Limitations to Seed Set in White Clover (*Trifolium repens* L.). II. The Influence of Light Intensity on Floral Development

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ABSTRACT

The influence of light intensity on post-initiation floral development in white clover (*Trifolium repens* L.) cv. Grasslands Huia was studied in a controlled environment growth room. Ramets of a single clone were grown at a range of light intensities from 40 to 200 μmoles $m^{-2}s^{-1}$. Ovary lengths, numbers of fully developed florets per inflorescence, and sizes of the ovules were found to be 18, 53 and 13% lower respectively when grown at the lowest light intensity than those in controls grown at the highest light intensity. A stain-clearing technique used to estimate embryo-sac fertility showed fertility to be 75% lower at the lowest light intensity than the highest. When plants were transferred into the open, and bee-pollinated, the seed number per floret was also lower in those previously grown at lower light intensities. A very high positive correlation (r=0.979) was found between apparent embryo-sac fertility and seed set in comparable inflorescences. This observation strongly suggests that the low seed set commonly reported under poor weather conditions might be, at least in part, attributable to decreased ovule fertility.

Additional index words: **floral development, floret number, ovule fertility.**

INTRODUCTION

Preliminary studies (Thomas, 1996) suggested that seed set in white clover in a 'poor' year might be the result of pre-fertilisation and/or post-fertilisation abortion of ovules, and that dull weather conditions might decrease seed yield by increasing seed abortion. Light intensity is known to be an important factor influencing flower development in white clover. Zaleski (1964, 1970) found that low intensities decreased the initiation of flower heads and led to complete or partial abortion of those that did form. Thomas (1961, 1987b) found, additionally, that short photoperiods favoured the abortion of inflorescences when plants were grown in warm conditions. Comparable effects have been reported in other species, such as *Lycopersicon* (Cooper, 1964).

The observation that drastic reduction of light intensity results in total abortion of young inflorescences leads to the obvious thought that a lesser reduction in light intensity, such as would occur during a 'poor summer', might lead to partial abortion and a concomitant reduction in ovule fertility. This in turn might, at least in part, be the cause of the lowered seed set often recorded in cooler, wetter, duller summers (eg van Bogaert, 1977).

The present investigation was therefore undertaken to test the hypothesis that light intensity influences post-initiation floral development in general, and ovule fertility in particular.

MATERIALS AND METHODS

Plant materials

Ramets of a single clone of white clover cv. Grasslands Huia were grown from stolon tip cuttings taken from stock plants. This clone had previously been the subject of a study in controlled environment conditions (Thomas, 1961) when it was referred to as New Zealand Government Stock clone A. Plants were grown in a mixture of sand and peat in 100 ml plastic pots. Each pot received 17 g of dolomite lime, 3.5 g of short term slow release fertiliser pellets (Osmocote), and 2.5 g of superphosphate. When established, these plants were maintained for a month in a glasshouse before being transferred to a growth room. During summer the maximum day/minimum night temperatures in the glasshouse were in the range of 28-33°C/15-17°C while during winter the temperatures were 20-23°C/13-15°C.

Experimental procedure

Two experiments were conducted in a controlled environment growth room with temperatures averaging 23 ± 1°C. The light source used, a combination of 15 Watt incandescent lamps and fluorescent tubes (Philips, TLD 58W/33, white), gave an intensity of approximately 200 μmoles $m^{-2}s^{-1}$ at leaf surfaces.

Experiment 1

Plants were transferred from the glasshouse into the growth room on 7 July 1987 and artificially induced to initiate inflorescences by exposing them to continuous light at approximately 200 μmoles $m^{-2}s^{-1}$ at their leaf surfaces for three weeks. Using the numbering system described previously by Thomas (1987a), in which the node bearing the youngest leaf primordium in the apical bud is designated as node 1 (N1), and successively older nodes are referred to as N2, N3 etc., the youngest leaf with

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unfolded leaflets was located at N7. At the end of this three-week period the oldest inflorescences were situated at N5 and N6, by which time the oldest ovules in their first-formed florets would have been well formed but probably still at the premeiotic stage (Thomas, 1987b). After this preliminary treatment, 12 plants were grown for four weeks in each of five different light intensities ranging from 40 to 200 μmoles $m^{-2}s^{-1}$ in continuous light. This was achieved by covering them with screens bearing one to four layers of shade cloth. At the end of the treatment, measurements were made of floral organs in the lowest florets on inflorescences at six different stages of development. These inflorescences were situated at nodes 7, 8, 9, 10, 11 and 12. The youngest of these inflorescences, at node 7, was that subtended by the youngest leaf with unfolded leaflets. Five florets from each of two flower heads for each stage of development per treatment were collected for measurements of the size of floral organs.

Experiment 2

As in Experiment 1, inflorescence initiation was induced by transferring plants from the glasshouse to the growth room where they were exposed to continuous light at an intensity of 200 μmoles $m^{-2}s^{-1}$ for three weeks. After the third week, on 12 January 1988, plants were transferred to five different light intensity treatments ranging from 40 to 200 μmoles $m^{-2}s^{-1}$ in the growth room. For each treatment, observations were made of the influence of light intensity on ovule number per carpel, ovule fertility and ovule dimensions. Ten flower heads were sampled for each treatment. When all the lowermost florets in an inflorescence reached anthesis (*ie.* when the inflorescences were situated at node 11), three of them were removed. In two of these the number of ovules in each carpel was counted, and their lengths and breadths recorded. The third excised floret was fixed in formalin-acetic-acid (FAA). Thus, for each treatment, ovule number per carpel was recorded for 20 florets (*viz.* 2x10) and ten florets were fixed in FAA.

The cytoplasmic state of the embryo-sacs of each of the FAA-fixed ovules was determined using the stain-clearing technique described by Stelly, Peloquin, Palmer and Crane (1984) which was modified as described by Pasumarty (1994) to give good clarity, resolution and contrast within white clover ovules. The fixed ovaries were stained with Mayer's haemalum and optically cleared with methyl salicylate. Embryo-sacs, in intact ovules dissected out of ovaries and mounted whole in methyl salicylate, were examined using Nomarski interference optics with a light green filter. The size and cytoplasmic condition of the embryo-sac and the presence or absence of polar, egg and synergid nuclei therein were recorded.

After the three florets had been removed from the selected ten flower heads per treatment, the plants were left for a few more days in the growth room until all the remaining florets on these heads showed white corolla colour. They were then transferred into a field plot of prolifically flowering white clover where they were cross pollinated naturally by bees. At 10-15 days after pollination, the ten flower heads per treatment were collected and stored for 4-5 days at 4°C. For each treatment, the number of florets per inflorescence was counted and the mean number of seeds in each of 30 fully developed florets from each flower head recorded.

RESULTS

Development of floral organs

The most rapid growth of different floral organs occurred at different stages of inflorescence development. In the lowermost florets on inflorescences of control plants growing at a light intensity of 200 μmoles $m^{-2}s^{-1}$, 71% of sepal growth and 50% of ovary growth had occurred by the time inflorescences were at node 8. Most growth of petals, filaments, and styles occurred at later stages of inflorescence development (Fig. 1.). The growth of ovaries appears to have been diauxic, two phases of higher growth rate being separated by a period of slower growth as the inflorescence moved from node 8 to node 9 (Fig.1.). Similar diauxic trends were observed in all but the very lowest light intensity treatments (Fig.2C). The effect of light intensity on development of floral organs varied from organ to organ (Fig.2). In all cases, growth was least at the lowest light intensity but in several cases there was also less growth at the highest intensity than at intensities of 56-120 μmoles $m^{-2}s^{-1}$. Once again, responses of petals, styles and filaments were similar and differed from those of sepals and ovaries. Petals and styles in florets at node 9 were larger at intensities of 56 and 90 μmoles $m^{-2}s^{-1}$, and filaments were longest at these intensities at nodes 8, 9 and 10. This apparent partial etiolation did not occur in sepals or ovaries at these nodes, but sepals showed a pronounced tendency towards partial etiolation at

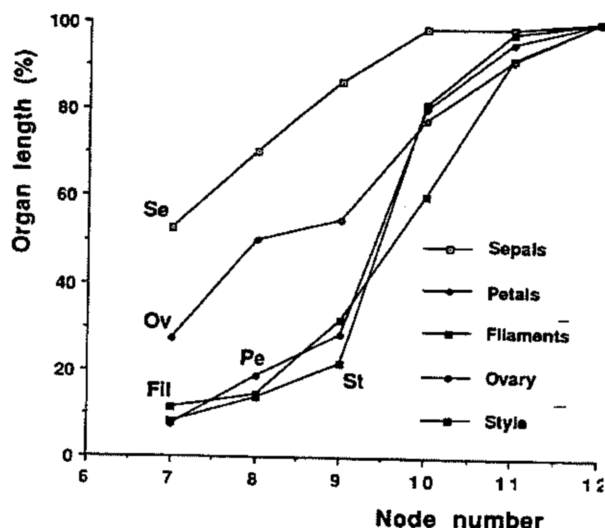

Fig. 1. The length of floral organs (expressed as % of maximum length attained at maturity) in the oldest florets of white clover inflorescences present at successive nodes of stolons of plants growing at 200 μmoles $m^{-2}s^{-1}$ light intensity in a controlled environment growth room.

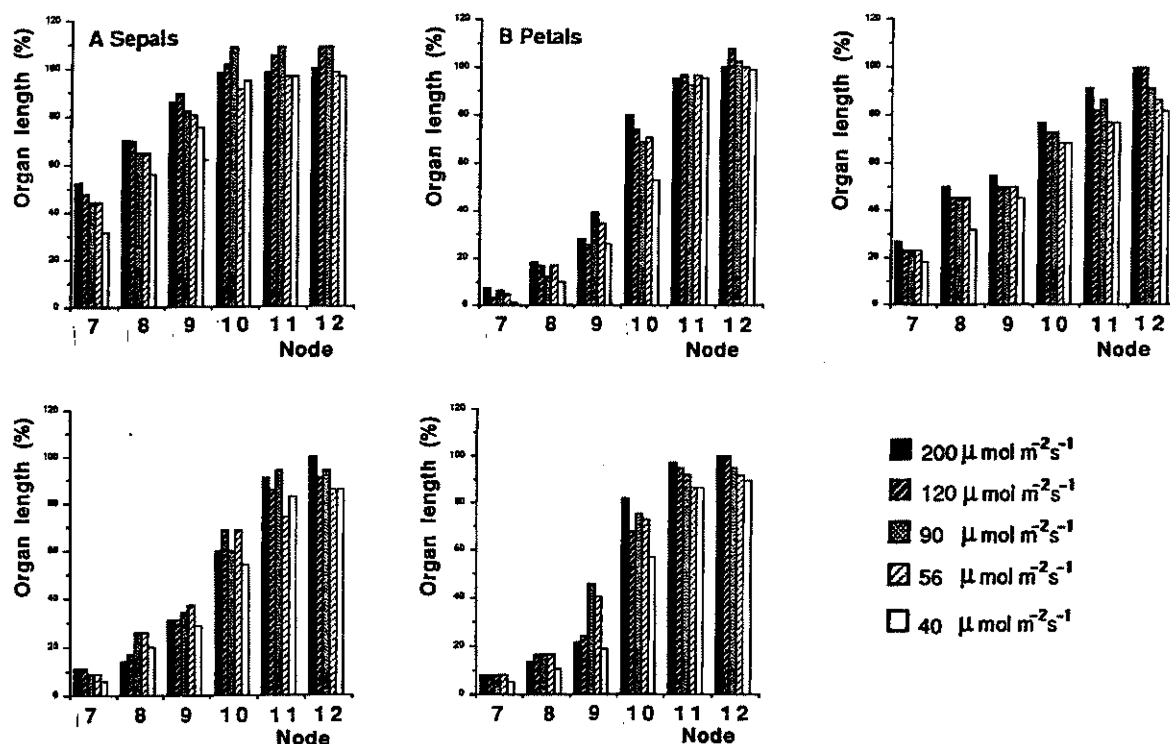

Fig. 2. The effect of light intensity on the length of white clover floral organs. The floral organ length (expressed as % of length at node 12 at 200 µmoles $m^{-2}s^{-1}$) was measured in the oldest florets of inflorescences at successive nodes of stolons of plants growing in five different light intensities in a controlled environment growth room. The range of standard errors was 1.1-4.4% for sepals, 0.1-3.3% for petals, 1.4-5.5% for ovaries, 0.9-4.0% for filaments and 0.8-7.7% for styles.

intensities of 90 and 120 µmoles $m^{-2}s^{-1}$ at nodes 10 to 12. In contrast, ovaries showed a very clear positive correlation between length and light intensity at all ages.

Ovule number and size

Light intensity had a significant effect on the number of ovules per carpel, and the ovules of plants grown under high light level (200 µmoles $m^{-2}s^{-1}$) were found to be slightly larger than those plants grown under low light intensities (Table 1). Lengths and widths were slightly greater at the proximal than the distal ends of the carpels (length 0.53 vs 0.42 mm respectively; width 0.36 vs 0.27 mm respectively). Similar trends were observed in all light regimes.

Ovule fertility

Most embryo-sacs examined appeared turgid and 'normal', containing four or five very clear nuclei towards their micropylar ends: viz. two synergid nuclei, an egg nucleus, and either a polar fusion nucleus or two separate polar nuclei. Antipodal nuclei were less clear. A high proportion of embryo-sacs appeared incomplete, however. In some cases fully grown embryo-sacs lacked nuclei while others were rather shrunken with no or few visible nuclei, even though most of the ovules in which they were situated appeared well grown, unshrivelled, and indistinguishable from those which contained well developed embryo-sacs as depicted by Pasumarty, Matsumura, Higuchi and Yamada (1993). Fully nucleate turgid embryo-sacs were classed as apparently fertile whereas those which lacked nuclei and/or were shrunken were clearly not so and therefore classed as sterile. There was a high positive correlation (r=0.969) between light intensity and apparent fertility, the percentage of apparently fertile ovules decreasing from 63 at the highest to 17 at the lowest intensity (Table 1).

Seed yield components

As expected, lower light intensities led to floret abortion and thereby reduced the number of florets per head which reached anthesis. Most striking, however, was the effect of reduced light intensity on the percentage of ovules setting seeds (Table 2). As with the percentage of fertile ovules in a carpel, the seed number per floret was higher in florets which developed at high light intensities. Although no count was made of the total number of seeds per inflorescence, this can be estimated by multiplying the average number of seeds per floret by the average number of florets per inflorescence (Table 2). It is clear that the total number of seeds per inflorescence was higher in those which developed at higher light intensities.

DISCUSSION

This investigation was undertaken to test the hypothesis that light intensity influences floral development in white clover, and the results obtained strongly support this. The effects on development of floral appendages, however, are not as great as might have been expected, bearing in mind that the very lowest intensities have in the past been found to lead to total abortion of

Table 1. Effect of light intensity on white clover average ovule number and size, and percentage of apparently fertile ovules.

Light intensity (µmoles $m^{-2}s^{-1}$)	Ovule number per carpel ±s.e.	Ovule size		Percentage of apparently fertile ovules
		Length (mm) ±s.e.	Width (mm) ±s.e.	
200	5.16±0.16	0.47±0.007	0.33±0.006	63
120	5.50±0.17	0.45±0.007	0.32±0.007	45
90	5.30±0.15	0.44±0.007	0.29±0.005	42
56	5.00±0.13	0.44±0.007	0.29±0.004	24
40	5.20±0.13	0.41±0.006	0.29±0.004	17

Table 2. Effect of light intensity on white clover seed yield components.

Light intensity (µmoles $m^{-2}s^{-1}$)	No. of fully developed florets per head	No. of seeds per carpel	% of ovules setting seed in fully developed florets	No. of seeds per head
200	55.1 a	3.56 a	63 a	194.0 a
120	47.9 b	3.05 b	55 b	145.1 b
90	42.9 c	2.54 c	48 c	108.0 c
56	25.7 d	1.60 d	31 d	41.1 d

Values followed by the same letter in the same column are not significantly different at $P<0.05$. In plants grown at an intensity of 40 µmoles $m^{-2}s^{-1}$ the total number of fully developed florets which formed per treatment was only 10-12, and these were all used to determine the percentage of fertile ovules.

inflorescences before emergence (Zaleski, 1970; Thomas, 1987b). In the present investigation, for instance, final size of ovaries, styles and stigmas was only 10 to 20% less at the lowest intensity used compared with the highest, and there was no difference between lengths of sepals and petals at these extremes (Fig. 2). It should be realised, though, that plants were all grown at the same high light intensity during the early stages of inflorescence development, and it was only after inflorescences were about to emerge from the shoot apical buds that they were transferred into the experimental treatments. This might well explain why there was little difference between the final lengths obtained by the earliest formed organs (sepals and petals) at the lowest and highest intensities, whereas greater differences were observed in the later formed organs.

The overall pattern of development of individual organs was also relatively little affected by light intensity. This pattern is shown clearly in Fig. 1, in which it can be seen that sepal growth began and ended ahead of the growth of other floral organs. In addition ovary elongation growth started ahead of that of petals, styles and stamen filaments and showed a tendency to diauxic growth, with a decline in rate between nodes 8 and 9. The growth curves of petals and styles were similar, showing a marked acceleration in growth rate between nodes 9 and 10, and the growth curve of stamen filaments parallelled those of petals and styles but lacked the marked acceleration phase between nodes 9 and 10. Comparison of floral development at different light intensities (Fig. 2) shows that the general pattern of growth was little affected, the only noticeable effect being a slight decrease in the acceleration rate of petal and style elongation between nodes 9 and 10 at low light intensities so that this phase extended from node 9 to node 11 at 40 µmoles $m^{-2}s^{-1}$.

It is interesting to note that the present results agree with those published earlier by Thomas (1987b, Fig. 3.6). The earlier account of floral development was a description of inflorescences grown throughout in full New Zealand natural summer sunlight at an intensity up to ten times as great as the highest artificial light intensity used in the present experiments, but, despite this difference, in both cases the petal and style growth rates accelerated between nodes 9 and 10 and ovary growth rate showed a tendency to decline between nodes 8 and 9.

Although there was little difference between final lengths of sepals and petals at the extreme high and extreme low light intensities (Fig. 2A-E), a clear partial etiolation response to intermediate light intensities occurred in sepals and, to a lesser extent, in petals. In both these cases the longest floral appendages formed at 120 and 90 µmoles $m^{-2}s^{-1}$. The increase in final lengths

might be explicable as being the result of increased cell expansion, as commonly observed in dark-grown seedlings. At the lowest light intensities the tendency towards increased cell expansion was probably offset by a decreased availability of photosynthate.

No measurements were made of cell size in this investigation, so it is not possible to distinguish between cell division and cell expansion as contributors to these differences in final size, but it is not unreasonable to assume that, as with other plant organs such as leaves (Denne, 1966), a phase in which cell division predominates is followed in floral organs by a phase of rapid cell expansion. The approximately sigmoid shape of the growth curves of petals and styles (Fig. 1) is then probably the result of the onset of a cell expansion phase between nodes 9 and 10.

Petals, styles and stamen filaments at node 9 were longest at intensities of 90 and 56 µmoles $m^{-2}s^{-1}$. This might be explicable on the basis of the duration of their cell division phase; the longer the phase was maintained, the more delayed the onset of rapid cell expansion would be. It is interesting that, unlike other organs, ovaries showed a positive correlation between light intensity and growth throughout their development, with no sign of partial etiolation effects. This suggests that cell expansion in ovaries might not be affected by light intensities to the extent that it is in the other organs.

The reduction in number of ovules per carpel from 5.6 to 5.2 at the lowest light intensity suggests that the ovule initiation process might also be affected by light intensity. Thomas (1987b) observed in plants of cv. Grasslands Huia growing outside in late November in Palmerston North that the first ovules were initiated on the inflorescence at node 5 when the youngest leaf with unfolded leaflets was at either node 7 or node 8. In the present investigation, the oldest inflorescences at the end of the three-week pretreatment period were at nodes 5 and 6. It is therefore probable in this case that ovule initiation was still incomplete at the end of the pretreatment period and must have reached completion during the treatment period. Effects of light intensity on ovule development in the present study were similar to those on ovary growth; ovules being about 12% smaller at the lowest light intensity than at the highest (Table 1). Embryo-sac development, however, was much more strongly affected, fertility being reduced from 63% at the highest light intensity to 17% at the lowest (Table 1).

The question which originally led to this investigation was 'why do white clover plants form so few seeds under poor weather conditions?' The results so far suggest that the cause may well be decreased fertility of ovules, and this suggestion is strongly supported by a comparison of apparent embryo-sac fertility (Table 1) with seed set in the same plants (Table 2). The correlation between apparent embryo-sac fertility and seed set in comparable inflorescences was very high (r=0.979). In three of the four light intensities for which data were obtained, though, the percentage of ovules setting seed was slightly higher than the percentage which was apparently fertile. This suggests that sterility was slightly overestimated, and that some ovules in which few visible nuclei could be discerned might really have been fertile. Despite this anomaly, the data leave little doubt that, given the combination of poor light conditions and good pollination, low seed set most likely resulted from a high level of ovule sterility. This situation is aggravated by the fact that lower light intensities also led to increased floret abortion (Table 2), the number of fully developed florets per inflorescence being reduced from 55.1 at the highest intensity to 25.7 at 56 µmoles $m^{-2}s^{-1}$.

In field conditions, whole plants of white clover do not receive levels of total daily incoming radiation as low as they did in this investigation. The young flower heads, however, develop at light intensities which vary with the structure of the foliage canopy from very high (perhaps about 50% of full light) in an open canopy, to a level as low as about 5% within a dense canopy (Brougham, 1958). It is clearly important to determine to what extent developing flower heads are influenced directly by low light intensity when they are growing on plants otherwise exposed to full light. Experiments designed to assess this will be described in a subsequent publication.

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REFERENCES

1. Brougham, R.W. 1958. Leaf development in swards of white clover. *New Zealand Journal of Agricultural Research* 1:707-718.

2. Cooper, A.J. 1964. A seasonal pattern of flowering of glasshouse tomatoes. *Journal of Horticultural Science* 39:111-119.

3. Denne, M.P. 1966. Leaf development in *Trifolium repens*. *Botanical Gazette* 127:202-210.

4. Pasumarty, S.V. 1994. A stain-clearing technique for observations within whole ovules of white clover (*Trifolium repens* L.). *Journal of Japanese Society of Grassland Science* 39:501-504.

5. Pasumarty, S.V., Matsumura, T., Higuchi, S. and Yamada, T. 1993. Cultivar variation for seed development in white clover (*Trifolium repens* L.) *Euphytica* 65:211-217.

6. Stelly, D.M., Peloquin, S.J., Palmer, R.G. and Crane, C.F. 1984. Mayer's hemalum-methyl salicylate: A stain-clearing technique for observations within whole ovules. *Stain Technology* 59:155-161.

7. Thomas, R.G. 1961. The influence of environment on seed production capacity of white clover (*Trifolium repens* L.).I. Controlled environment studies. *Australian Journal of Agricultural Research* 12:227-238.

8. Thomas, R.G. 1987a. Vegetative growth and development. In: White Clover (ed. M.J. Baker and W.M. Williams), 31-62, CAB International, UK.

9. Thomas, R.G. 1987b. Reproductive development. In: White Clover (ed. M.J. Baker and W.M. Williams), 63-123, CAB International, UK.

10. Thomas, R.G. 1996. Limitations to seed set in white clover (*Trifolium repens* L.).I. Preliminary observations. *Journal of Applied Seed Production.* 14: 59-66.

11. Van Bogaert, G. 1977. Factors affecting seed yield in white clover. *Euphytica* 26: 233-239.

12. Zaleski, A. 1964. Effect of density of plant population, photoperiod, temperature and light intensity on inflorescence formation in white clover. *Journal of the British Grassland Society* 19:237-247.

13. Zaleski, A. 1970. White clover seed production. In: White Clover Research (ed. J. Lowe), 147-155. Occasional Symposium No. 6, British Grassland Society.