Limitations to Seed Set in White Clover (*Trifolium repens* L.).
I. Preliminary Observations

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ABSTRACT

Ovule growth in white clover (*Trifolium repens* L.) was measured and compared at intervals of one or more days after anthesis in florets that had either been pollinated by bees, manually pollinated by 'rubbing', or remained unpollinated. Many ovules in pollinated florets aborted before maturing into seeds. Ovules were classed as 'early-aborting' if they did so without increasing in length by more than 23% and 'late-aborting' if they increased in length by 33% or more before abortion. In cv. S.184 growing in England, about 22% of ovules aborted early and 25% aborted late. In cvs. Grasslands Huia and Grasslands Tahora growing in New Zealand, there was about 15% early abortion but only about 7% late abortion. The evidence presented, including observations of ovule growth in unpollinated inflorescences and 'rubbed' self-pollinated inflorescences in which two florets set seed, strongly suggests that early abortion might result from ovule sterility, whereas later abortion is clearly the result of post-fertilisation failure of embryo development, perhaps as a result of competition between ovules for nutrients.

Additional index words: **ovule sterility, abortion, pollination.**

INTRODUCTION

The number of ovules in a carpel of white clover greatly exceeds the average number of seeds set per floret (see Thomas, 1987). Atwood (1940) noted that the number of seeds formed in a carpel which usually contains five or six ovules rarely averages more than two, and Dessureaux (1951) found a mean seed number per floret of only about 2.5 in Ladino clover plants which had an average of about six ovules per carpel.

The causes of this low seed production per floret are not known, despite such knowledge being highly desirable as a basis for the development of improved management practices for seed production. In chronological sequence, factors which could theoretically be responsible for such a low percentage of ovules developing into seeds are:

(i) ovule sterility
(ii) inadequate pollination
(iii) pollen sterility
(iv) poor stigma receptivity
(v) poor fertilisation
(vi) post fertilisation abortion of developing seeds

With regard to these, no information was available before the present study concerning ovule sterility or the possible effects of low stigma receptivity and fertilisation failure. Studies of environmental influences on flower head development (Thomas 1961, 1981) however, suggest that pollen sterility is probably not a factor leading to low seed set.

Because low seed yields are often correlated with poor weather conditions (eg. van Bogaert, 1977) it is frequently suggested that low yields result from low bee foraging activity, despite the equal possibility that bad weather might affect any or most of the other five factors listed above.

Various observations have suggested that the number of seeds which mature might be limited by the availability of nutrient for seed development. Under 'low nutrient' conditions it is speculated that competition between florets might lead to the abortion of developing seeds. Atwood (1940), for instance, found that seed number per floret was significantly higher in flower heads from which all but ten florets had been removed than in intact heads. Similarly, Clifford (1985) reported that small flower heads often yield approximately the same number of seeds as large, and Binek (1983) found that seed number per flower head was increased by removing about half the flower heads from plants.

The assumption behind the nutrient competition hypothesis is that nutrient shortage results in post-fertilisation abortion of developing seeds. It is important to realise, however, that most of the observations on which this hypothesis is based could be explained equally well by proposing that fertility of ovules is adversely affected by poor nutrient conditions. Data available do not allow us to distinguish between these two alternatives.

Despite the widely held beliefs that seed set is limited by bee foraging activity and post-fertilisation abortion of developing seeds, there is virtually no direct evidence in support of either of them. As a start to clarifying the factors which do cause the low seed set in white clover, the present study was undertaken to ascertain the extent to which failure to produce seeds is clearly the result of post-fertilisation abortion of developing seeds, and to what extent it results from early abortion of ovules which is possibly caused by lack of fertilisation.

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MATERIALS AND METHODS

Three sets of observations were made on plants of white clover (*Trifolium repens* L.). The study began using plants of the relatively small-leafed cultivar S.184, and was extended to include observations on plants of the two cultivars Grasslands Huia and Grasslands Tahora.

Cv. S.184

The plants of cv. S.184 were growing with perennial ryegrass in an old mixed sward at New Milton, in Hampshire, England during August 1986. The sward had been mown to a height of approximately 2 cm four weeks previously. During the period of observation, the weather was overcast and bee foraging activity low.

Flower heads on ten plants were tagged the day before anthesis of their oldest florets was anticipated, and then left exposed to foraging bees. Flower heads were harvested over a period of three weeks from the time of tagging and measurements made of ovules dissected out of carpels from all florets.

Cvs. Grasslands Huia and Grasslands Tahora

Plants of cvs. Grasslands Huia and Tahora were grown as spaced plants in experimental field plots at Massey University, Palmerston North, New Zealand in December 1986/January 1987. Observations were made after a period of fine sunny weather. Twenty flower heads were collected from each cultivar a few days after pollination (as judged by the degree of reflexion of their youngest florets). Ovules present in each floret on each flower head were then counted and measured.

Effect of pollination and fertilisation

In December 1989/January 1990 flower heads of further field-grown plants of cv. Grasslands Huia were individually caged to prevent pollination by bees. The cages, measuring 40 x 40 x 40 mm, were made of thin transparent clear plastic sheet which was perforated by holes with a diameter of 3.2 mm at a density of 4 cm^2. The cages were thus well ventilated and flower heads placed in them received photosynthetically active radiation at a level only about 6% below that falling on uncaged heads, as determined using a Li-cor LI-188 B light meter.

Flower heads were placed in cages just before the onset of anthesis, when the oldest florets were just beginning to show petal colour. In some cases once the heads reached anthesis they were 'rubbed' between thumb and fingers (as described by Atwood, 1941) to bring about self-pollination. Other control heads remained 'unrubbed' and unpollinated. Measurements of ovule and carpel size were made over a period of 8-10 days from anthesis in both 'rubbed' and 'unrubbed' heads, as shown in Fig. 1.

Ovule measurements

Ovule measurements throughout these investigations were made using an eyepiece micrometer in a dissecting microscope with a magnification of x 32. In all cultivars, ovules which remained at the same size (<0.43mm long) as those in fully developed but unpollinated carpels, or which were somewhat smaller because of shrinkage, were classed as early-aborted. Those which had clearly started post-fertilisation growth and were >0.56mm long, but were both lagging behind others in the developing fruit and were becoming chlorotic, were classed as late-aborted.

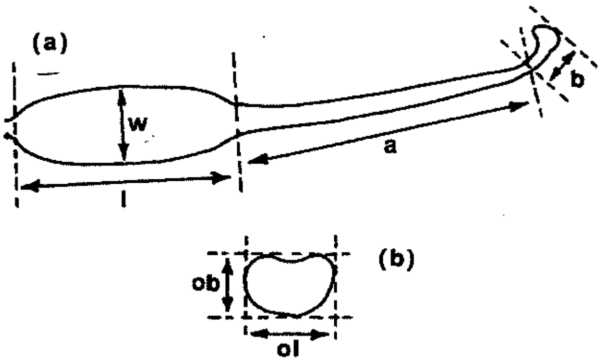

Fig.1. Dimensions of white clover (a) carpels and (b) ovules. Measurements were made of ovary length (l), ovary width (w), style length (a+b), seed and ovule length (ol) and seed and ovule breadth (ob)

RESULTS

Ovule growth during seed development in cv. S.184

Size of unfertilised ovules at anthesis

As a basis for determining the stage of development at which ovules stop growth after fertilisation, it is necessary to know the extent to which they grow without pollination and fertilisation and to know their 'normal' rate of growth after fertilisation has taken place. A study of ovule development in cv. S.184 was made to ascertain this.

Measurements of ovule sizes made at two-day intervals after anthesis showed that they reached a maximum length of about 0.46 mm before fertilisation, and remained at about this length for eight days after anthesis in the absence of pollination. When the lengths of fully grown but unfertilised ovules were compared eight days after anthesis at the basal (proximal) and stylar (distal) ends of carpels, the basal ones were found to be slightly larger (Table 1), but little difference was found between their average lengths in upper and lower florets on the same flower head. Almost all the unfertilised carpels examined contained only four ovules. To simplify presentation of data, the few which contained five or more have been omitted.

Post-fertilisation growth of ovules into seeds

After cross-pollination, growth of ovules in cv. S.184 carpels varied greatly. Just under half of them grew rapidly and developed fully into seeds. The growth of these is shown in Fig. 2. No differences were detected between the post-fertilisation growth rates of seeds in upper and lower florets, despite their differences in initial size shown in Table 1.

Table 1. Ovule length (mm) in unfertilised white clover flower heads at each of the four positions within carpels of upper and lower florets eight days after anthesis. 1 = basal ovule, 4 = stylar ovule. Standard errors are given in parentheses.

	Ovule position			
	1	2	3	4
Upper florets	0.46(0.030)	0.43(0.020)	0.42(0.030)	0.42(0.015)
Lower florets	0.46(0.025)	0.43(0.018)	0.42(0.022)	0.43(0.021)

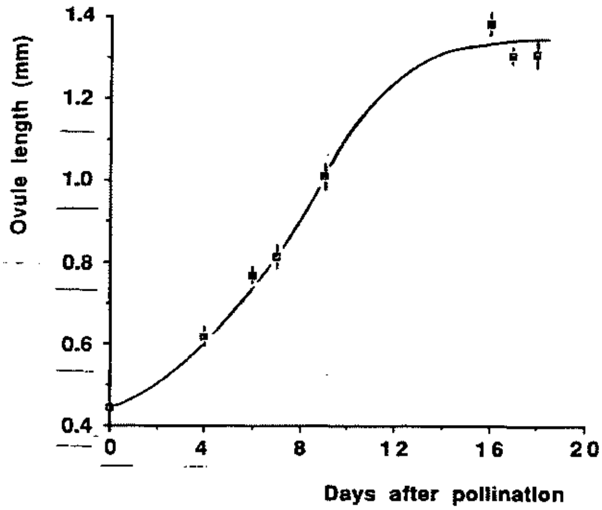

Fig. 2. Average length of the longest ovule in each carpel of white clover cv. S.184 flower heads. Forty ovules were measured from each of three inflorescences at each age shown. Vertical bars represent ± standard error.

Ovule abortion

Just over half the ovules in cross-pollinated flowers aborted. The percentage which stopped growth at various stages up to 18 days after pollination is shown in Fig. 3. From this figure two groups of ovules are apparent: the 18.6% of ovules which clearly did not grow at all after pollination (early-aborting, at a length <0.43mm) and a larger group, comprising 28.2% of ovules, which grew to lengths of 0.57 mm or more before aborting (late-aborting). There was also a smaller intermediate group consisting of the 8.6% of ovules which aborted at lengths from 0.46 to 0.53 mm which could not be assigned with certainty to either the early- or late-aborting categories.

By the eighteenth day, many early-aborted ovules had shrivelled to less than half their initial length and were often so small that they were difficult to detect. The late-aborting ovules grew rapidly at first, but then stopped growth, became chlorotic, and died before reaching full size.

The percentage of 'early' and 'late' aborted ovules of cv. S.184 was recorded separately for all carpels in the lower, middle, and upper florets of the flower heads and for each position within each carpel (Table 2). In this table the previously described intermediate group has been added to the early-aborting category because unpollinated ovules were later found occasionally to continue growing up to lengths of 0.53 mm to 0.56mm after anthesis (see Figs. 4-7).

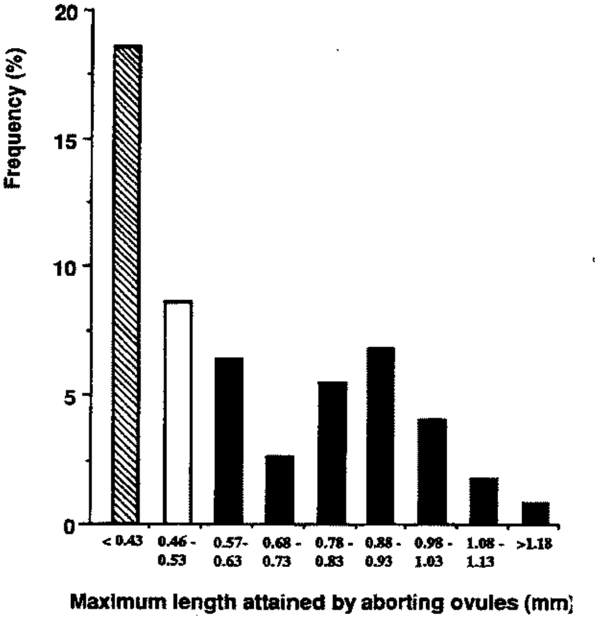

Fig. 3. The maximum lengths attained by aborting ovules of white clover cv. S.184. Frequencies are expressed as a percentage of the combined populations of aborting (244) and non-aborting (196) ovules from six flower heads. Frequency of early-aborting ovules is shown as hatched, intermediate ovules as unshaded and late-aborting ovules as black

More abortion of both types ('early' and 'late') occurred in upper florets than in lower, but there were no clear differences in the frequency of abortion relating to the position of ovules in the carpels. The total abortion in the upper florets was 67% compared with only 28% in the lower florets.

The total number of ovules which aborted in each carpel ranged from zero to four. Only about 2% of mature carpels contained no seeds.

Frequencies of aborted ovules per carpel caused by early abortion and by post-fertilisation abortion are shown in Table 3. The similarity between the two is remarkable.

Ovule and seed abortion in cv. Grasslands Huia and cv. Grasslands Tahora

Observations made of ovule development in fertilised flower heads of plants of cvs. Grasslands Huia and Grasslands Tahora growing in New Zealand are summarised in Table 4.

Comparison of the data in Table 4 with those in Fig. 3 shows the percentage of early abortion in these

Table 2. Percentage abortion of ovules in different positions within the carpels and within the flower heads of white clover cv. S.184. Basal (proximal) ovules in carpels are numbered 1 and stylar (distal) ovules 4.

(a) 'Early' abortion

	Ovule position in carpel				
	1	2	3	4	Average
Upper florets	25	25	37	25	27.7
Middle florets	37	25	19	19	26.7
Lower florets	6	25	12	13	12.5
Average	22.7	25.0	22.7	19.0	

(b) 'Late' abortion

	Ovule position in carpel				
	1	2	3	4	Average
Upper florets	44	12	50	50	39.0
Middle florets	19	25	25	25	23.5
Lower florets	12	19	19	12	15.5
Average	25.0	18.7	31.3	29.0	

Table 3. Percentage of carpels with 0, 1, 2, 3 or 4 aborted ovules and developing seeds as a result of (a) early abortion and (b) post-fertilisation abortion in white clover cv. S.184. (Records were made for all florets on each of ten flower heads).

	No. of ovules aborted per carpel					
	0	1	2	3	4	Total
(a) Early abortion (%)	38.3	36.2	14.8	8.6	2.1	100
(b) Post-fertilisation abortion (%)	37.5	35.4	14.6	12.5	0.0	100

Table 4. Frequencies (%) of ovule abortion in ovaries of white clover cvs. Grasslands Huia and Grasslands Tahora.

	Length of aborted ovules (mm)			
	<0.43	0.46-0.53	0.56-0.73	>0.77
Grasslands Huia	14.0	0.6	2.0	2.0
Grasslands Tahora	13.1	3.7	4.5	4.1

plants of cvs. Grasslands Huia and Grasslands Tahora to have been slightly lower than that in cv. S.184 growing in England, but not markedly dissimilar. However, there was much more late abortion in cv. S184 than in cvs. Grasslands Huia and Grasslands Tahora. In all three cultivars, the frequency distributions showed a clear 'trough' between the early- and late-aborting categories.

Effect of pollination and fertilisation on ovule and carpel growth

The previous observations show that the growth of many ovules stops very soon after pollination. To gain some understanding as to whether those which aborted early had or had not been fertilised, experiments were carried out using cv. Grasslands Huia to determine in more detail the extent to which unfertilised ovules continue growth after pollination.

Ovule growth without pollination

Fig. 4 shows the growth of ovules at different positions in unpollinated carpels. The number of ovules per carpel ranged from six to eight. Data are only

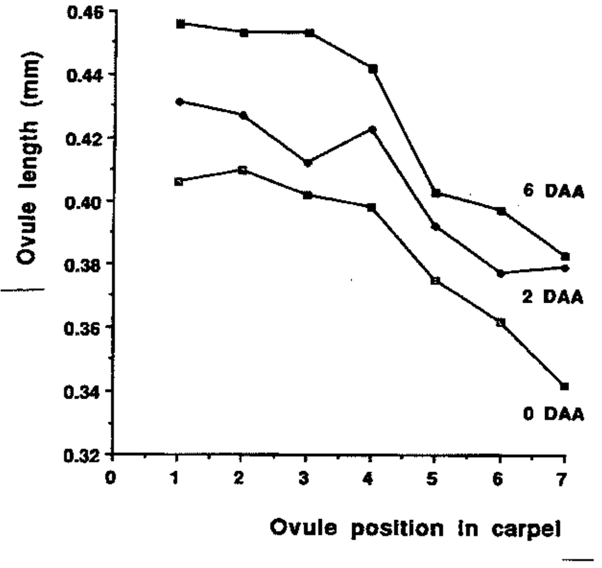

Fig. 4. The relationship between lengths of ovules and their positions within carpels 0, 2 and 6 days after anthesis (DAA) in unpollinated florets of white clover cv. Grasslands Huia. Ovule positions in a carpel are numbered sequentially from 1 (basal end) to 7 (stylar end). Each point on the graph represents the average of ten measurements.

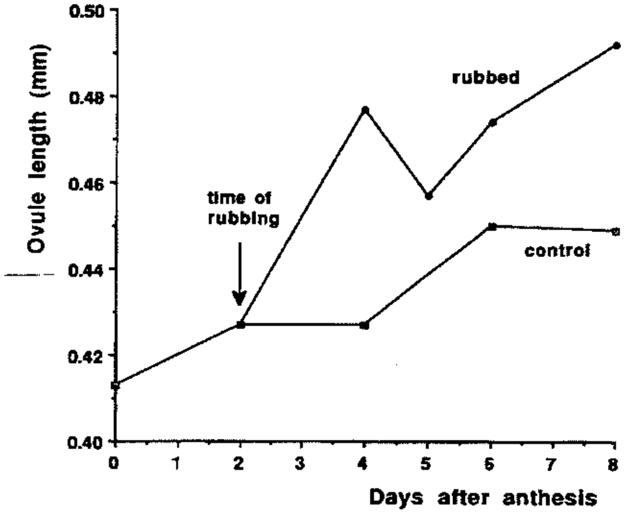

Fig. 6. The growth of ovules of white clover cv. Grasslands Huia in response to self-pollination of flower heads by 'rubbing'. Each point for both control (unpollinated) and treated ('rubbed') flower heads represents the mean length of the three most basal (proximal) ovules in each of ten carpels.

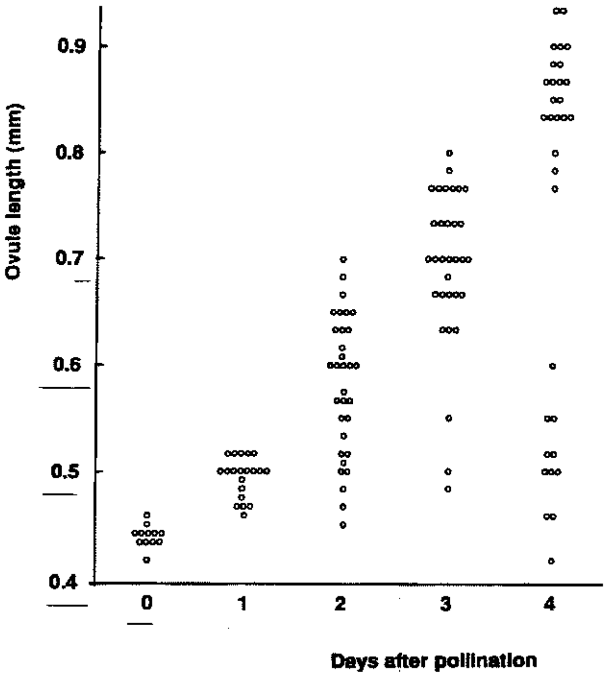

Fig. 5. The effect of cross-pollination by bees on early stages of ovule growth in white clover cv. Grasslands Huia. Each point represents the length of one ovule in the basal half of a carpel.

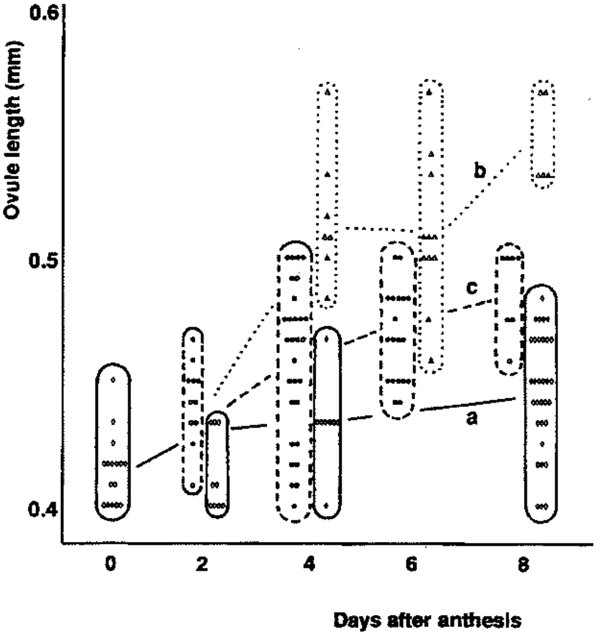

Fig. 7. The effect of self-pollination of white clover cv. Grasslands Huia florets on the growth of their three most basal (proximal) ovules. Ovules in control florets which remained unpollinated (a________) are compared with those in self-pollinated florets which set some seed (b.......) and which set none (c-------). Flower heads in groups (b) and (c) were manually self-pollinated by 'rubbing' two days after anthesis. Each point on the graph represents one ovule. Ovules which were clearly growing into seeds six and eight days after anthesis are omitted from the figure.

presented for carpels containing seven ovules.

All ovules grew slightly over a six-day period from anthesis (see Figs. 5 and 6 also). At anthesis, ovules nearer the bases of the carpels were larger than those nearer the styles, averaging a length of 0.41 mm compared with 0.34 mm. All ovules grew to a similar extent over the six-day period so that at the end of that time basal ovules remained larger with an average length of 0.45mm than those nearer the styles which had an average length of 0.38 mm. The latter did not 'catch up'. On the sixth day a few ovules at the extreme stylar end were beginning to lose chlorophyll and shrivel. Over the next two days, shrivelling of increasing numbers of ovules began near the style and progressed basipetally. The ovule nearest the base of the carpel always stayed plump and green the longest.

Effect of cross pollination on ovule growth

Ovule growth was followed in detail over a four-day period after cross pollination by bees. Fig. 5 shows the length of each ovule measured in carpels dissected at daily intervals after pollination. Ovule breadth increased in a similar fashion. Within 24h of pollination, ovule size had increased markedly to reach an average length of 0.49 mm from an initial average length of 0.43 mm. Growth rates of ovules in the pollinated carpels varied greatly however, some increasing their length by 50% within two days while others grew little. During the second and third days after pollination it became possible to distinguish two groups of ovules: one in which ovules were growing rapidly into seeds and another in which they grew much more slowly. All ovules remained plump and green for three days after pollination but all those in the slow-growing group had stopped growing, turned pale, and begun to shrivel by the fourth day. Some of these aborting ovules shrank between the third and fourth days.

The largest ovules were always towards the basal end of the carpel, never occurring at the two most stylar positions. This is in keeping with the observation (Fig. 4) that the basal ovules are the largest in unpollinated carpels. The ovules which grew least and ultimately aborted though, were randomly located within the carpels, occurring with equal frequency at all positions.

Effect of self pollination on ovule growth

From Fig. 5 it is apparent that ovules which aborted in cross-pollinated florets mostly grew to a slight extent before doing so, even when they aborted early. This raised the question as to whether these aborting ovules had been fertilised and aborted very soon thereafter, or whether pollination can trigger the growth of ovules which remain unpollinated. To answer this question, unpollinated flower heads were self-pollinated by rubbing them between thumb and forefinger in the expectation that no fertilisation would occur. Growth of ovules in most of these inflorescences is compared with growth in control unpollinated ovules in Fig. 6. The rubbing treatment received by the flower heads slightly stimulated ovule growth. The average length attained by ovules in 'rubbed' flower heads eight days after anthesis was 0.49 mm compared with 0.45 mm in unrubbed controls. None of these ovules developed into seeds; all of them began to turn pale and shrivel about six days after pollination, suggesting that they had remained unfertilised.

By chance however, the air temperature soared abnormally to exceed 30°C in the vicinity of the flower heads on one day during the investigation, and a few of the florets which were self-pollinated on that day each set a single seed. The growth of the three most basal ovules - (ie those with the most uniform size; see Fig. 4) - in these florets is compared in Fig. 7 with the growth of others in adjacent florets on the same flower heads which produced no seeds. In the florets which produced one seed, the ovules which failed to grow into seeds grew larger than those in neighbouring florets which formed no seeds at all. Four days after pollination two ovules had grown to lengths of 0.70 and 0.75 mm and were clearly developing into seeds. After a further two days, two other ovules had reached lengths of 1.0 and 1.1 mm.

Effect of pollination on carpel growth

Carpel dimensions (style length and ovary length, breadth and thickness) were measured in all dissected florets as described in Materials and Methods. Results showed that in the unpollinated florets and unfertilised self-pollinated florets none of these dimensions showed any significant increase after anthesis. Style length

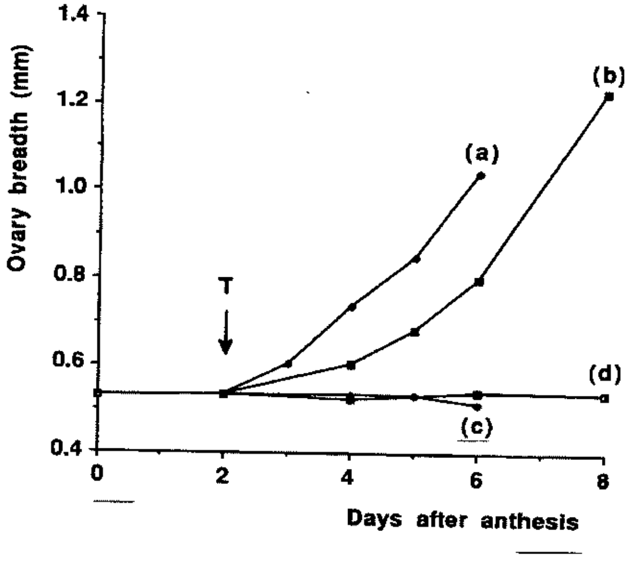

Fig. 8. The effect of pollination and seed set on ovary breadth in white clover cv. Grasslands Huia. Pollination (T) occurred two days after anthesis. Measurements of breadth are shown for florets which were cross-pollinated by bees and had good seed set (a), self-pollinated by 'rubbing' but had slight seed set (b), self-pollinated by 'rubbing' but had no seed set (c) and remained unpollinated and had no seed set (d). Each point in (a), (c) and (d) represents the mean of five of the lowermost carpels on each of four flower heads. Each point in (b) represents the mean of only one lowermost carpel on each of the only two self-pollinated flower heads which set seed.

remained unchanged in all carpels but ovary size increased in all which contained developing seeds. Increase in ovary breadth (Fig. 8) was parallelled by increases in length and thickness. Florets reflexed rapidly in both self-pollinated and cross-pollinated flower heads.

DISCUSSION

This study of ovule growth and development in white clover has revealed a number of aspects of potential significance to seed production, and provides a basis for beginning to understand why seed set in this plant is often so low.

White clover carpels contain from four to eight ovules depending on cultivar and growing conditions. In a very young carpel ovules are initiated first at the basal end (see Thomas, 1987) with the result that at anthesis the basal ovules of a carpel are always older and larger than those at the stylar end (Table 1; Fig. 4). In the present study, ovules in florets that remained unpollinated continued to grow very slightly for up to six days after anthesis, but the size difference between the basal and stylar ovules was retained (Fig. 4).

When florets of cv. S184 were cross pollinated in natural conditions in England, their ovules grew to reach a maximum size about 16 days after pollination (Fig. 2). This agrees with the finding of Hyde, McLeavy and Harris (1959) that maximum fresh weight and viability of seeds in Government Stock white clover in New Zealand were reached about 15 days after fertilisation. Full seed growth requires fertilisation, but ovule growth was stimulated slightly in cv. Grasslands Huia by 'rubbing' flower heads to self-pollinate them, even though there were no indications that they were fertilised and all of them died about eight days after anthesis (Fig. 6). It is not clear whether this slight stimulation was caused by pollination, rupture of the membrane covering the stigmatic surface (Thomas, 1987), or bruising of flower parts. It is clear though, that when self-pollination led to the growth of a single seed in a carpel, the growth of all the other ovules in the carpel was significantly increased (Fig. 7). While it is possible that this stimulated growth was the result of fertilisation, which must then have been followed by very early post-fertilisation abortion, it is noteworthy that none of the ovules in the carpels in which fertilisation had apparently occurred grew as little as most of the ovules in self-pollinated carpels which formed no seeds. It seems more probable therefore, that growth of one ovule into a seed stimulated the growth of the other ovules in the same way that growth of fertilised ovules led to a general stimulation of ovary growth (Fig. 8).

Ovules in cross-pollinated carpels either grew fully to form seeds or they aborted at some stage before this. Growth of all ovules in such carpels was stimulated slightly (Fig. 5) but some, here termed 'early-aborted', died without having grown much more than 10% in length, whereas others, termed 'late-aborted', frequently doubled or trebled their lengths before aborting. Early-aborting ovules stopped growing between 24 and 48 h after pollination (Fig. 5), although they did not die until between the 72nd and 96th hours. Early abortion occurred in between 15 and 26% of ovules in all cultivars and environmental conditions studied; *viz.* in cv. S.184 in England, and in cvs. Grasslands Huia and Grasslands Tahora in New Zealand.

At this stage it is not possible to decide the cause of this early abortion. Three obvious possibilities are:

1. ovules are fertile but not fertilised by pollen,
2. ovules are fertile and are fertilised, but the embryo aborts at a very early stage of development,
3. ovules are sterile.

None of these three can be eliminated on the basis of our present knowledge. The low bee activity apparent around the plants of cv. S.184 studied in England led to seeds being set in nearly 98% of florets (Table 3), which implies that almost every floret was pollinated at least once. Whether more visits by pollinators would have increased the seed set is unknown, and the relationship between the number of visits and number of seeds set requires elucidation.

The second possibility, that embryos abort very early after zygote formation, is supported by the observation that all ovules started to grow before early abortion after cross-pollination. It must be remembered however, that a comparable slight stimulation of growth of ovules which were probably unfertilised occurred in carpels which formed seeds following self pollination (Fig. 7).

It is thus premature to conclude that this second possibility is correct, and the third possibility, that some of the early-aborting ovules might have been sterile, seems just as likely.

Late abortion of ovules was certainly a post-fertilisation event in most cases. Embryos were readily discernible in all larger aborted ovules, but more detailed microscopic examination would be needed to ascertain whether they are present in the smallest late-aborting ovules.

The difference between the frequency of late abortion in cv. S.184 in England and that in the New Zealand-grown Grasslands cultivars is noteworthy. Whether the higher frequency of late abortion in cv. S.184 was caused by the duller cooler conditions prevailing in England at the time the observations were made, or whether the cause was genetic, is not known. It does seem likely however, that nutrient availability to fertilised ovules was one factor limiting their development. Observations which support this include:

- late abortion occurred in 30% of the more stylar ovules in cv. S.184 compared with only 22% of those nearer the supply of nutrient at the base of the carpel (Table 2);
- there was a higher frequency of both late and early abortion in the uppermost than the lowermost florets in flower heads (Table 2); and
- ovules nearer the bases of ovaries produced larger seeds on average than those at stylar ends (but this could equally have been caused by the basal ovules being larger before fertilisation as shown in Fig. 4).

In contrast, the relationship between early abortion and nutrient supply is less obvious. Although ovules began to die in basipetal sequence from the stylar to the basal ends of ovaries when they remained unfertilised in unpollinated or self-pollinated carpels, early abortion in cross-pollinated carpels occurred randomly along the lengths of the carpels and was not greater nearer the stylar ends where the nutrient level would be expected to be lowest.

CONCLUSIONS

This study has shown that up to 50% and more of the ovules in a white clover carpel can abort without developing into mature seeds. In three different cultivars growing in two different sets of climatic conditions, 15 to 24% of ovules aborted early. The possible causes of this early abortion, including embryo-sac sterility and inadequate pollination, are under investigation. The frequency of later, post-fertilisation, abortion ranged from 4% in cv. Grasslands Huia growing in warm sunny conditions in New Zealand (Table 4) to an average of 26% in cv. S.184 growing in cooler duller conditions in England. To what extent this difference might be the result of climatic, rather than genetic differences can only be speculated, but the evidence that late abortion is linked with nutrient availability to fertilised ovules points to the possible importance of climatic factors such as low light intensity as causes of low seed yields.

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