Thesis submitted for the degree of
M.Agr.Sc. in the University of New
Zealand. 1939.

INHERITANCE OF GROWTH HABIT AND

CALYX MARKING IN

SUBTERRANEAN CLOVER.

(Trifolium subterraneum Linn.)

BY

" BULK "

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SECTION I.

INTRODUCTION.

Subterranean clover is an annual legume which perpetuates itself in pasture by reseeding. After flowering the peduncle turns downward, and the head enters the soil. From this habit the common and specific names have been derived.

(1) Origin of the Species.

The original home of the species is stated to be Southern Europe, Western Asia to India and North Africa (Cheeseman 1925). An account of the accidental introduction of the species into Australia gives the period as 1880-1890 (Gardner & Dunne, 1933). From there, probably, it spread to New Zealand. Though the actual time of its arrival cannot be stated, it was recorded by Cheeseman in 1906 near Auckland, where for many years it has been known as "Mangere" clover.

(2) Varietal Diversity and Pure Lines.

A considerable diversity exists within the species, numerous varieties or strains having been recognised. These strains have been named according to districts and localities in which they were found. As a result there are a number of synonyms.

That these strains are pure lines has been demonstrated both in Australia and New Zealand. Harrison (1933) has recorded that the seed from a single plant of the "Burnerang" strain was multiplied to twenty pounds in two years without a single variant appearing. Harrison (1935) also states that "....Tallarook has bred true to type over a period of years in the Burnley Pasture Plant Research Field. The flowers are normally self-fertilized and each generation of plants has great stability of type, though growing side by side with other strains". The Grasslands Division of the Plant Research Bureau of New Zealand has conducted large scale single plant trials and no variants, in numerous strains

tested, have appeared.   That the strains are pure lines is to be expected as the florets of the species are self-fertilized, in fact cleistogamous (Donald & Neal-Smith 1937: Hunter 1931). Donald & Neal-Smith consider that "...mutation is the likely primary cause of the range of distinct types existent".

(3)   Growth Habit.

Most strains of Subterranean Clover possess a prostrate growth habit.   This is typical of Dwalganup, Nangeela, Mt.Barker and Tallarook strains, shown in Figs. 1 - 4.   One strain, Burnerang (Fig. 5), has a characteristic erect, bunchy habit.

(4)   Calyx Marking.

The calyces of many strains possess a red band at the base of the lobes.   This banding is characteristic of the Mt. Barker and Burnerang strains (Figs. 6 and 7).   Other strains, such as Dwalganup, Nangeela and Tallarook, possess no marking on the calyx tube.   These unmarked types are shown in Figs. 8, 9 and 10.

(5)   Scope of Investigation.

The primary purposes of the project have been to study -

(a)   The inheritance of growth habit.

(b)   The inheritance of calyx marking.

As far as is known at present the two habits are of equal value from a pasture point of view.   Presence or absence of calyx marking may appear of little moment, but for an understanding of the genetical make-up of a species such preliminary studies as these are necessary.   Whichever growth habit be found more valuable, knowledge of its mode of inheritance must be obtained in any attempted building of new strains by breeding.   The value of knowledge of the inheritance of a qualitative character such as calyx-marking lies in its possible linkage with less easily recognised characters.

(6)   Method of Approach.

Hybridization of strains, followed by a study of the F.1 and

F.2 generations has been the method of study.   This involved

emasculation and hand pollination.

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The Parent Strains and their Growth Habits.

Fig. 1. Dwalganup.(S.1).

Typical prostrate habit.

Fig. 2. Nangeela.(S.3).

Typical prostrate habit.

Fig. 3. Mt.Barker. (S.4).

Typical prostrate habit.

Fig. 4. Tallarook. (S.5).

Typical prostrate habit.
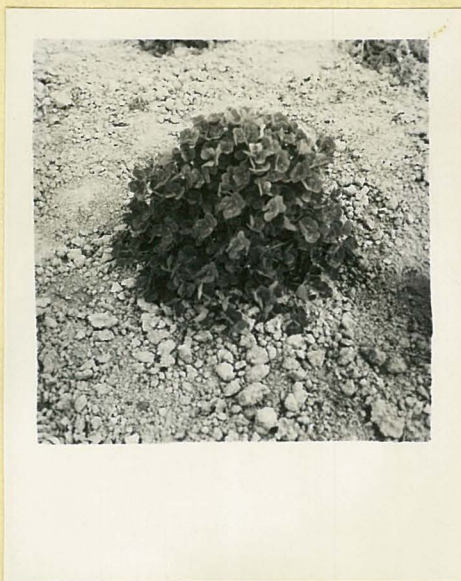
Fig.5. Burnerang. (S.2).

Typical erect habit.

Fig.6.Mt.Barker.(S.4).

Distinct red band at
base of calyx lobes.

Fig.7.Burnerang.(S.2).

Calyx marking paler than
Mt.Barker but distinct.

Fig.8.Dwalganup.(S.1).

No calyx marking.

Fig.9.Nangeela.(S.3).

No calyx marking.

Fig.10.Tallarook.(S.5).

No calyx marking.

SECTION II.

TECHNIQUE.

Usually, the infloresence contains three florets, but four are not uncommon and five are produced at times. The florets are small, 10 - 15 m.m. in length, and hybridization in the field is rendered extremely difficult, if not impossible.

(1) Symbols used for Parent Types.

Symbols were adopted for the parent plants and strain names dispensed with. The symbols used were:

S.1   denoting Dwalganup strain.

S.2   "   Burnerang   "

S.3   "   Nangeela   "

S.4   "   Mt. Barker   "

S.5   "   Tallarook   "

(2) Culture of Parent Plants.

On 15/5/37 a number of seeds of each of the five strains were sown. Finally one seedling from each was selected as a parent plant and grown in an eight inch pot. McMillan (1937) stated that at Canberra mildew caused some difficulty with potted subterranean clover plants. No trouble was experienced during this study, however. McMillan also stated that wilting will interfere with seed setting. Accordingly, some spare plants were subjected to wilting and the influence on seed setting noted. It was found that wilting at the time of pollination, or even several days later, caused the failure of seed-setting in most florets.

Severe aphid infestation occurred on two occasions. This was treated with Nicotine sulphate according to Cunningham's (1935) recommendation. The method completely destroyed aphids on each occasion.

(3) Scheme of Crossing.

The scheme followed was that of diallel crossing, in which each parent was crossed with each of the others. Crosses were

made in both directions i.e. a parent was used both as a male
and a female.   The crosses were designated as X1 to X 10,
while their respective reciprocal crosses were X 1A to X 10A.
The system of allocation of cross-numbers is shown in Table I.
Crossing was carried out over a period of two months.   This
period is longer than necessary, but a fairly long period must
be covered when early and late-flowering strains are to be
crossed.   Differential treatment, such as in time of sowing, or
in lengthening the day for late strains by artificial lighting,
would shorten the period.

TABLE I.

Showing the system of allocating cross numbers
and the crosses involved.

Parents			Cross Number
Female		Male	
S.1	x	S.2	X 1
"		S.3	X 2
"		S.4	X 3
"		S.5	X 4
S.2	x	S.1	X 1A
"		S.3	X 5
"		S.4	X 6
"		S.5	X 7
S.3	x	S.1	X 2A
"		S.2	X 5A
"		S.4	X 8
"		S.5	X 9
S.4	x	S.1	X 3A
"		S.2	X 6A
"		S.3	X 8A
"		S.5	X 10
S.5	x	S.1	X 4A
"		S.2	X 7A
"		S.3	X 9A
"		S.4	X 10A

(4) Method of Emasculation.

Hybridization involved emasculation and hand pollination.
At the time emasculation was commenced, descriptions of suitable
means of performing the operation had not been seen. No record
of any such hybridization in New Zealand was available. McMillan's
article came to hand shortly after this, in which he described
a method which was apparently satisfactory. Nevertheless, his
method of scraping out the anthers with a blunt needle proved to
be tedious and not particularly safe, as it was necessary to
remove the anthers at a time when they would rupture easily.

A more satisfactory method was evolved which, though slow,
was faster than that of McMillan and definitely safer. This
method, which was used throughout the work, consisted in opening
the floret and removing the anthers with fine pointed forceps.

The small nature of the florets necessitated some form of
magnification and, as both hands must be free, a binocular
dissecting microscope, giving a magnification of 25 X, was
employed. The type of binocular microscope which is worn on a
head-band probably would prove most useful.

Good lighting of the florets undergoing emasculation was
necessary. For this purpose a 100 watt pearl type lamp, in an
opaque shade, was used. This allowed bright illumination of
the object, while the operator's eyes were protected from the
light.

The peduncle was held firmly on the bench top with the aid
of drawing pins, and was protected from injury by means of strips
of rubber placed under the heads of the pins. Black paper, such
as photographic goods are packed in, was found to be of great
assistance when placed under the florets on the bench top. This
made the floral parts more easily distinguished. All the florets
in a head were utilized except those in which anthers had burst
naturally or during emasculation. Fig.11 shows the general
arrangement during emasculation.
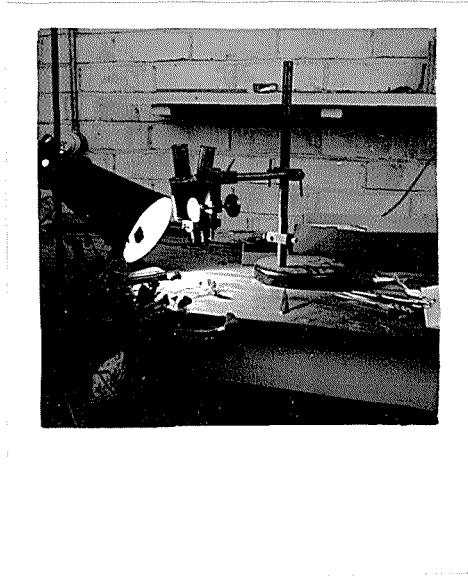
Fig. 11. Showing the general arrangement of equipment during the emasculation or pollination operations.

Opposite calyx lobes were grasped, each with a pair of forceps, and peeled back towards the base of the floret. The keel was then opened and the lobes turned back. In some cases this could be done directly; in other cases the wings first had to be turned back. This depended on the position of the floret on the bench. At times, for more easy manipulation, a wing or one or more calyx lobes could be removed entirely with no subsequent ill-effects on the floret.

Once the keel was opened it was not difficult to grasp the anthers and remove them with one pair of forceps, while holding the floret with the other pair. The anthers might be removed singly or in one or more bunches, this again depending on the position of the floret. Care had to be taken not to injure the style or stigma. The anthers were counted as removed to ensure that all had been taken out, and the forceps were sterilized in alcohol after each operation. It was found necessary to

emasculate the florets at a stage one to two days before the
anthers would dehisce normally. At this stage the corolla
is sufficiently developed to about equal the calyx lobes in
length, or no more than slightly project beyond them. The
anthers are then firm and resistant to rupturing. After emascul-
ation had been completed, the keel, wings and calyx lobes were
replaced in position over the style for its protection. The
infloresence was then distinguished by tying a small label to
its peduncle and recording on it the "S" number of the plant,
number of florets emasculated, and the date. Duplicate entries
were made in a note book kept for the purpose. Bagging of the
emasculated florets was considered to be unnecessary as the
risk of accidental pollination is negligible.

(5) Other Methods of Emasculation.

Since completing emasculation by the forceps method other
methods have come under notice, and one method has been tried
which must be considered as more efficient than that used.

Kirk (1930) used a suction method for emasculating florets
of Melilotus. Suction pressure was obtained from a water tap
to which a suction pump was attached. A glass nozzle with a
small bore was attached.

Savage (1935) used the suction pressure from the manifold
of an idling car engine, and utilized an hypodermic needle in
place of the glass nozzle.

Hill (1937) used a car wind-shield wiper to obtain suction
pressure for emasculating sugar-cane flowers.

Kirk's method, with Savage's modification of an hypodermic
needle was experimented with in the year following the main
emasculation work. The method proved highly satisfactory,
and considerably faster than the forceps method. The anthers
are plucked off and carried away by the air-stream as the needle
is moved about from anther to anther. Danger of bursting anthers
is eliminated almost entirely, and manipulation of the flower
is more satisfactory generally.

Oliver (1910) used a jet of water for depollinating florets, but his method does not appear at all satisfactory, especially as the anthers were permitted to dehisce before the operation.

That the method used was reasonably satisfactory, however, can be seen from Table IV (page 18) which shows that 69% of emasculations followed by hand pollination were successful.

(6) Method of Pollination.

Pollination immediately following emasculation proved to be largely unsuccessful since emasculation was performed at an early stage.   It was necessary, therefore, to wait until such a time as the stigma became receptive.   This time was at least one day after emasculation, while pollination two days after emasculation was still successful.

A direct method of pollination was employed in which the anthers from the desired male parent were brushed over the stigma. The anthers from a floret of the male parent were grasped in a bunch with the forceps, and applied directly to the stigma of the female parent.   The stigma had been prepared by folding back the floral parts of a previously emasculated floret. Anthers were chosen which had recently burst, and it could be seen readily whether pollen adhered to the stigma, as the operation was carried out under the microscope.   The floral parts were then replaced over the pollinated stigma and labelling completed by adding to the previous data the "S" number of the male parent and the date of pollination.

To enable the male parent in any cross to be recognised at a glance, a colour system was employed.   A colour had been allotted to each of the five parents, and, whenever a parent was used as a male a piece of wool of its corresponding colour was tied around the peduncle of the pollinated infloresence.   Only one male parent was used to pollinate the florets of any one infloresence.

(7) Harvesting and Sowing the Seed from the
    P.1 and F.1 Plants.

Harvesting of seed from the P.1 plants extended over a period
from mid-December to early January.   The seeds from which the
F.1 plants were to be grown were sown in boxes on the 27th April
in the glasshouse.   Germination was satisfactory, seed from five
of the crosses germinating 100%, seed from one cross germinating
50% and the average being 85%.   The F.1 seedlings were planted
out on the 22nd August 1938, in rows, and were spaced 3 feet apart
each way.   This wide spacing was to allow good development and
at the same time to permit of each plant being harvested separately.
Despite this precaution it was necessary to trim the trailing
stems back at times to prevent the plants growing one into another.

Since the pure-line status of the strains had been well
established, it was considered unnecessary to study a large L.1
population.   But, for purposes of comparison, 10 L.1 plants
from each parent were put out.   The plants finally put out
consisted of these 50 L.1 plants and 121 F.1 plants from the
various crosses and their reciprocals.

The F.1 plants were harvested separately on the 12th January
1939.   Climatic conditions for ripening and harvesting had not
been good, the weather being wet and dull.   As the number of
plants would be very large it was impracticable to study an F.2
from every cross or even from every F.1 plant of a particular
cross.   The F.2 plants to be studied had to be limited to a
number raised from several F.1 plants only of suitable crosses.
Suitable crosses were X 5 and X 5A   (Parents: Nangeela (S.3) and
Burnerang (S.2)) for growth habit study, and X 8 and X 8A
(Parents: Nangeela (S.3) and Mt. Barker (S.4)) for calyx marking
study.   Seed from two F.1 plants of both the cross and reciprocal
was to be used in raising an F.2 large enough for study but not
too large for the land available.

One box was sown with seed from each of the following F.1

plants :- X 5/2, X 5/5, X 5A/4, X 5A/5, X 8/2, X 8/3, X 8A/1, X 8A/5 *   These sowings were made on 27/1/39, a fortnight after harvesting.   Early sowing was made   so that it might be possible to complete the project in the time available, particularly where floral characters were involved.

(8) Germination of Hybrid Seed.

McMillan has stated that his efforts to germinate subterranean clover seed immediately after harvest failed, and that a definite rest period appeared necessary.   This proved to be the case with seed from the F.1 sown on 27/1/39.   Germination was exceedingly disappointing.   Seed from three plants only had germinated to an extent of over 10% after a month had elapsed.   The three F.1 plants which produced more than 10% of seedlings are as follows, with germination figures in parenthesis : X 8/2 (80%), X 8A/1 (15%), X 8A/5 (40%).

Seeds from the remaining F.1 plants of the above crosses were placed on moist filter paper on 13/3/39.   Germination was negligible in each case.   Apparently such seeds, though mature, required a period of "after-ripening" before they would be capable of germinating.   This after-ripening possibly may be connected with the activity of enzymes within the seed or the production of phytohormones.   It was thought that if suitable means could be adopted, this after-ripening process might be accelerated.   No facilities were available for testing the effect of storage at different temperatures along the lines of Hyde's (1936) work with "new season's" Algerian oats.   There was the possibility that enzymes, or their products could be supplied to the seeds.   It happened that experimental retting of linen flax was proceeding in the vicinity.   In this retting process the flax undergoes fermentation in a tank of water and a strongly smelling liquor is produced which has an acid reaction.

*   "5/2" indicates plant No.2 of the F.1 of X 5, and so on.

With the idea in mind that such liquor might contain enzymes, organic acids or other products of fermentation, it was decided to try out the effect of such liquor on germination. Unfortunately little seed was available for an exhaustive trial, seed from the F.1 being too valuable for such purposes. However, 100 seeds from a plant (X 10A/3) were used. On 7th March 50 of these were placed on filter paper moistened with tap water, and 50 on paper moistened with retting liquor. In 5 days 50% of the liquor treated seeds had germinated, and none of the water treated. After 10 days, no more of the seeds had germinated in either case and the experiment was discontinued. Though no conclusive result had been reached, an indication was given that such treatment accelerated germination. All seedlings raised after this were first germinated on filter paper and then pricked out into boxes. This ensured that the maximum number of plants per box could be grown. Since the retting liquor had had no deleterious effect on germination, and indications were that it was beneficial, the filter paper used after this in germination of seeds was moistened with the liquor.

It has been suggested, in literature describing the commercial preparation "Hortomone A", which is used to some extent in rooting cuttings, that it might be of some value in accelerating germination of seeds. Accordingly a solution of Hortomone A was used, 1 part in 320 parts of water. This strength had been recommended in the literature for use on soft cuttings, though it was pointed out that such a concentration was harmful to some soft cuttings. One hundred seeds from two F.1 plants of two crosses were soaked overnight (28/3/39) in a 1 in 320 solution of Hortomone A, and a similar series in tap water. The following morning the seeds were removed, rinsed, and placed on filter paper moistened with water. After 6 days the following

germination percentages were obtained:

Plant No.	Hortomone A treated.	Water treated.
X 7/1	16	21
7A/1	9	24
10/2	20	51
10A/2	17	28

From these results it can be seen that Hortomone A, at this concentration, and for the period used, depressed germination of subterranean clover seeds. No lasting depression was caused, however, as final figures were much the same in each case. The maximum possible germination percentages were not obtained, as when sufficient seeds to fill a box had germinated the experiment was discontinued. The test indicated also that seeds were becoming "after-ripened", since germination of water moistened seeds was fair.

When sufficient seedlings to fill a box with the progeny of each of the four plants used in this test had been obtained, seeds from one more F.1 plant of each cross and its reciprocal were germinated and pricked out in boxes. On 13/6/39 bulked seed of the F.1 of each of two crosses and their reciprocals were sampled and placed on filter paper to germinate. The crosses and reciprocal crosses were X 5, X 6, X 5A, X 6A. Seed from F.1 plants of X 5 and X 5A had previously shown very low germination (27/1/39). In this case, however, germination proved to be excellent, as is shown in Table II (page 14).

TABLE II.

Showing the results of germination on filter
paper of F.1 seed. Seed of similar origin had pre-
viously shown very poor germination.

Cross No.	X 5	X 5A	X 6	X 6A
% Germination 20/6/39	82	75	50	51
% Germination 28/6/39	94	83	67	83
% Hard seeds 28/6/39	4	12	24	16
% Germination 5/7/39	100	96	94	98

The "hard" seeds (seeds with an impermeable testa) were
scratched following the count on 28/6/39. Additional germination
after this date has been due mainly to germination of these
scratched seeds. Scratching was effected by rubbing the seeds
between sheets of sand-paper.

Of the original box sowings of seed from F.1 plants, only
those of X 8/2 and X 8A/5 provided sufficient F.2 plants for study.
Seed of X 7A/2 had germinated in sufficient quantity, in a later
sowing, to produce a reasonably large F.2. The bulk of the F.2
finally grown consisted of progeny of the following F.1 plants:
X 7/1, X 7/5, X 7A/1, X 7A/2, x 7A/4, X 8/2, X 8A/5, X 10/2,
X 10/3, X 10A/2, X 10A/5 and of the bulked F.1, of X 5 + 5A and
X 6 + 6A. Seeds of most of these were germinated on filter-
paper, pricked out into boxes, and finally planted in the plot.
Some F.2 families were grown early enough to plant out in the
autumn of 1939, but other families were not planted out till the
spring. Since the spring was dry these latter plants made poor

growth, but sufficient for the purposes of the study.

(9) Photography.

All photographic work has been carried out by the writer.
The camera used was a Voigtlander with an f.4.5 lens.   Since
no enlarging apparatus was available, a supplementary lens was
utilized after some experimenting. This lens, which fitted over
the original lens, enabled close-up photographs to be made of
florets.   Contact prints made from negatives produced with the
aid of this supplementary lens have an image $1\frac{1}{3}$ x natural size.
The photographs of single plants were taken at a distance of
three feet from the object.

SECTION III.

RESULTS.

(1) Seed Setting of the P.1.

Cross fertility is shown in Tables III, IV and V. From these it is evident that seed yield differed widely in the various crosses, but that the fertility of the parents, whether used as male or female, varied little on the basis of total seeds produced by each parent.

TABLE III.

Seed setting of the P.1.

Showing number of Florets emasculated

and pollinated, and number of seeds harvested from

the parent plants in the various crosses.

Parents.		No. florets emascul- ated & pollinated.	No. seeds harvested.
Female.	Male .		
S.1	S.2	16	13
"	S.3	11	9
"	S.4	15	6
"	S.5	12	9
S.2	S.1	7	5
"	S.3	9	7
"	S.4	10	6
"	S.5	8	6
S.3	S.1	12	10
"	S.2	16	11
"	S.4	8	3
"	S.5	14	11
S.4	S.1	7	4
"	S.2	10	4
"	S.3	11	7
"	S.5	9	8
S.5	S.1	12	9
"	S.2	8	8
"	S.3	12	6
"	S.4	10	7
Totals		217	149

TABLE IV.

(Data from Table III.)

Fertility of the Parents when used as Females.

Female Parents	S.1	S.2	S.3	S.4	S.5	Totals
No. of florets of each parent emasculated and pollinated.	54	34	50	37	42	217
No. of seeds harvested from each parent.	37	24	35	23	30	149
Seeds harvested expressed as percentage of emasculations.	68.5	70.5	70.0	62.1	71.4	68.6

TABLE V.

(Data from Table III.)

Fertility of the Parents when used as Males.

Male Parents	S.1	S.2	S.3	S.4	S.5	Totals
No. of florets pollinated by each male parent.	38	50	43	43	43	217
No. of seeds harvested after pollination by each male parent.	28	36	29	22	34	149
Seeds harvested expressed as percentage of pollinations.	73.6	72.0	67.4	51.1	79.1	68.6

Tables IV and V show that S.4 (Mt.Barker) has been least fertile and S.5 (Tallarook) most fertile whether used as a male or female. The differences are not great, however, and since cross-fertilization does not take place naturally, the results cannot be taken as a guide to field fertility.

(2.) a. General Observations on the F.1.

There appeared to be an expression of heterosis in at least some of the hybrid families. This observation is limited however, by the small number of F.1 plants within any cross or reciprocal and also by the uneven fertility of the plot soil. Plants within any particular cross or reciprocal were similar. In general appearance the hybrids were about intermediate between their parents e.g. in proportion of stem and leaf. In leaf marking the F.1 plants approached the parent possessing the most intense markings e.g. S.3 (Nangeela) had a pronounced white crescent on its leaflets, and other strains had less distinct markings. The F.1 plants from a cross of S.3 with any other parent approached S.3 in intensity of marking. Similarly the characteristic chocolate base of the leaflets of S.5 (Tallarook) appeared in the F.1 of crosses/of S.5 with any other parent.

b. General Observations on the F.2.

Since the F.2 studied was limited to progeny from suitable crosses, the observations apply only to the F.2 from X 5, X 6, X 7, X 8, X 10 and their reciprocals. Segregation in the F.2 was evident for such characters as stemminess and leafiness, leaf marking, calyx marking, growth habit, time of flowering etc. The ranges of variation were similar in families derived from the F.1 plants of the same crosses or reciprocals.

(3) a. Growth Habits of the F.1.

Every F.1 plant from all crosses and reciprocal crosses was of prostrate habit. This is shown in Figs. 12 to 25.

Fig.12. Cross 1.
Dwalganup x Burnerang.
(Prostrate x Erect).
Progeny prostrate.

Fig.13 Cross 1A.
Burnerang x Dwalganup.
(Erect   x Prostrate).
Progeny Prostrate.

Fig.14. Cross 5.
Burnerang x Nangeela.
(Erect   x Prostrate).
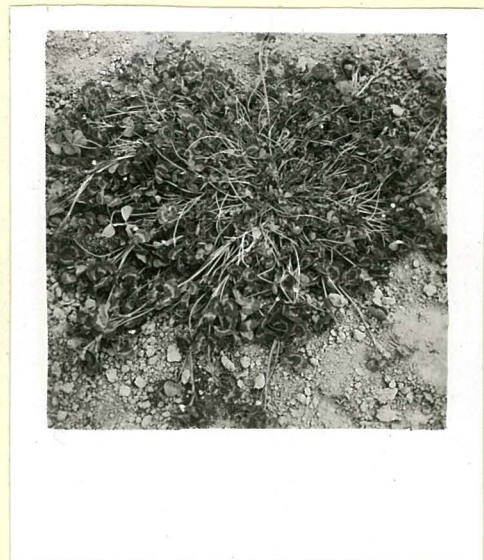Progeny prostrate.

Fig.15. Cross 5A.
Nangeela x Burnerang.
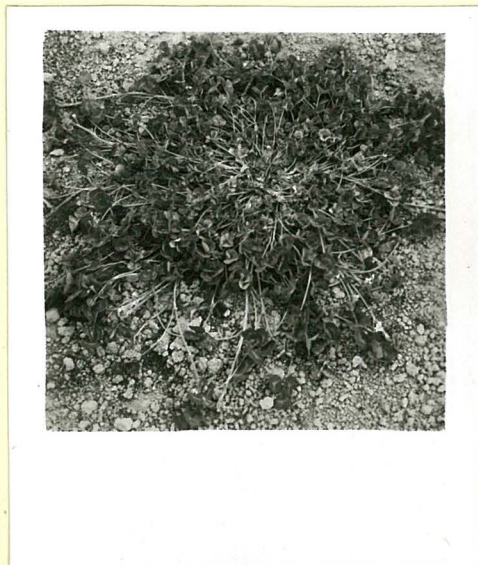(Prostrate x Erect).
Progeny prostrate.

Fig.16. Cross 6.
Burnerang x Mt.Barker.
(Erect   x Prostrate).
Progeny prostrate.

Fig. 17.   Cross 6A.
Mt.Barker x Burnerang.
(Prostrate x Erect).
Progeny prostrate.

Growth Habits of the F₁ Generation.

Female Parents are mentioned first in each case.

Fig.18.   Cross 7.
Burnerang x Tallarook.
(Erect   x Prostrate).
 Progeny prostrate.

Fig.19.   Cross 7A.
Tallarook x Burnerang.
(Prostrate x Erect).
 Progeny prostrate.

Fig. 20.   Cross 2A.
Nangeela x Dwalganup.
(Prostrate x Prostrate).
 Progeny prostrate.

Fig.21.   Cross 3.
Dwalganup x Mt.Barker.
(Prostrate x Prostrate).
 Progeny prostrate.

Fig. 22.   Cross 4.
Dwalganup x Tallarook.
(Prostrate x Prostrate).
 Progeny prostrate.

Fig. 23.   Cross 8.
Nangeela x Mt.Barker.
(Prostrate x Prostrate).
 Progeny prostrate.

Fig. 24.   Cross 9.
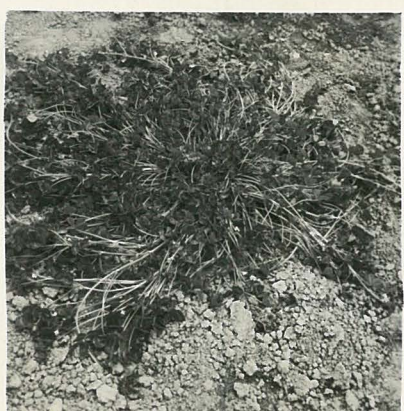Nangeela x Tallarook.
(Prostrate x Prostrate).
 Progeny prostrate.

Fig. 25.   Cross 10.
Mt.Barker x Tallarook.
(Prostrate x Prostrate).
 Progeny prostrate.

Dominance proved to be complete, no types intermediate between prostrate and erect appearing.

  b. Growth Habits of the F.2.

  There were 507 F.2 plants from the following F.1 hybrids of prostrate parentage: X 8/2, X 8A/5, X 10/2, X 10/3, X 10A/2, X 10A/5. All F.2 plants grown from these hybrids were of prostrate habit. Segregation for other characters took place as has been explained.

  Of F.2 plants derived from the F.1 of crosses of prostrate and erect habits, 327 were planted out. F.1 plants which produced this F.2 were X 7/1, X 7/5, X 7A/1, X 7A/2, X 7A/4. Seed from the F.1 of X 5 and 5A, and X 6 and 6A had been bulked for a germination test as previously described. Forty plants were raised from each of these bulked samples.

  Segregation for prostrate and erect habits took place as is shown in the following Table VI.

TABLE VI.

Showing segregation for growth habit in the F.2 of crosses involving parents of prostrate and erect habits.

F.2 raised from F.1 plant	Number in F.2 population.	Number of prostrate types in F.2.	Number of erect types in F.2.
X 7/1	70	57 53	13 17
X 7/5	69 17·25	59 52	10 17
X 7A/1	70	54 53	16 17
X 7A/2	38 95	27 28	11 10
X 7A/4	80	63 60	17 20
Bulked X 5 + 5A	40	28 30	12 10
Bulked X 6 + 6A	40	33 30	7 10
Totals	407 192	321 + 15 306	86 — 16 102

The erect types appearing in the F.2 were quite as
distinct as the original S.2 type. Fig.32 shows a general
view of segretation for growth habit.

Fig.32. Showing a general
view of segregation for growth
habit in the F.2 of a cross
(X 7A) of prostrate and erect
types.

(4) a. <u>Calyx Marking of the F.1</u>.

Banded calyx proved to be dominant, all F.1 progeny
from crosses of marked and unmarked parents, or marked and
marked, being banded. The F.1 of crosses of unmarked types
were unmarked. All F.1 plants within a cross were similar,
and the cross resembled its reciprocal. Figs. 26 to 31 show
typical florets from the F.1 of representative crosses.

It proved difficult to judge whether dominance was com-
plete since the expression of the dominant character appeared
to be influenced by environmental factors. Light appeared to
exert an influence on intensity, and may control the expression

Calyx Markings of the F$_4$ Generation.

Female Parents are mentioned first in each case.

Fig.26. Cross 3.
Dwalganup x Mt.Barker.
(Unmarked x Marked).
Progeny marked.

Fig. 27.   Cross 6.
Burnerang x Mt.Barker.
(Marked   x Marked).
Progeny marked.

Fig.28.   Cross 8.
Nangeela x Mt.Barker.
(Unmarked x Marked).
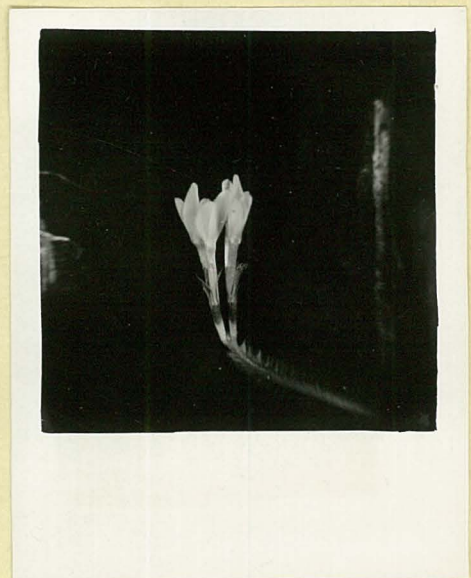Progeny marked.

Fig.29.   Cross 10.
Mt.Barker x Tallarook.
(Marked   x Unmarked).
Progeny marked.

Fig.30. Cross 2.(Centre).
Dwalganup x Nangeela.
   (Left)   (Right).
(Unmarked x Unmarked).
Progeny unmarked.

Fig.31.   Cross 9.
Nangeela x Tallarook.
(Unmarked x Unmarked).
Progeny unmarked.

of the character, since young buds as yet hidden between
the stipules did not show the character. If the genetical
make up of the plant were suitable such buds developed the
band of colour when they reached the light.   Similarly
the marking of calyx tubes developed entirely in the shade
of foliage did not reach the intensity of colour of those
developed in full light.   Also, S.2 had a marking fainter
than that of S.4.

    b. Calyx Marking of the F.2.

     Segregation for calyx marking took place in the F.2.
Amongst the marked segregates there were obvious differences
in intensity of marking, while unmarked were distinct.   In
the F.2 of a cross involving unmarked S.3 and marked S.4 it
was noticed that in some types otherwise classified as
"unmarked" there were individuals in which the calyx lobes
presented a reddish appearance, which sometimes extended
slightly down the tube itself.   This coloration of the lobes
is considered to be quite distinct from the banding of the
tube at the base of the lobes, since some banded calyces
had their lobes green with no trace of reddish coloration.
Similarly, some unbanded calyces had their lobes coloured.

    The proportions of marked and unmarked types appearing
in the F.2 are shown in Table VII (see page 23.)

TABLE VII.

Showing segregation for calyx marking in the
F.2 of crosses involving parents of marked   and
unmarked types.

	F.2 raised from F.1 plant.	Number in F.2 population.	No. of marked types in F.2.	No. of unmarked types in F.2
S3 × S4 —	X 8/2	80	65 60 + 5	15 20
S4 × S3	X 8A/5	39	28 30 - 2	11 9 0
S4 × S5	X 10/2	70	54 53 + 1	16 17 0
	X 10/3	69	52 52 =	17 37 0
S5 × S4	X 10A/2	70	45 53 - 8	25 17 0
S5 × S4	X 10A/5	109	73 81 - 8	36 27·0
S5 × S2	7A/1	70	42 53 - 11	28 17
			160	89 27 1
	Totals	507	359	148

The intensity of marking varied from a shade as deep as
that of S.4 to a shade as pale as that of S.2.

S5   marked
S4   unmarked
S2

× 7A   ?

SECTION IV.

DISCUSSION.

(1) Emasculation, Pollination and Seed-setting.

From Tables IV and V on page 18, it has been seen that 69% of emasculated florets produced seeds. Probably the unfruitful emasculations (31%) were due to faulty pollination technique as much as to emasculation method. Emasculation may be performed at any period during the day, but it is probable that the morning before the heat of the day, would be most suitable. At this time the maximum number of florets are in a suitable condition, whereas later in the day many anthers mature rapidly, as atmospheric temperature rises in the glasshouse. Practically all emasculation done in this project has been carried out between 7 p.m. and 10 p.m., but this time was utilized from necessity rather than from its superiority.

Kirk's method of emasculation, which has been described on page 8, with Savage's modification of a hypodermic needle, has been discussed in the same section. It has been remarked that the method appears to be superior to the one used.

The success or failure of pollination could be ascertained at the end of five or six days after the operation by viewing the ovaries of pollinated florets with the microscope. If successful, the ovary would have commenced to enlarge, and would be green and plump. Also, unfertilized florets withered and died while fertilized florets folded back along the peduncle.

McMillan, in work carried out at Canberra, had found that florets were intolerant of injury. For instance, he states that "...whenever the calyx was injured the flower died". In this study, however, the flowers proved to be very tolerant of injury. It has been described how the calyx lobes were folded back or some removed completely. The same applied to parts of the corolla. Possibly climatic conditions were sufficiently different from those at Canberra to influence

results here.

(2) <u>Germination</u>.

It has been shown that the seeds of Subterranean Clover must undergo a period of "after-ripening" before they are capable of germinating. Probably the few seeds in a sample which germinated were those first formed, and so had had a sufficient time for after-ripening between maturity and sowing. Since it was possible that the pecularity was confined to seeds of the F.1, trials were made with seed of the L.1 of the various parents. Results in this test of L.1 seed were similar to those of F.1 seed.

Reference to notes previously made showed that the F.1 plants of a cross or its reciprocal had flowered within a few days of each other, so that the differences in germinating capacity of seed from similar genotypes could not be due to this factor. If the pure-line status of the strains is accepted, then each plant of the F.1 of any one cross or its reciprocal must be indentical genetically. The differences in germinating capacity then, must be attributed to external factors -- the environment.

From the trial of the effect of retting liquor on germination, which has been described on page 12 , it appeared that the liquor had an accelerating effect on germination of seeds in the particular dormant stage encountered. Though inconclusive, since no replications in the trial were possible, the results indicate that future work along these lines will be useful. Any method which enabled a worker to germinate such seeds immediately after harvest would be valuable, particularly where it was desired to raise two generations in one year in the glasshouse, and so speed up the work of selection following hybridization.

Regarding results with "Hortomone A" it has been seen
that the concentration used depressed germination, but had
no lasting effect. While it has not been possible to obtain
information on the active substance or substances present in
this commercial preparation, it is probable that the inhibition
of germination is connected with the concentration. Went and
Thimann (1937) have shown that a high concentration of auxin
inhibits root growth, and that extremely low concentrations
may accelerate root growth.

(3) Growth Habit.

It has been stated that the strains of Subterranean Clover
are pure lines. Hence the strains breed true for growth
habit, and individuals of a strain must be homozygous for the
factors influencing the habit. The F.1 populations, derived
from crosses of five distinct strain types, show that the
prostrate habit is dominant over erect habit. The factorial
basis of inheritance cannot be determined, however, from the
F.1. For this reason an F.2 population, derived from the
F.1 of suitable crosses, has been studied. In this F.2 pop-
ulation segregation has taken place for growth habits, the
erect type of S.2 reappearing. From a study of the ratios
obtained in the F.2, and from the type of F.1, an hypothesis
may be submitted that growth habit is inherited in a simple
Mendelian manner, erect type being recessive to prostrate.
To test this hypothesis, it is necessary to decide whether the
ratios obtained depart significantly from the 3 : 1 ratio
expected in an F.2 of this nature. Use has been made, there-
fore of the test for "goodness of fit" of a Mendelian ratio.
Sinnot & Dunn (1932) present a method of doing this which
involves determining the Standard Error of the proportion of
one type within the F.2 sample. To find the standard error
of one type the following formula is used:

Standard Error of the proportion $= \sqrt{np \ (1 - p)}$

where n = size of sample

and p = proportion of a type expressed

as a decimal.

To apply this to the prostrate type proportion of the F.2 population derived from the F.1 plant X 7/1 :-

n = 70

$p = \dfrac{57}{70} = 0.8$

$$S.E. \ of \ p = \sqrt{70 \times 0.8 \times 0.2}$$
$$= \sqrt{11.2}$$
$$= 3.34$$

Barring any influence but sampling errors in the seed of the F.1 from which the population arose, the chances are 0.9974 that the true number of prostrate types in every 70 lies between 70 ± 3 x S.E. of p, or between 67 and 47. The expected number of prostrate types, on the basis of the hypothesis, is 53 ($\frac{3}{4}$ x 70). This lies between 67 and 47 and there is, therefore, no significant departure from a 3 : 1 ratio.

Table VIII, set out below, shows each of the ratios treated according to the method described above.

TABLE VIII.

F.2 growth habit ratios examined for "goodness of fit".

F.1 plant which produced an F.2	X 7/1	X 7/5	X 7A/1	X 7A/2	X 7A/4	X5+5A Bulked	X6+6A Bulked
No.of F.2 plants (n)	70	69	70	38	80	40	40
No.of prostrate types obtained in F.2 (p)	57	59	54	27	63	28	33
Standard Error of p	3.3	2.9	3.5	2.8	3.7	2.9	2.4
Range of prostrate proportion (p $\pm$ 3 x S.E. of p)	47-67	50-68	43-65	19-36	52-74	19-37	26-40
No.of prostrate types expected in F.2 ($\frac{3}{4}$ x n)	53	52	53	29	60	30	30
Whether departing significantly from a 3 :1 ratio.	No	No	No	No	No	No	No

From these results there is little doubt that growth habit is inherited in a simple Mendelian manner, the expression of the characters being controlled by a simple factor pair. If the dominant allelomorph is distinguished as "P" and the recessive as "p", then the factorial constitution determining the growth habit of the homozygous prostrate type may be written as PP, while that of S.2 is pp. The hybrid from a cross between prostrate and erect is then Pp (prostrate), while the F.2 from selfing Pp consists of 1PP: 2Pp : 1pp, or on a phenotypical basis 3 prostrate : 1 erect.

It has been remarked in the introduction that Donald & Neal-Smith consider the likely primary cause of the ranges of distinct types within the species to be mutation. Sinnot & Dunn remark that "One characteristic of new gene mutations is that they are generally recessive to the wild type or to the condition from which they arose." It is probable, therefore, that the erect type of Subterranean Clover has arisen as a recessive mutant of the wild or prostrate type.

All possible F.2 populations have not been studied, but there is no reason to doubt that similar results would arise in all cases of hybridization of Burnerang and prostrate types.

(4) Calyx Marking.

Just as the strains breed true for growth habit so they breed true for the presence or absence of calyx marking. The strains are therefore homozygous for the factors determining presence or absence of calyx marking. The F.1 demonstrates that the marked state is dominant over the unmarked state. In crosses between marked and unmarked types the F.1 is marked, while in the F.2 the unmarked type reappears in proportions which suggest a ratio of 3 marked : 1 unmarked. The perfect ratio is not obtained, but in one case (X 10/3) the ratio obtained is as close as possible (52 : 17, Table VII).

The ratios obtained have been examined statistically for "goodness of fit" in the same manner as the growth habit ratios were examined.

Table IX, set out below, shows each of the ratios treated according to the method described previously.

TABLE IX.

F.2 calyx marking ratios examined for "goodness of fit".

F.1 plants which produced an F.2.	X8/2	X8A/5	X10/2	X10/3	X10A/2	X10A/5	X7A/1
No. of F.2 plants (n).	80	39	70	69	70	109	70
No. of marked types obtained in F.2 (p).	65	28	54	52	45	73	42
Standard Error of p.	3.6	2.9	3.5	3.6	4.0	4.9	4.1
Range of marked proportion (p ± 3 x S.E. of p).	54-76	19-37	44-65	41-63	33-57	58-88	30-54
No. of marked types expected in F.2 ($\frac{3}{4}$ x n).	60	29	53	52	53	82	53
Whether departing significantly from a 3 : 1 ratio.	No	No	No	No	No	No	No

In the one F.2 population studied (from X 7A) with S.2 as the marked parent, there was segregation for presence or absence of colour, but apparent segregation for intensity occurred too. In the cross of S.2 (marked) and S.4 (marked) all the F.2 were marked as expected. But segregation for intensity took place, depth of colour varying from a shade as deep as that of S.4 to one as pale as that of S.2. This range is much wider than any found in the L.2 of S.4, and suggests that modifying factors have segregated. In the F.2

of crosses of S.4 and unmarked parents (S.3 and S.5) there
appeared to be segretation for intensity. The variation was
too great to be attributed to environmental factors, since
it was much wider than in the L.2 of S.4.

It is evident, then, that presence or absence of calyx
marking is controlled by a pair of principal factors while
modifying factors influence its intensity. If the dominant
allelomorph for presence of marking is "M", then the recessive
allelomorph is "m". S.2 and S.4 are represented by MM,
unmarked parents by mm, and the first hybrid generation as Mm.
The F.2 will then segregate into genotypes the proportion
of which will approximate 1MM : 2Mm : 1mm, or on a pheno-
typical basis, 3 marked : 1 unmarked.

SECTION V.

SUMMARY OF RESULTS.

(1) The inheritance of growth habit and calyx marking in Subterranean Clover has been studied by hybridizing five strains and raising an F.1 and F.2 generation.

(2) Growth habit is inherited on a simple Mendelian basis, prostrate habit being dominant over erect.

(3) Calyx marking is inherited on a simple Mendelian basis with the marked state dominant over the unmarked.

(4) Modifying factors influence the intensity of marking.

(5) Subterranean Clover seeds require a period of "after-ripening", following harvest, before they are capable of germination.

(6) A method of emasculation has been described together with an improved method.

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