
Tallarook × 458 F_2	Normal	43	41·9	32·5	54·5
Tallarook × 458 F_2	Dwarf	23	10·6	2·0	16·5
Tallarook × Clare F_2	Normal	20	42·2	26·5	51·5
Tallarook × Clare F_2	Semi-dwarf	8	26·1	19·5	35·5
Tallarook × Clare F_2	Dwarf	41	11·0	3·5	21·0 (one 30·5)

Data were not available to investigate the relationship between vegetative abnormalities and seed set. However, some dwarf plants in both crosses set seed readily and some vegetatively normal plants set relatively little seed. This supports the view that vegetative development and reproductive efficiency are virtually independent.

Other abnormalities were observed in F_2 plants of Tallarook × 458 grown by Dr. P. S. Nutman in studies on *Rhizobium*. Apart from dwarfs similar to those observed in the field, two dwarf plants produced leaflets which were very aberrant.

TABLE 8

INDEPENDENCE OF VEGETATIVE ABNORMALITY AND POLLEN SCORE IN F_2

Abnormal Pollen Score (%)	Tallarook × 458 F_2		Tallarook × Clare F_2		
	Normal	Dwarf	Normal	Dwarf	Semi-dwarf
< 10	13	1	3	2	—
20–40	24	3	1	3	—
50–70	24	4	9	17	6
80–100	1	3	10	14	2
Empty anthers	11	3	—	—	—
Total	73	14	23	36	8

The length was approximately twice the width, compared with the normal length: width ratio of about 0·9. One of these dwarfs had empty anthers and set no seed, the other had about 80 per cent. normal pollen and set some seed (Plate 1). In

TABLE 9

CHIASMA FREQUENCY OF SOME PARENTS AND F_1'S

Parents and F_1 Crosses	No. of Cells	Mean Chiasmata per Cell	Chiasmata per Observed Bivalent
Tallarook*	19	15·9	2·02
458*	23	17·7	2·22
Clare	23	16·0	2·00
Wenigup	26	17·0	2·13
Burnerang × Wenigup	18	13·4	1·87
Tallarook × Clare	36	14·4	1·68
Tallarook × Wenigup	43	13·8	1·94
Tallarook × 458*	18	12·1	1·79

* Material collected in field, remainder from glasshouse.

addition one plant of this cross was not only dwarf but did not flower during a 13 months life, despite exposure to a 20-hr day, under conditions which allowed all other plants in the glasshouse to flower.

(*d*) *Meiosis*

Meiotic behaviour was studied in four parent lines and the four F_1 hybrids which showed the greatest pollen sterility. Chiasma frequency was significantly reduced in each of the hybrids, indicating a degree of non-homology between the chromosomes or the genes of the parental lines (Table 9). The existence of chromosomal non-homology was confirmed by the metaphase associations (Table 10; Plate 2). The four parent lines each had a mean of approximately two chiasmata per bivalent and, except for occasional univalents in Tallarook, had regular pairing with eight bivalents. The hybrids had from 1·4 to 1·8 chiasmata per potential bivalent, 1·7 to 1·9 per observed bivalent, and pairing was far from regular. One of the hybrids, Tallarook × 458, had a high degree of failure of pairing; the other three showed evidence of structural changes indicating the presence of translocation heterozygotes.

TABLE 10

METAPHASE PAIRING IN SOME PARENTS AND F_1'S

Parents and F_1 Crosses	No. of Cells	Metaphase Pairing							
		1 IV 6 II	1 IV 5 II 2 I	1 III 6 II 1 I	1 III 5 II 3 I	8 II	7 II 2 I	6 II 4 I	5 II 6 I
Tallarook*	38		—	—	—	35	3	—	—
458*	117		—	—	—	117	—	—	—
Clare	23		—	—	—	23	—	—	—
Wenigup	26		—	—	—	26	—	—	—
Burnerang × Wenigup	18	6	—	5	1	4	2	—	—
Tallarook × Clare	36	6	—	9	2	12	5	2	—
Tallarook × Wenigup	43	17	3	9	2	10	1	1	—
Tallarook × 458*	51	—	—	—	—	36	13	1	1

* Material collected from field, remainder from glasshouse.

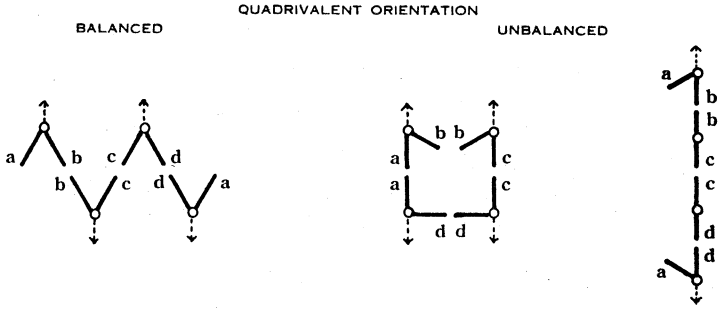
This failure of pairing and structural hybridity leads to numerical and segregational unbalances in the products of meiosis, which could account for some of the observed pollen sterility.

Numerical unbalance results from random movement of univalents at meiosis. In the absence of polarized segregation 50 per cent. of the pollen grains will have a deficiency or an excess of chromosomes. Random segregation of univalents in both meiotic divisions was indicated by second anaphase (A II) counts of 10:8::7:7 and 8:7::9:8 in Tallarook × 458; 10:10::6:6 in Burnerang × Wenigup; and 7:7::9:9 in Tallarook × Wenigup. There was no evidence of misdivision of univalents which would add to the unbalance by the production of broken chromosomes.

Segregational unbalance resulting from duplications or deficiences of parts of chromosomes can follow certain types of anaphase separation of trivalent or quadrivalent associations. Unbalanced segregations of a trivalent and univalent are, in most cases, also numerically unbalanced. However, quadrivalent segregation

usually results in numerical balance and the segregational balance depends upon the orientation of the quadrivalent (Plate 2; see also Sinnott, Dunn, and Dobzhansky 1950, p.255).

Figure 4 presents the types of quadrivalent orientations and the proportions of balanced and unbalanced types for each of the three hybrids. These data are insufficient to permit any differentiation between the hybrids on this basis, but, in general, about 80 per cent. of the quadrivalents result in segregational and numerical balance and consequently do not contribute to pollen sterility. The joint contributions of numerical and segregational unbalance of these F_1 hybrids (Table 11) accounts for less than 40 per cent. of the observed pollen sterility.



F_1's		
5	BURNERANG X WENIGUP	1
6	TALLAROOK X CLARE	–
15	TALLAROOK X WENIGUP	5
26		6

Fig. 4.—Quadrivalent orientation and proportion which yield balanced and unbalanced products in the three translocation heterozygotes.

Summarizing we may say that there is evidence of non-homology, including translocations, of chromosomes of different varieties. The reduction in chiasma frequency per observed bivalent indicates the presence of rearrangements in several of the chromosomes, especially as the "Schultz-Redfield" effect (see White and Morley 1955) may operate to obscure such an effect, since rearrangements decreasing chiasma frequency in one chromosome or portion thereof tend to be accompanied by compensating increases in chiasma frequency in other portions of the chromosome complement. However, the structural rearrangements are insufficient to account for all, and probably less than half, of the observed pollen sterility.

IV. Discussion

This investigation has disclosed degrees of genetic incompatibilities in a rather limited set of crosses. These may be expressed in a variety of ways of which we have found reduced pollen fertility, reduced fertilization, embryo abortion, and vegetative breakdown. In general, these symptoms were correlated between crosses but within F_2 progenies most of the abnormalities appeared to be segregating independently.

Not all crosses showed symptoms of sterility barriers but when reproductive abnormalities occurred in a cross, all types of abnormality were usually present. Vegetative abnormalities only occurred in crosses showing severe reproductive abnormalities but pronounced reproductive failure was not invariably accompanied by vegetative breakdown.

Meiotic irregularities indicated structural chromosome changes between some parental varieties. Clare and Wenigup differed from Tallarook by at least one translocation. Similarly Burnerang and Wenigup differed by a translocation. The behaviour of the F_1 Tallarook $\times$ 458 indicated that some structural rearrangements had occurred but no translocations were observed.

TABLE 11

CONTRIBUTION OF NUMERICAL AND GENIC UNBALANCE TO POLLEN STERILITY (CALCULATED FROM TABLE 10 AND FIG. 4)

F_1	Numerical Unbalance (%)	Segregational Unbalance (%)	Total Unbalance (%)	Observed Pollen Sterility (%) (see Table 1)
Burnerang × Wenigup	22	7	29	85
Tallarook × Clare	25	3	28	80
Tallarook × Wenigup	19	9	28	80
Tallarook × 458	15	—	15	68

We have seen that these structural changes, as indicated by meiotic behaviour in the F_1, contribute to pollen sterility. However, their contribution in every case accounts for less than 40 per cent. of the observed pollen sterility (Table 11). Therefore most of the observed sterility is probably genic in origin or is determined by cryptic structural differences such as suggested by Stebbins and Vaarama (1954) in *Elymus* × *Sitanion* hybrids.

There are three kinds of evidence that support the view that the sterility is genic rather than chromosomal in origin.

Firstly, the variations in pollen fertility within and between anthers suggests a system of balance which is easily upset by small variations in the environmental conditions. A system so finely adjusted is likely to depend on balance between genes since the more gross structural effects would determine more generalized symptoms.

Secondly, predictions of the pollen fertility of crosses were not always fulfilled. For example, a study of the pollen fertility of the cross Tallarook $\times N_1E$ suggests that these varieties are fairly closely related, whereas Tallarook and Dwalganup are not (this is supported by Tallarook $\times$ Mt. Barker and Mt. Barker $\times$ Dwalganup crosses). From this one might predict that an N_1E and Dwalganup cross would have much pollen sterility. However, the pollen fertility of this cross was high. Such a situation is unlikely if the parents differ structurally.

Thirdly, the lack of correlation between abnormalities within F_2's suggests that finely balanced physiological systems were affected. If gross deficiencies or duplications were present one would expect more drastic symptoms.

While none of this evidence is conclusive it suggests that the observed sterility derives not only from chromosomal differences between varieties but also from disturbances to physiological equilibria determined by the balance of the individual genes.

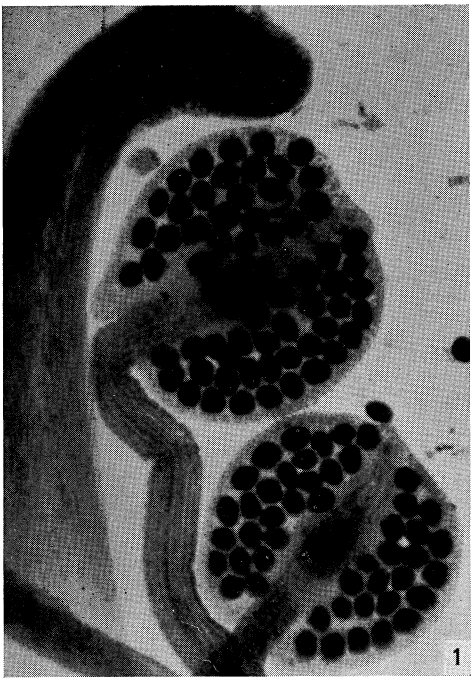
Unfortunately the geographical origin of the varieties in question is not known. Certain physiological studies in progress suggest that Tallarook, Mt. Barker, and Burnerang derive from areas which are climatically very different from those in which Wenigup, Clare, Yarloop, and the early-flowering varieties were originally evolved to their present forms. Crosses between Tallarook and the last three varieties mentioned certainly suggest genetic differences of a high order. Therefore the divergence is probably of long standing and their respective origins probably geographically remote. But the cross between Tallarook and N_1E, for example, i.e. between varieties which differ widely in maturity and in many morphological and physiological attributes, shows relatively little hybrid sterility. Therefore at least some types of physiological and morphological divergence need not be associated with a high degree of hybrid sterility. Obviously a clear picture of the role of geographic isolation in this case of subspeciation must await results of further work now being undertaken.

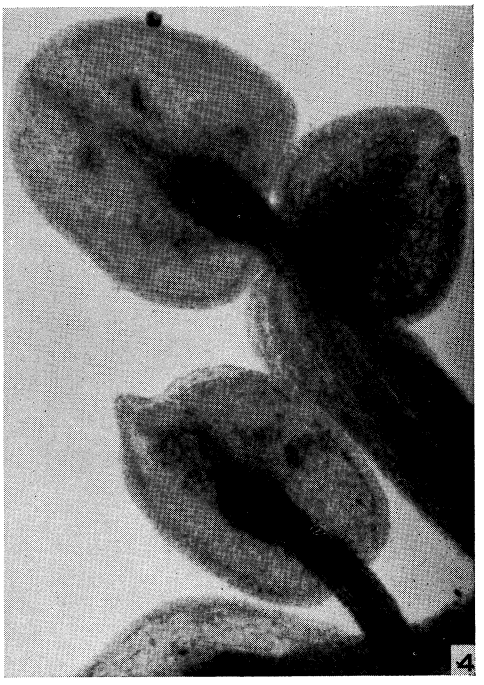
The accumulation of genic and structural differences of the order observed is very likely to have required large numbers of generations during which varieties were reproductively isolated. The period of 50 to 70 years in which the species has existed in Australia is probably quite inadequate. Therefore these observations support the contention of Aitken and Drake (1941) that the majority of the varieties existing in Australia were introduced as such. Nevertheless, the possibility should not be overlooked that occasional mutation or hybridization, followed by selection, may have resulted in some of the recognized strains.

The present range of *T. subterraneum* in Australia, and the agronomic value of the species, is largely determined by the combinations of characters existing in the available varieties. Although the species, and even single varieties, have been successful and extremely valuable in many habitats, there are strong possibilities that recombinations of characters will extend the range and increase productivity. The species is therefore of great potential value to the agronomist and plant breeder.

The importance of hybrid sterility in a programme of selection is difficult to evaluate. Extreme genetic divergence is probably an important barrier to hybridization. Nevertheless, crosses between widely divergent varieties (e.g. Tallarook and Clare) are possible, and these provide a wide range of recombinations. There is a possibility that in some desirable crosses the only balanced segregates will resemble one parent or the other, with no intermediate types. As seed production is likely to be an attribute of importance to the survival and use of the species there may be a strong case for rigid selection for seed yield during the early stages of a breeding

SUBSPECIATION IN TRIFOLIUM SUBTERRANEUM





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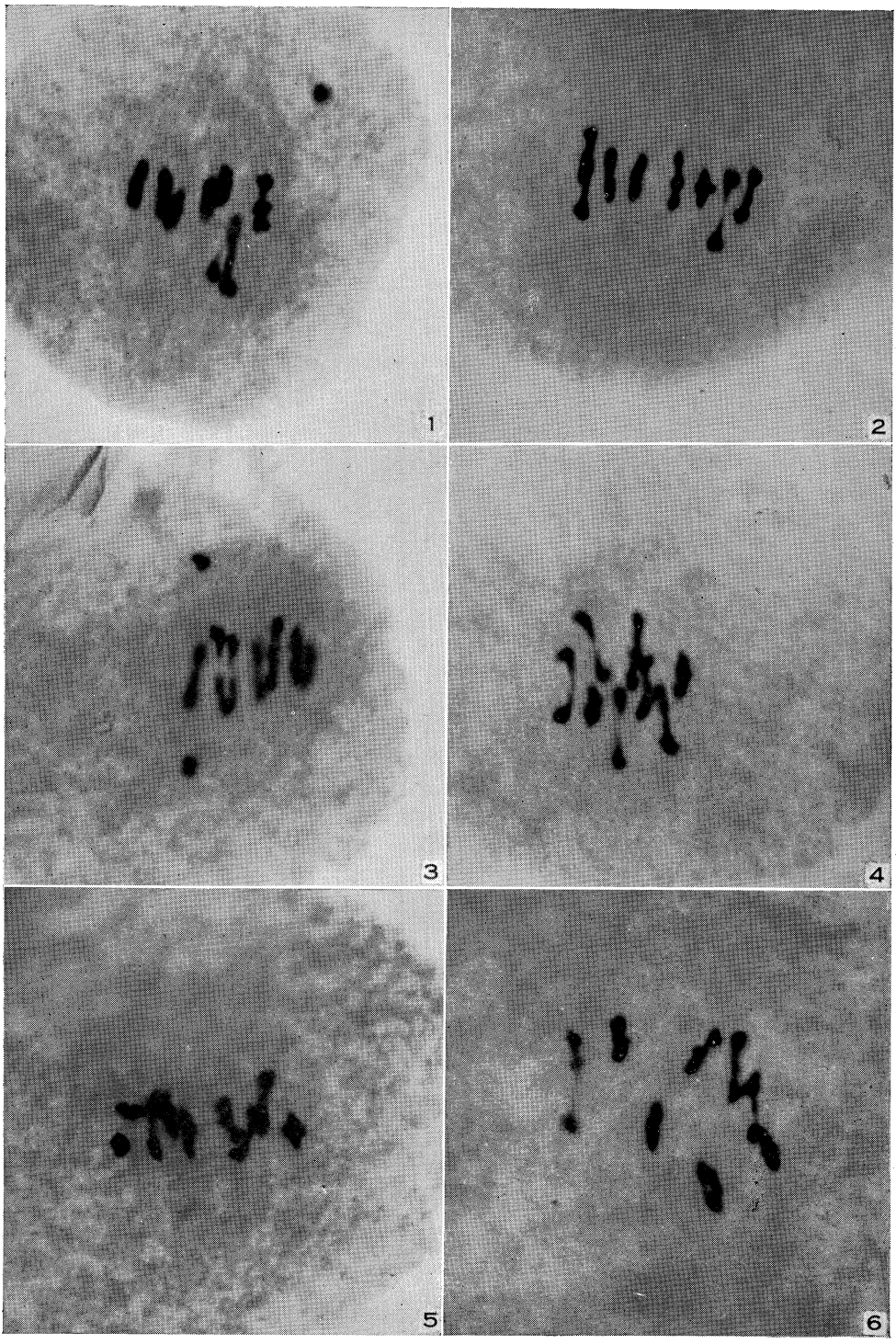

programme. However, in plots exposed to natural selection there would be a fairly rapid elimination of plants of reduced fertility. Presumably such selection would lead to new genetically balanced and fertile strains.

V. Acknowledgments

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Explanation of Plates 1 and 2

Plate 1

Pollen grain development in some anthers of F_2 *T. subterraneum* hybrids. All × 65.

Fig. 1.—Normal anthers from Tallarook × Yarloop.
Fig. 2.—Reduced pollen fertility in Tallarook × Clare.
Fig. 3.—Extreme infertility in Tallarook × Clare. Note variation between anthers.
Fig. 4.—Empty anthers in Tallarook × 458. Note lack of differentiation. (cf. Figs. 2 and 3.)

Plate 2

First metaphase configurations of some F_1 *T. subterraneum* hybrids. All × 1750.

Fig. 1.—Tallarook × Clare showing trivalent and univalent.
Fig. 2.—Tallarook × Wenigup showing quadrivalent.
Fig. 3.—Tallarook × Wenigup showing quadrivalent and two univalents. This type of quadrivalent orientation leads to segregational unbalance.
Fig. 4.—Burnerang × Wenigup showing quadrivalent.
Fig. 5.—Tallarook × Wenigup showing quadrivalent and two univalents.
Fig. 6.—Tallarook × Wenigup showing quadrivalent.