Limitations to Seed Set in White Clover (*Trifolium repens* L.). IV. Effect of Canopy Density and Artificial Shading in the Field.

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ABSTRACT

A field experiment using clonal material of white clover cv. Grasslands Huia was conducted to clarify the relative importance of pre-fertilisation ovule sterility and post-fertilisation ovule abortion as components of seed yield in New Zealand, and the extent to which these are influenced by light intensity, by comparing the effects of canopy density and simulated overcast weather conditions under conditions of optimal pollination. Flower heads which developed in a dense canopy produced 39% fewer seeds per head than those formed in an open canopy. Seed yield was also strongly affected by artificially shading the plants so that they received only 45% of incoming radiation before or after pollination. Some of this reduction was brought about by an increase in the number of florets aborting in all treatments, but much of it was caused by low seed set per pod. Observations of the cytoplasmic state of embryo sacs indicated that the flower heads that developed in dense canopies produced only 54% normal embryo sacs, compared with 71% in open canopies. Artificial shading of open canopies before pollination reduced the ovule fertility to 63%. There was a close correlation between the level of embryo sac fertility and the percentage of ovules setting seed. The relative importance of these factors and the advantage of the practice of defoliation at the time of "closing" for seed production are discussed. The results clearly demonstrated that within a dense clover canopy, light intensities can be as low as 1% and that the changing angle of the sun during the day changes the level of light reaching beneath the clover foliage canopy.

***Additional index words*: canopy shade, overcast weather, ovule fertility, pollen fertility, pollen load, premature seed abortion.**

INTRODUCTION

While the primary factor influencing seed yield in white clover must be considered to be number of flower heads per unit area (Zaleski, 1961), the proportion of ovules which develop into seeds can also have a significant secondary effect. Under apparently good growing conditions, it is common for less than half of the ovules to develop into mature seeds. In cv. Grasslands Huia for instance, in which the number of ovules per floret has been reported to be 5.5 (Thomas, 1981), the average number of seeds per floret in good growing conditions can be as low as 2.2 (Clifford, 1979). Such poor seed development has often been linked with duller, cooler summers (van Bogaert, 1977; Romero, 1985; Pasumarty, Matsumura, Higuchi and Yamada, 1993b; Pasumarty, Higuchi and Murata, 1995) and has also been shown to be accentuated by denser foliage canopies and artificial shading (Pasumarty and Thomas, 1990).

Three factors have been suggested as probable contributors to low seed set in duller conditions: poor pollination as a direct result of low bee activity (van Bogaert, 1977; Bourdot and Butler, 1981), pre-fertilisation ovule sterility (Thomas and Pasumarty, 1996), and post-fertilisation abortion of developing seeds (Robbie, 1989; Pasumarty and Thomas, 1990; Khrbeet, Marshall and Hides, 1994; Marshall, James and Potter, 1996).

While the essential role of pollination as a factor influencing seed set is incontrovertible, the relative importance of pre-fertilisation ovule sterility and post-fertilisation ovule abortion is not so clear. Under glasshouse conditions in New Zealand, Pasumarty and Thomas (1997) found that shading individual developing inflorescences to a level of 1% of incoming radiation for six days reduced ovule fertility by 16% and that under optimal pollination conditions this reduction accounted fully for the reduction of seed set observed. Post-fertilisation abortion was minimal in these experiments. In contrast, Robbie (1989), Khrbeet *et al.* (1994) and Thomas (1996b) all demonstrated clearly that post-fertilisation abortion of developing seeds can be a major factor contributing to reduced seed yield in the United Kingdom, even though such an effect seems to be smaller in New Zealand (Thomas, 1996b). Differences in natural light levels at the two locations might account for this.

The present investigation was therefore undertaken to clarify the relative importance of pre-fertilisation ovule sterility and post-fertilisation ovule abortion as components of seed yield in the field in New Zealand, and the extent to which these can be influenced by light intensity.

MATERIALS AND METHODS

The experiment was carried out in a field previously used for a summer forage crop at the Seed Technology Centre, Massey University, Palmerston North, New Zealand (latitude 40° 23' South, longitude 175° 37' East). The soil type was a Tokomaru silt loam of moderate fertility. The land was ploughed, harrowed and rolled on 23 May 1988. Stolon tip cuttings of clones A and C of white clover cv. Grasslands Huia (Thomas, 1979) were collected from stock plants and were grown in a 1:1 mixture of sand and peat in 1000 cm^3 plastic pots. The plants were maintained for one month in a glasshouse and then transferred outside for a further two months before being planted out in the field at two densities on 5 August 1988. After planting out, the herbicide Paraquat was applied to all the plots by hand spraying between rows of plants at the rate of 3 l ha^{-1} on 9 September 1988. The

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experimental area received a spring application of 150 kg ha^{-1} of 30% potassic superphosphate on 28 October 1988. Details of prevailing temperature, precipitation, and sunshine hours are presented in Table 1.

The experiment was laid out in a randomised complete block design with four experimental treatments, replicated four times. Ramets were planted in rows, in which clones A and C were planted alternately to ensure an adequate source of compatible pollen for cross-pollination. Within the rows all plants were spaced 15 cm apart, but inter-row spacing differed between the treatments. The four experimental treatments were:

1. Open Canopy: Inter-row spacing of 60 cm; unshaded.
2. Open Canopy with pre-fertilisation shade (Pre.f.Shade); Inter-row spacing of 60 cm.

Overcast weather conditions during peak flowering were simulated by artificially shading plants for one month from 16 December 1988 to 16 January 1989 with neutral shade cloth. These plants received only 45% of incoming radiation. Immediately after removing the shade, 25 flower heads in which the corollas of the oldest florets were just showing white were tagged per replicate.

3. Dense Canopy: Inter-row spacing of 15 cm; unshaded.
4. Open Canopy with post-fertilisation shade (Post.f.Shade); Inter-row spacing of 60 cm.

Twenty five randomly selected flower heads per replicate were tagged on 16 January 1989 just before anthesis of their oldest florets. After they had been pollinated (as judged by the degree of reflexion of their florets) the plants were artificially shaded with shade cloth from 27 January to 15 February 1989 so that they received only 45% of incoming radiation.

Ovule fertility

On 20 January 1989, four florets were collected at random from each of 25 tagged flower heads for each treatment and fixed in formalin/acetic/alcohol (FAA). The ovaries were excised from the florets after fixation and stained with Mayer's haemalum before being optically cleared with methyl salicylate (Pasumarty, 1994). The cytoplasmic state of the embryo sacs and presence or absence of polar, egg and synergid nuclei of stained ovules were examined using Nomarski interference microscopy. Embryo sacs lacking nuclei were classified as sterile (Pasumarty, Matsumura, Higuchi and Yamada, 1993a).

Table 1. Weather data for Palmerston North: mean values for each month over the experimental period 1988-1989.

Month	Temperature (°C) Mean daily Max	Temperature (°C) Mean daily Min	Rainfall (mm)	Sunshine (hours)
August	13.8	5.7	94	141
September	15.6	9.4	144	68
October	17.1	10.2	98	138
November	19.8	10.9	63	182
December	22.9	13.4	57	225
January	24.1	15.2	92	223
February	23.1	12.9	75	193

Pollen fertility

On 20 January 1989, two lowermost florets were sampled from each of five inflorescences selected randomly from the 25 in each treatment to assess the pollen fertility. Anthers from each of these florets were squashed in Snow's alcoholic carmine (Snow, 1963) and the percentage sterility of a sample of 100 pollen grains from each floret was determined on the basis of the staining response.

Pollination

For adequate pollination, three honeybee hives were placed approximately 100 m away from the experimental plot. To check the effectiveness of pollination, 25 florets which had just been fertilised (as judged by the degree of reflexion of their florets) were selected randomly from dense and open canopies of shaded and unshaded plots on 16, 21 and 29 January 1989 to assess the pollen load (number of pollen tubes per ovary). In each, the placental region of the ovary was dissected out and stained for 10-15 minutes in aqueous aniline blue dissolved in 0.1M K_3PO_4. The stained placental regions were mounted in a few drops of aniline blue stain and examined under a fluorescence microscope for observation of fluorescent callose plugs (Nakanishi and Hinata, 1973).

Number of florets per inflorescence

Tagged flower heads were harvested from plants of clone C in each treatment on 15 February and floret number per inflorescence was counted from 10 randomly selected ripe inflorescences per treatment.

Number of seeds per head

Two harvests were made of tagged flower heads from plants of clone C to assess the seed number per floret. The first harvest was carried out ten days after pollination (as judged by the degree of reflexion of the florets) and the second one at the time of final harvest (15 February). At the first harvest, ten tagged flower heads were selected randomly per treatment and stored at 4 °C until required for counting. The mean number of seeds per floret was then recorded in 20 randomly collected florets (10 upper and 10 lower) from each of the ten stored inflorescences per treatment. At the second harvest, 12 florets (6 upper and 6 lower) were randomly collected from each of the remaining 15 tagged inflorescences per treatment. To count the number of seeds per floret, the pods were placed on Polaroid photographic paper and X-rayed using a Faxitron Hewlett-Packard X-ray machine (25 KVA, 1 min exposure time).

Seed weight

Using the bulked samples of seed for each treatment, four weight counts of 100 seeds each were made. The 1000-seed weight was standardised to 10% moisture content.

Photon flux beneath clover canopy

Photosynthetically active radiation (PAR) was measured by means of quantum sensors attached to three LI-COR LI-188B light meters. After calibrating these against each other, one sensor was placed above the foliage canopy to measure the level of PAR falling on the foliage of white clover growing in the trial field, and the other two were located at two different positions at ground level within the canopy to measure the PAR beneath the foliage. The measurements were made at hourly intervals from 6.00 h to 19.00 h, NZ standard time on five different days and on each occasion the two sub-canopy light sensors were placed in different locations. On four of the five days the weather was clear with intermittent clouds (sunny) and on the other the sky was cloudy all day (overcast).

RESULTS

Pollen fertility

Pollen sterility was greatest in the flower heads developed in dense canopies, (Table 2); 16% of these pollen grains being sterile compared with only 5% in open canopies and 8% in those plants which were artificially shaded.

Pollen load

When the number of pollen tubes present in the placental region of the carpel was counted, 89-92% of the carpels were found to have more than ten pollen tubes in both dense and open canopies, suggesting that the florets had received more than sufficient compatible pollen to fertilise the ovules.

Ovule number and fertility

The treatments had no significant effect on the number of ovules per floret, but strongly influenced the number of embryo sacs which developed normally (Table 2). Only 54% of embryo sacs were normal in flower heads which developed in dense canopies compared with 71% in open canopies. Pre-fertilisation

Table 2: Effect of treatments on ovule number per floret, percentage of ovules setting seed, and percentage of fertile ovules per floret.

Treatment	Ovule no. per floret	Pollen fertility (%)	Fertile ovules (%)	Ovules setting seed (%)
1. Open canopy (full light)	4.52 ±0.09[3]	95 ±0.9	71 (0.966)[5]	67.6
2. Open/pre.f.s[1]	4.53 ±0.05	92 ±1.8	63 (0.903)	56.2
3. Dense canopy (full light)	4.31 ±0.04	84 ±2.0	54 (0.931)	49.3
4. Open/post.f.s[2]	4.52 ±0.09[1]	—[4]	—[4]	49.1

[1] Open/pre.f.s = open canopy with pre-fertilisation shade (i.e plants were artificially shaded before fertilisation)
[2] Open/post.f.s = open canopy with post fertilisation shade (i.e plants were artificially shaded after fertilisation)
[3] Data on ovule number per floret obtained from treatment 1. Values are the means ± s.e;
[4] — = not recorded
[5] Correlation (r) values between seed set and fertile ovules are given in parentheses

Table 3: Effect of treatments on seed yield components.

Treatment	Number of fully developed florets per inflorescence	Seed number per fully developed floret		Calculated seed number per head	1000 seed weight[5] (mg)	Seed weight per head (mg)
		10 DAP[4]	30 DAP[4]			
1. Open canopy (full light)	103 a[1] (3.9)	3.39 a (0.17)	3.06 a (0.07)	315	0.573 a (0.02)	180.5
2. Open/pre.f.s[2]	94 a (3.6)	2.61 b (0.21)	2.55 b (0.13)	240	0.588 a (0.01)	141.1
3. Dense canopy (full light)	90 b (2.1)	2.31b (0.12)	2.13 b (0.08)	192	0.476 b (0.01)	91.4
4. Open/post.f.s[3]	—[3]	3.39 a (0.17)	2.22 b (0.06)	229[7]	0.530 a (0.003)	120.8

[1] Values followed by the same letter between treatments (in same column) are not significantly different at P<0.05; standard errors are given in parentheses
[2] Open/pre.f.s = open canopy with pre-fertilisation shade (i.e plants were artificially shaded before fertilisation)
[3] Open/post.f.s = open canopy with post fertilisation shade (i.e plants were artificially shaded after fertilisation)
[4] DAP = days after pollination; [5] 10% moisture content
[6] — = Not recorded: plants equivalent to those in treatment 1
[7] floret number per inflorescence in open canopy was used to estimate this value

shading of plants in open canopies reduced the ovule fertility from 71% to 63%.

Number of florets per head

The flower heads developed in an unshaded open canopy had the highest number of fully developed florets per inflorescence (Table 3). In dense canopies and in plants which were artificially shaded before pollination, there was a 9 to 13% reduction in the number of fully developed florets (Table 4). The effect of post-fertilisation shade was not recorded.

Table 4: Percentage reduction in various seed yield components relative to those in open unshaded control plants in treatment 1.

Treatment	Percentage reduction in floret number	seed number per floret	seed number per head	seed weight per head (mg)
2. Open/pre.f.s[1]	8.7	16.7	23.8	21.6
3. Dense canopy (full light)	12.6	30.3	39.0	49.3
4. Open/post.f.s[2]	—[3]	27.4	27.6	33.5

[1] *Open/pre.f.s = open canopy with pre-fertilisation shade (i.e. plants were artificially shaded before fertilisation)*
[2] *Open/post.f.s = open canopy with post fertilisation shade (i.e plants were artificially shaded after fertilisation)*
[3] *— = Not recorded*
Values are calculated for each treatment with respect to the control treatment (open canopy). See caption of Table 3 for further details.

Number of seeds per floret

The number of seeds per floret was 30% higher in the open canopy than in the dense canopy (Table 3). Pre-fertilisation shading of open canopy plants reduced the seed number per floret by 17%, and post-fertilisation shading reduced it by 28%. Although the total number of seeds per inflorescence was not counted, this was estimated by multiplying the average seed number per floret by the average number of fully developed florets per inflorescence. The results of such calculations (Table 3) show that the total number of seeds per head was highest in the open canopy and most heavily reduced (by 39%) in the dense canopy. Artificially shading the plants before or after pollination also reduced the number of seeds per flower head, by 24 and 28% respectively (Table 4). From ten days after pollination to the final stage of seed development (30 DAP), the proportion of seeds lost was very slight in all treatments except that receiving post-fertilisation shade (Table 3). In that instance there was a reduction of about 18.5% in the number of ovules developing into mature seeds, from 67.6 to 47.1% (Table 2), as a result of late abortion of developing seeds.

Correlation between seed set and ovule fertility

For treatments 1 to 3, the number of seeds per floret was closely correlated with ovule fertility: 71 and 68% respectively in the open canopy, 54 and 49% in the dense canopy, and 63 and 56% in the pre-fertilisation shaded open canopy (Table 2).

Seed weight

Open canopy seeds were heaviest while those from the dense canopy were lightest. Artificially shading the plants before or after pollination had no significant effect on 1000-seed weight (Table 3).

Photon flux beneath clover canopy

The level of PAR falling on the canopy surface over a daily 13-hour period is shown in Figure 1a. The average level of PAR on a sunny day was 1110 μmoles m^{-2} s^{-1} compared with about 350 μmoles m^{-2} s^{-1} on the overcast day. The percentage of PAR penetrating the canopy differed over the period of a day and between sunny and overcast conditions, being higher on the overcast day (1.8-3.4%) and lower on sunny days (0.2 – 2%). On the sunny days percentage penetration showed a sharp peak at 12.00 h, NZ standard time, when the sun was at its highest point.

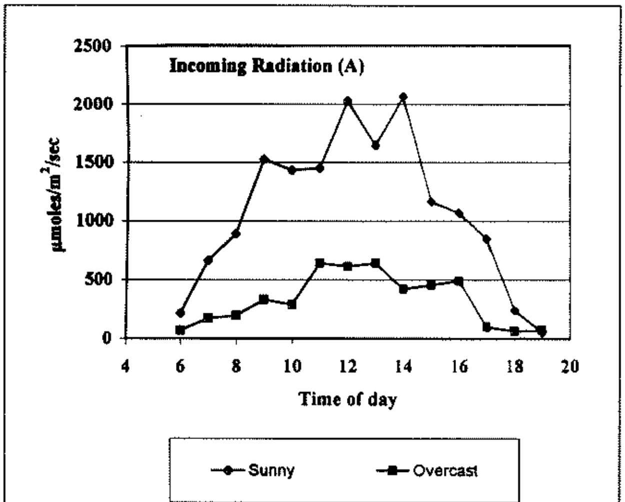

Figure 1a. The level of photosynthetically active radiation (PAR) falling on the canopy surface over a daily 13-hour period.
Figure 1b. The effect of time of day on the percentage of PAR penetrating white clover foliage canopies. Hourly values are the means of eight observations made on four sunny days and two observations for the overcast day in the month of January 1989, in Palmerston North.

DISCUSSION

The percentage of ovules setting seed in white clover is affected firstly by the level of ovule sterility induced by pre-fertilisation conditions, and secondly by the degree of post-fertilisation ovule abortion. Even under seemingly optimal conditions, about 20% of ovules are sterile, but following favourable pollination all or most of the remaining fertile ovules develop into mature seeds (Thomas, 1996a; Pasumarty and Thomas, 1997). The major fundamental determinant of seed set potential is thus the level of ovule sterility.

The present investigation has confirmed the very high correlation between seed set and ovule fertility reported previously when plants were grown in full light during the post-fertilisation phase, the proportion of ovules setting seed and of fertile ovules being 67.6 and 71.6% respectively in open canopies in full light throughout, 49.3 and 54.0% in dense canopies in full light throughout, and 56.2 and 63.0% in pre-fertilisation-shaded open canopies (Table 2). Thus in each situation the level of seed set was confirmed as being largely determined by the level of ovule fertility. A 55% reduction in the light intensity received by developing inflorescences in an open canopy during the pre-fertilisation phase had a lower adverse effect than allowing early inflorescence development to proceed in dense canopies, ovule fertility being reduced by 8% in the former instance compared with 17% in the latter.

These findings are explicable in terms of the observation that light has a direct effect on early stages of inflorescence development, acting via a photomorphogenically active regulating system such as that mediated by phytochrome (Pasumarty and Thomas, 1997). Flower heads of white clover emerge as inflorescence buds from the stolon apex in the axils of the youngest leaves (Thomas, 1981). Over the next few days these continue to grow and are gradually raised above the stolons by elongation of their peduncles. In dense canopies this post-emergence growth continues for a few days in heavy shade before the flower heads are raised above the foliage. Brougham (1958) reported light intensities beneath the foliage canopy of white clover to be as low as 5% of incoming radiation, and measurements during the present investigation revealed even lower intensities of less than 1% of incoming PAR (Figure 1b). The reduction in the level of PAR received by developing inflorescences in a dense canopy was thus far greater than the 55% reduction brought about by artificial shading, and this would account for the stronger effect of canopy density on ovule sterility.

Under suboptimal post-fertilisation conditions some developing seeds abort in the first few days after fertilisation as a result of sudden arrest in embryo growth following normal early development of the embryo (Pasumarty *et al.*, 1993a). In the present investigation the level of post-fertilisation abortion was strongly increased with an artificially reduced level of solar radiation. Such a reduction had a greater effect on this phase of seed development than on the pre-fertilisation phase. Artificial shading of open canopies during this post-fertilisation phase led to an 18.5% reduction in the number of ovules developing into mature seeds, from 67.6 to 49.1% (Table 2), as a result of ovule abortion. This degree of post-fertilisation ovule abortion was closely similar to that found in a previously reported field study by Pasumarty and Thomas (1990) into seed set in cv. Grasslands Pitau in which artificial post-fertilisation shade led to a 17% decrease in seed number per floret.

The degree of post-fertilisation abortion differs in different situations however, ranging from almost nil in well pollinated, open canopies in full natural light in New Zealand (Thomas and Pasumarty, 1996; Pasumarty and Thomas, 1997) to quite high levels in other cases (Robbie, 1989; Pasumarty *et al.*, 1993a; Khrbeet *et al.*, 1994; Marshall *et al.*, 1996; Thomas, 1996b). These differences in degree of post-fertilisation ovule abortion are probably ascribable to differences in levels of photosynthate available for "seed provisioning" as suggested by Clifford (1986) and Thomas and Pasumarty (1990).

The importance of light intensity as a factor affecting post-fertilisation ovule development is supported also by the results of a study of the relationship between sunshine hours and seed set in field grown white clover plants in Japan (Pasumarty *et al.*, 1995). In that study a regression analysis demonstrated a strong positive correlation between seed set and sunshine hours over the two-week period following anthesis. Such an effect of light intensity could well also explain the differences observed in post-fertilisation ovule abortion in different geographical locations. Thomas (1996b), for instance, found as much as 25% post-fertilisation ovule abortion in wild S.184 clover growing in the United Kingdom compared with only 4% in cv. Grasslands Huia and 12% in cv. Grasslands Tahora growing in New Zealand. It is uncertain to what extent these differences are the result of genetic differences as opposed to environmental influences, but it is certainly possible that they arose as a result of differences in light intensity.

Previously reported photon flux density levels which range from 350-820 µmoles m^{-2} s^{-1} for glasshouse experiments in Aberystwyth, UK (Khrbeet *et al.*, 1994) to 400 m^{-2} s^{-1} in a growth cabinet experiment in Japan (Pasumarty *et al.*, 1993a) are lower than those recorded in Palmerston North, New Zealand. In the present investigation, levels of incoming PAR in midsummer reached 2377 µmoles m^{-2} s^{-1} and the mean level of incident radiation received by white clover foliage canopies over the period from 6.00 h to 19.00 h on a clear day was about 1110 µmoles m^{-2} s^{-1} while only on an overcast day was it reduced to about 350 µmoles m^{-2} s^{-1} (Fig.1a). Thus the irradiance levels recorded in a growth cabinet in Japan and in a glasshouse in the United Kingdom were similar to the mean hourly levels occurring under overcast conditions in the field in New Zealand, and would on the basis of the present observations, therefore, be expected to lead to a significantly higher degree of post-fertilisation ovule abortion than in New Zealand.

The present investigation shows clearly a dual effect of light intensity on potential seed yields of white clover which is summarised in Figure 2. The percentage of fertile ovules is determined during the pre-fertilisation phase. The pre-fertilisation conditions thus set the maximum number of seeds that is possible per floret. This is then strongly influenced by the level of light falling directly on young developing inflorescences. Intense shading of inflorescence buds within a dense canopy reduced the proportion of fertile ovules from 71% to 54%, and a much smaller reduction in light intensity brought about by artificially halving the level of solar radiation received by open canopies reduced the proportion from 71% to 63%. During this pre-fertilisation phase also, reduced light intensity falling directly on young developing inflorescences decreased the number of floret buds which developed fully to maturity from 103 (in a fully lit open canopy) to 94 (with pre-fertilisation shade) and 90 (in a dense canopy) (Table 3). Thus the combined effect of increased floret abortion and ovule sterility in dense canopies during the pre-fertilisation phase was a 39% reduction of seed number per flower head, from 315 to 192.

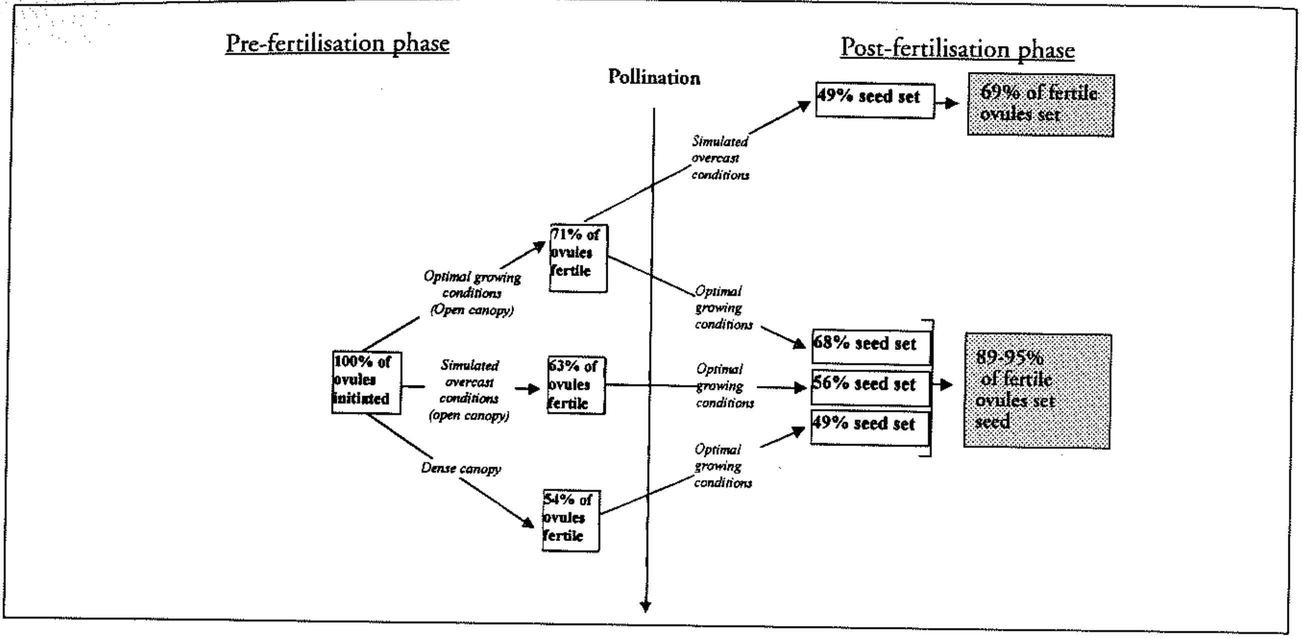

Figure 2. Relationship between growing conditions, pre-fertilisation ovule fertility and post-fertilisation abortion.

After fertilisation, the proportion of the fully pollinated fertile ovules which develop into mature seeds is dependent on the level of incoming solar radiation. Full light during the post-fertilisation phase in this experiment led to a very high proportion, ranging from 89 to 95%, of fertile ovules developing into mature seeds regardless of the pre-fertilisation level of ovule sterility. There seemed to be little limitation to "seed provisioning". With a 55% reduction in the level of incoming radiation, however, the proportion of fertile ovules which developed into mature seeds was reduced to 69%, presumably as a result of limited photosynthate availability.

In conclusion, the results of this investigation provide an explanation of the justification for the practice of defoliation of white clover seed crops prior to flowering (Clifford, 1979) and the advantages of growing such crops in locations with very high sunshine hours throughout the crucial period from flower head emergence to seed maturation. Duller, wetter weather during late spring and summer will diminish ovule fertility and increase floret abortion by leading to lush dense canopies in which developing inflorescence buds will be heavily shaded. Conversely, brighter and drier growing conditions will enhance ovule fertility and floret development by producing more open canopies in which flower heads will be fully exposed to light during the critical early stages of their development. This knowledge points to the possible advantage of delaying "closure" for seed production when weather conditions favour rapid lush growth. In applying this practice, however, care would need to be taken not to extend the delay beyond the point when flower density declines, or peak flowering is delayed for pollinators, or seed harvest would become late and difficult. Once flower heads have been fertilised, continued sunny weather would maximise the development of ovules into seeds.

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