Overwintering and spring growth of white clover (Trifolium repens L) - development and importance of leaves

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presented by
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I GENERAL SUMMARY

Under favourable conditions during the growing season, the proportion of white clover (Trifolium repens L.) in grass/clover mixtures often increases, whereas a low proportion of clover and, thus, a low yield are observed in spring. It was assumed that processes during winter and spring are responsible for these fluctuations during and between the growing seasons and, thus, for its lack of persistence. The aim of this study was to investigate the influence of climate, competition and genotype on overwintering and the regrowth of white clover in spring. The special focus of the study was on leaf development at low temperature and the role of leaves during winter and spring.

A) In a field experiment, swards of two white clover varieties (AberHerald and Huia), differing in winterhardiness, temperature requirements for growth and carbohydrate reserves, were investigated. Overwintering, spring growth and yield of white clover in pure stands and in competition with perennial ryegrass (Lolium perenne L.) were examined over three years. This experiment was conducted within the scope of a collaboration (COST Action 814) among several European research groups, all of which made similar experiments under very different climatic conditions.

B) In the experiments in the field and in a controlled environment, the effect of two white clover varieties (AberHerald and Huia), grown with and without competition with perennial ryegrass (Lolium perenne L.), on the leaf development of individual white clover plants was examined. We also tested the hypothesis that leaves are important for successful overwintering and spring growth by comparing plants that were defoliated throughout the winter with undefoliated plants.

Results:

1) Annual yield and yield in spring was about 27% higher for AberHerald than for Huia when grown in competition with perennial ryegrass and 10% higher when grown as a monoculture.
2) Leaf emergence and leaf size were very temperature-dependent, and AberHerald had more and bigger leaves than Huia due to the more intense meristematic activity. Leaves of AberHerald were about 40% larger than those of Huia in early and late spring.

3) Carbohydrate content in the stolons was clearly higher for AberHerald than for Huia. This effect was measured in autumn (+8 mg TNC/100mg stolon dry mass) and, to a lesser extent, in spring (+5 mg TNC/100mg stolon dry mass).

4) In general, competition with perennial ryegrass reduced growth rates and the size of all the organs of white clover. The number and size of the leaves as well as the number of nodes and buds were negatively affected by competition.

5) Both varieties generally lost only few buds and nodes, the clear exception being white clover plants when they were defoliated throughout the winter. These plants lost about 45% of their initial number of buds and nodes.

6) Plants with no leaves in winter showed lower rates of leaf growth and, thus, fewer (-300%) and much smaller leaves (-350%).

Leaves were found to be very important for overwintering and the competitive ability of white clover. Thus, the persistence of clover and its proportion in swards in spring may be increased by preserving leaves during winter and spring; this can be achieved by breeding and/or management.
II ZUSAMMENFASSUNG


B) In Feldversuchen und in einem Klimakammerversuch wurde die Blattentwicklung von Weissklee bei tiefen Temperaturen untersucht. Im Feld wuchsen die beiden Weisskleearten, AberHerald und Huia, als Einzelpflanzen, ohne oder mit Konkurrenz von englischem Raigras. Es wurde die Hypothese geprüft, ob Blätter für eine erfolgreiche Überwinterung und den Aufwuchs im Frühling von Bedeutung sind. Zu diesem Zweck wurden Weissklee-Pflanzen während des ganzen Winters entblättert und mit nicht entblätterten Pflanzen verglichen.
Resultate:

1) Der Jahresertrag und der Ertrag des ersten Aufwuchses waren in der Mischung ca. 27% und in der Monokultur ca. 10% höher für AberHerald als für Huia.

2) Die Blatterscheinung und die Blattgrösse waren stark von der Temperatur abhängig. AberHerald hatte mehr und grössere Blätter (ca. 40% im Frühling) als Huia bewirkt durch eine höhere meristematische Aktivität.

3) AberHerald hatte im Herbst (+8 mg TNC/100 mg TS der Stolonen) und, weniger ausgeprägt, auch im Frühling(+5 mg TNC/100 mg TS der Stolonen) signifikant höhere Kohlenhydratreserven als Huia.

4) Im allgemeinen erniedrigte die Konkurrenz von englischem Raigras die Wachstumsraten und die Grösse aller Organe des Weissklees. Die Anzahl Blätter, die Blattgrösse, aber auch die Anzahl Nodien und Wachstumspunkte wurden durch die Konkurrenz verringert.


6) Entblätterte Pflanzen hatten niedrigere Wachstumsraten und deshalb weniger (-300%) und viel kleinere Blätter (-350%) als nicht entblätterte Pflanzen.

III GENERAL INTRODUCTION

White clover (*Trifolium repens* L.) is the most important pasture legume in regions with a humid temperate climate. In Switzerland, 70% of the agricultural land is covered by meadows and pastures, most of which are situated in hilly regions and at higher altitudes. The advantages of clover are high forage quality and its contribution to the nitrogen supply of the sward by symbiotic nitrogen fixation. In order to profit from these advantages, 25 to 40% of the sward should be clover. However, fluctuations in clover yield and proportion during and between growing seasons are often observed and cannot be explained fully by management. White clover often increases its proportion in summer if conditions are favourable. Thus, it was considered that adverse climatic conditions, especially in winter and spring, are responsible for its lack of persistence.

Limited tolerance to freezing and, thus, extensive death of stolons may be a reason for clover's decline. Damage to white clover during winter can reduce its proportion considerably (Eagles and Othman, 1981; Harris et al., 1983). Without a protecting covering of snow, cold winds and frost usually killed the clover in coastal regions of Great Britain and Scandinavia (Ollerenshaw and Haycock, 1984). In regions with prolonged snow cover, plants are protected from freezing temperatures, but they still have to endure a long period without photosynthesis and, thus, can not produce carbohydrates. Sagisaka (1995) confirmed the direct relationship of the rates of metabolism of carbohydrate reserves and the life span of white clover under snow. Thus, limited carbohydrate reserves may be another cause of clover death and the observed decline in the proportion of clover.

Genotypes differ largely in their ability to survive the winter. Northern types are usually more winterhardy than southern types (Haycock and Ollerenshaw, 1982; Juntilla et al., 1990). Since white clover is wide-spread and is found even at the Arctic Circle, limited winterhardiness of white clover may indicate the wrong genotype at the wrong site (Rhodes, 1991). Nevertheless, winterhardiness is an important characteristic of white clover genotypes,
because spring and annual yield are related to the number of stolons and buds that survive the winter (Harris et al., 1983; Collins et al., 1991). Growth is largely influenced by temperature. During the summer, white clover has a higher relative growth rate than grasses and, hence, increases its proportion; however, during winter, under cool temperatures, lower relative growth rates are observed (Mitchell, 1956; Woledge, 1988; Woledge et al., 1989). In addition, leaves of white clover had a lower position in the sward relative to grasses during winter and spring (Woledge et al., 1990). This is thought to explain its poor ability to compete with grasses in spring and can lead to a long-term decrease in white clover (Spedding and Diekmahns, 1972; Brock, 1974). It is suggested that an improvement in growth at low temperature may increase the competitive ability of white clover and, hence, its persistence.

The growth rates of genotypes differ at low temperatures (Eagles and Othman, 1981; Ollerenshaw and Haycock, 1984). Northern types often have lower growth rates than southern types. In particular, Mediterranean clover (Ladino types) grows very fast at low temperatures, but its winterhardiness is limited. Under our climatic conditions a combination of winterhardiness and faster growth at low temperature is desired. A negative correlation between winterhardiness and growth at low temperature has been found in some genotypes (Eagles and Othman, 1981) but not in all (Haycock and Ollerenshaw, 1982; Ollerenshaw and Haycock, 1984). Growth and winterhardiness differed in their response to climatic conditions at the site of origin rather than to latitudes. Thus, it was possible to select a genotype with a high degree of winterhardiness and fast growth at low temperature based on material collected from hilly regions near Zurich. The new variety AberHerald, bred at the Welsh breeding station in Aberystwyth, UK, had higher spring and annual yields in Wales than Huia, due to low stolon death and high rates of leaf expansion (Rhodes et al., 1994).

Carbohydrates are considered to be important for the physiology of overwintering and for regrowth processes. Carbohydrates accumulate in autumn and are consumed during winter; moreover, changes in the composition of the carbohydrates during cold hardening are observed (Vez, 1961; Harris et al., 1983; Winkler and Nösberger, 1985; Lüscher, 1989). Higher
concentrations of carbohydrates in legumes were often accompanied by a higher degree of winterhardiness (Levitt, 1980; Harris et al., 1983; Rosnes et al., 1993; Collins and Rhodes, 1995). A clear positive relationship between the life span of white clover under snow and the amount of carbohydrate reserves in autumn was shown by Sagisaka (1995). In the experiment of Frankow-Lindberg et al. (1995), spring yield of white clover was positively correlated to the carbohydrate reserves in autumn but even more so at the beginning of regrowth. Regrowth in spring may be influenced by starch reserves (Lüscher, 1989). An experiment in a controlled environment at low temperature showed that white clover plants with higher carbohydrate concentrations in the stolons produced heavier leaves per bud, but had similar relative growth rates due to fewer buds. Baur-Höch (1988) showed that starch plays a major role in regrowth when the remaining leaf area is small after a severe defoliation. Plants may have only a few remaining leaves at the beginning of regrowth after a harsh winter. Thus, varieties with high carbohydrate reserves should be at an advantage for overwintering and regrowth in spring.

Overwintering and regrowth of white clover, as related to carbohydrates, is complex and influenced by climate, competition and genotype. In order to detect the processes that are important for successful overwintering and regrowth of white clover, a joint multi-site research programme, „Overwintering and Spring Growth of White Clover“, was initiated in 1991. Within the framework of the „European Cooperation in the Field of Scientific and Technical Research (COST) 814 Programme“ („Crop adaptation to cool and wet regions in Europe“), it was possible to examine the overwintering and spring growth of white clover at different sites in Europe with very different climates. Several countries were involved in starting the joint experiment in 1992 (Mikkeli, Finland; Nancy, France; Freising-Weihenstephan, Germany; Aberystwyth, Great Britain; Uppsala, Sweden and Zurich, Switzerland). The experiment is now being conducted at 12 sites in Europe. The aim of this field experiment is to examine the influence of different climates on overwintering, regrowth and yield of two white clover varieties, AberHerald and Huia, differing in winterhardiness, in their temperature requirements for growth and in their
carbohydrate reserves. The results of the experiment in Zurich are presented in Part IV.

Part V discusses leaf development and its significance for overwintering and regrowth. The aim of several experiments in the field and in a controlled environment was to test the temperature-dependence of leaf development and to determine whether leaves are important for overwintering and the competitive ability of white clover.

The results show that growth at low temperature, especially fast rates of leaf expansion, are important for the competition of white clover with perennial ryegrass. We suggest that this was the key advantage of AberHerald, because a lack of leaves decreased growth rates and increased death rates of buds and nodes.
IV OVERWINTERING AND SPRING GROWTH OF WHITE CLOVER UNDER DIFFERENT CLIMATIC CONDITIONS

1 Summary

In the presented field experiment the overwintering and spring growth of two white clover varieties (AberHerald and Huia), grown as monocultures and in mixtures with perennial ryegrass (Lolium perenne L.), were examined for three years. We selected two varieties, differing in winterhardiness, temperature requirements for growth and carbohydrate reserves, to determine whether (i) improved winter survival, (ii) increased growth under low temperature and (iii) high levels of carbohydrate reserves increase the yield in spring and reduce seasonal fluctuations of white clover. This experiment was conducted within the scope of a collaboration (COST Action 814) among several European research groups under very different climatic conditions. Our results are:

1) Annual yield and yield in the spring of the second growing season was about 10% higher for AberHerald than for Huia when grown in monoculture and clearly higher (about 27%) when grown in mixture with perennial ryegrass.

2) AberHerald had greater leaf area per terminal bud in spring, because its leaves were 20% to 50% larger than those of Huia.

3) Carbohydrate reserves of AberHerald were clearly higher than those of Huia in autumn and, to a lesser extent, in spring. The specific weight of the stolons was 1.5 times that of Huia.

4) AberHerald and Huia lost similar number of stolons and buds, (0 to 20%) but only few.

5) The influence of climate among years and sites is discussed.

We conclude that the advantage of AberHerald over Huia may be related to its faster rate of leaf expansion or to its greater number of carbohydrate reserves, while loss of stolons and buds had only a minor effect on persistence.
2 Introduction

White clover (*Trifolium repens* L.) is the most important pasture legume in cool, temperate climates. The advantages of white clover are its excellent forage quality and its ability to fix nitrogen. However, one of the major problems in the management of grass/clover mixtures is fluctuation in the yield of white clover during and between growing seasons. A low clover proportion in the yield is often observed in spring. Thus, the fluctuation may be related to overwintering processes, especially to the death of stolons and buds of white clover in winter. Cold and frost can cause extensive damage to white clover. Harris et al. (1983) observed a marked reduction (30 to 60%) in stolon length in winter as a result of low temperatures. Collins et al. (1991) recorded a decline of 40 to 60% in stolon length, and Eagles and Othman (1981) found that 30 to 100% of the stolons died during winter. The damage during winter depended on the variety and considerably reduced the proportion of white clover in the following growing season. Thus, winterhardiness is an important characteristic of white clover genotypes, because spring and annual yield are related to the number of stolons and buds that survived the winter (Harris et al., 1983; Collins et al., 1991).

Regrowth of white clover in spring is often delayed as compared with regrowth of the companion grasses (Williams, 1970). This is considered to be a competitive disadvantage (Davies and Young, 1967; Spedding and Diekmahns, 1972) and may be an important reason for the low yield of white clover in mixed swards in spring. In general, white clover has a lower growth rate than grasses under cool temperature (Mitchell, 1956; Mitchell and Lucanus, 1960); the growth rate of white clover varies considerably, depending on the genotype (Eagles and Othman, 1981; Ollerenshaw and Haycock, 1984). In climates with cold winters it is an advantage to have winterhardy and fast-growing varieties at low temperature. From native plant material from the hilly regions near Zurich, it was possible to select a variety with a high degree of winterhardiness and fast growth at low temperature. In our experiment, this new variety, AberHerald, was compared with the standard variety Huia.
Carbohydrate reserves are supposed to play a major role in overwintering and regrowth processes. The total amount and composition of carbohydrate reserves are known to change during cold hardening of plants. In experiments since 1961, it was found that carbohydrates in white clover accumulated in autumn and were used up in winter (Vez, 1961; Harris et al., 1983; Winkler and Nösberger, 1985; Lüscher, 1989). Higher concentrations of carbohydrates in different legume species and in white clover varieties are often accompanied by a higher degree of winterhardiness (Levitt, 1980; Harris et al., 1983; Rosnes et al., 1993; Collins and Rhodes, 1995). In Sagisaka’s experiment (1995), a clear relationship between carbohydrate reserves in autumn, basal metabolic rate and the life span of plants under snow was observed. These plants are dependent on carbohydrate reserves, because they can not produce carbohydrates by means of photosynthesis.

Carbohydrates influence regrowth processes, especially in spring. In a low temperature experiment in a controlled environment white clover plants with higher total nonstructural carbohydrate (TNC) concentrations produced heavier leaves per bud, but the whole plant had similar relative growth rates (Lüscher, 1989). Baur-Höch et al. (1990) showed that starch is important for regrowth when the remaining leaf area is small after a severe defoliation. The situation in spring after a harsh winter when plants have only little remaining leaf area, is comparable to the situation after defoliation. Under these conditions, varieties with high TNC concentrations in the stolons should have a higher growth potential. Increased growth of white clover in the early spring can improve its competitive ability relative to the companion grasses.

In the field experiment presented here, overwintering and spring growth of two white clover varieties, grown as monocultures and in mixtures with perennial ryegrass (*Lolium perenne L.*), were studied for three years. Two varieties, differing in winterhardiness, temperature requirements for growth and carbohydrate reserves, were selected to determine whether (i) improved winter survival, (ii) increased growth at low temperature and (iii) high levels of carbohydrate reserves can increase white clover yield in spring and reduce seasonal fluctuations. Our experiment is part of a research project in collaboration with groups from 12 European countries (COST Action 814).
whose aim is to answer these questions by conducting experiments under very
different climates, ranging from temperate to sub-Arctic regions.
Our results show that annual yield and yield in the spring of the second growing
season were higher for AberHerald than for Huia. This increase in yield may be
related to higher carbohydrate reserves and/or to a faster production of leaf
area early in spring, while winterhardiness played only a minor role in the
persistence of white clover.
3 Material and Methods

3.1 Experimental site

The experiment was carried out in Eschikon (47°27' N and 8°41' E) near Zurich. Eschikon is situated at 550 m asl and annual precipitation is 1100 mm (long-term average). The soil is 19% clay, 40% silt and 41% sand and has a pH of 6.1 (measured in water).

Air temperature (yellow spring thermistors, series 400) was measured 2 m above the ground at a weather station 200 m from the experimental site. Monthly mean temperatures are means of daily temperatures (24 h). Frost days have a minimum air temperature below 0°C.

The weather varied considerably during the three winters of the experiment. The second winter was the coldest with unusually frequent frosts in November and April (Table 1). March was very mild (Figure 1). The first and third winters had unusually warm periods in January and February respectively. Spring was rather warm in the first year and cold in the third year, especially in March.

Table 1 Monthly mean temperatures, number of frost days and number of days with snow cover at Eschikon near Zurich during the experimental period (1992 - 1995) and long-term average (1978 - 1992).

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean temp [°C]</td>
<td>Frost days [no.]</td>
<td>Snow days [no.]</td>
<td>Mean temp [°C]</td>
</tr>
<tr>
<td>Oct.</td>
<td>7.0</td>
<td>2</td>
<td>0</td>
<td>6.9</td>
</tr>
<tr>
<td>Nov.</td>
<td>5.6</td>
<td>4</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Dec.</td>
<td>-0.1</td>
<td>24</td>
<td>0</td>
<td>3.0</td>
</tr>
<tr>
<td>Jan.</td>
<td>2.8</td>
<td>11</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Feb.</td>
<td>-1.1</td>
<td>25</td>
<td>13</td>
<td>-0.2</td>
</tr>
<tr>
<td>Mar.</td>
<td>3.8</td>
<td>17</td>
<td>10</td>
<td>7.7</td>
</tr>
<tr>
<td>Apr.</td>
<td>10.0</td>
<td>1</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>May</td>
<td>14.0</td>
<td>0</td>
<td>0</td>
<td>12.2</td>
</tr>
<tr>
<td>June</td>
<td>15.8</td>
<td>0</td>
<td>0</td>
<td>16.4</td>
</tr>
<tr>
<td>July</td>
<td>16.0</td>
<td>0</td>
<td>0</td>
<td>20.8</td>
</tr>
<tr>
<td>Aug.</td>
<td>16.7</td>
<td>0</td>
<td>0</td>
<td>18.4</td>
</tr>
<tr>
<td>Sept.</td>
<td>12.2</td>
<td>0</td>
<td>0</td>
<td>12.7</td>
</tr>
</tbody>
</table>
Figure 1 Maximal air temperatures (A) and minimal air temperatures (B) (weekly means) measured 2 m above ground at Eschikon near Zurich during the three winters of the experiment (1992/93 - 1994/95).

3.2 The experiment

The experiment was set up at the beginning of June 1992 and lasted until April 1995. The experimental plots measured 7 m x 3 m, including a 50 cm border on each side. The experimental design was a Latin square with four replicates and four treatments. The four treatments were the two white clover varieties, AberHerald and Huia, each sown as a monoculture or in mixture with perennial ryegrass (cv. Préférence). The monocultures were broadcast with 6 kg( ha)^{-1} white clover seeds capable of germinating and the mixtures with 20 kg( ha)^{-1} perennial ryegrass and 3 kg( ha)^{-1} white clover seeds capable of germinating. The germination rate was 63% for AberHerald and 81% for Huia. Swards were cut four times in the first year and five times in the growing seasons 1993 and 1994. Cutting height was 5 cm. The plots were fertilised each spring with 80 kg( ha)^{-1} P_2O_5 and 240 kg( ha)^{-1} K_2O applied as Foskal (15% P / 30% K) and KCl (60% K). All the plots received nitrogen fertiliser as ammonium-nitrate (27.5% N). At the beginning of each regrowth nitrogen was applied at a rate of 25 kg( ha)^{-1} nitrogen in the first year and at a rate of 60 kg( ha)^{-1} nitrogen in the following two growing seasons.
In autumn 1993 the proportion of perennial ryegrass decreased to 5% in the mixture with AberHerald. To ensure a larger proportion of grass, perennial ryegrass plants (sown in quick pots and propagated in the greenhouse for six weeks at 18/13°C day/night temperatures) were transferred to the mixture plots at a planting distance of 10 cm x 10 cm after the first cut in 1994. Unsown plant species were removed manually throughout the experiment. Plots were treated with Benomyl (5 g(m)^{-2}) in spring 1993 and 1994 to reduce the risk of Fusarium root rot complex (Fusarium spp.).

3.3 Data collection and analysis
3.3.1 Yield of white clover and ryegrass during the growing season
The fresh weight of the harvested plants was determined at each cut (Table 2 A) on a sub-plot of 1 m² of each plot. Dry mass and species proportion (white clover, perennial ryegrass, unsown species) were determined from a sub-sample of at least 300 g fresh weight. Dry mass yield was calculated on the basis of the fresh weight yield of the sub-plot and the determined dry mass. The proportion of unsown species was always below 1% of dry mass yield and, therefore, not presented.

3.3.2 Samplings of the whole plants during winter and spring
Destructive harvests were made in three winters. For these harvests a sub-plot, one third of the whole plot, was reserved. Four cores, 12 cm in diameter and 10 cm deep at intervals of at least 30 cm, were randomly taken five times during the winter from each sub-plot to determine the dry mass and morphological characters of different plant parts (Table 2 B). After the white clover plants were washed and separated from the ryegrass the following measurements were made: dry mass of clover of a) roots, b) stolons, c) laminas (> 0.6 stages of Carlson (Carlson, 1966)) and d) buds, petioles and leaflets (< 0.6 stages of Carlson (Carlson, 1966)). Furthermore, we counted the buds of clover, the tillers of ryegrass and the laminas of clover (> 0.6 stages of Carlson; Carlson, 1966). Lamina area (Li-3000, Li-Cor, Lincoln, Nebraska, USA) and stolon length of white clover were measured.
Buds were defined as terminal buds if internode elongation had occurred (at least 1 internode visible) and as axillary buds if internode elongation was not detectable and the first leaf had reached a developmental stage of at least 0.3 Carlson (Carlson, 1966). The leaf area of individual white clover leaves was calculated by dividing the sampled leaf area by the number of sampled laminas.

<table>
<thead>
<tr>
<th>Harvest:</th>
<th>1.</th>
<th>2.</th>
<th>3.</th>
<th>4.</th>
<th>5.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A growing season 1993</td>
<td>18 May</td>
<td>22 June</td>
<td>22 July</td>
<td>1 Sept.</td>
<td>19 Oct.</td>
</tr>
</tbody>
</table>

3.3.3 Determination of carbohydrate reserves in the stolons

At each winter harvest (Table 2 B) two additional cores were taken from the plots to collect stolons for the determination of carbohydrate reserves. After washing the stolons they were immediately frozen in liquid nitrogen, freeze-dried and ground to pass a 1 mm screen (cyclotec 1093 sample mill (Tecator/Udy)).

Carbohydrates were determined according to a modified method of Fischer et al. (1997). The sugars were extracted from 10 to 15 mg of stolon powder in 1 ml 80% ethanol for 30 min at 80°C. After centrifugation (12'000 g, 10 min), the sediment was resuspended in 1 ml 80% ethanol, and the extraction was repeated. To measure sugar, 0.1 ml of the combined ethanol extracts was diluted with 0.4 ml H₂O and incubated for 30 min at 95°C in 5 ml anthron solution (500 mg anthron and 10 g thio-urea in 1 l 66% sulphuric acid). Colour developed after 30 min in the dark and was measured with a spectrometer (Beckman DU-65) at a wave length of 620 nm.

After the two ethanol extractions the sediment was used to solubilize starch in 0.5 ml H₂O and 0.1 ml 5% termamyl (source: Novo Industri AS, Bagsvaerd, DK) solution for 15 min at 95°C. Starch breakdown products were completely
broken down to glucose by adding 0.5 ml buffer 1 (70 ml 1 M NaOH diluted with 950 ml H₂O; adjusted to pH 4.33 with acetic acid), and 0.08 ml amyloglucosidase solution [10 mg amyloglucosidase in 1 ml buffer 2 (buffer 1; adjusted to pH 4.6 with acetic acid)] and incubated for 30 min at 60°C. The samples were brought to 10 ml with H₂O and then filtered.

For the glucose measurement, 0.05 ml of the sample solution was diluted with 0.95 ml H₂O, 0.6 ml buffer 3 [0.05 ml ATP (300 mg ATP-Na₂H₂ and 300 mg NaHCO₃ in 6 ml H₂O), 0.05 ml NADP (60 mg NADP-Na₂H in 6 ml H₂O) and 0.5 ml buffer (14 g triaethanolamin-hydrochlorid and 250 mg MgSO₄·7H₂O in 80 ml H₂O); it was adjusted to pH 7.6 with 5 N NaOH and brought to 100 ml with H₂O] and 0.01 ml hexokinase-glucose-6-phosphate-dehydrogenase (Boehringer). The solutions were measured with a spectrometer (Hitachi U2000) at a wave length of 340 nm.

Total non-structural carbohydrates (TNC) was calculated as the sum of sugar and starch.

3.3.4 Statistics

The statistical analyses were carried out with the SAS computer program (Statistical Analysis System, SAS Institute, Cary, North Carolina, USA). Analyses of variance were carried out according to the General Linear Model procedure. Duncan tests were used to determine significant differences among treatment means.
4 Results

4.1 Yield and its seasonal distribution

Total annual yield (clover and ryegrass) (Figure 2) reached about 1000 g/m² dry mass for monocultures and 1200 g/m² total dry mass for mixtures. Total annual yield was equal in both growing seasons, irrespective of the clover variety. However, clover content changed drastically during the experiment (Figure 3), AberHerald being a very strong competitor. AberHerald increased its proportion in mixtures during the first growing season from 46 to 95% and remained at about 92% during the second growing season, while Huia increased its proportion from 44 to 75% during the first growing season and from 56 to 84% during the second growing season.

AberHerald had higher clover yields than Huia during both growing seasons (Figure 3). This was especially pronounced in mixtures where AberHerald had 22 and 32% higher clover yields than Huia in the first and second growing season respectively. AberHerald in monoculture had 10% higher yields than Huia during both growing seasons. During the first growing season, AberHerald
had a higher clover yield than Huia at the second, third and fourth cuts and at the first and second cuts of the second growing season. During both growing seasons, grass yield decreased continuously from cut to cut (Figure 3). Clover yield, however, remained stable until late summer or autumn.

**Figure 3** Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on yield of white clover (—) and ryegrass (---) during two growing seasons. Mean values of four replicates; error bars=standard error of mean (mostly hidden by the symbols).

### 4.2 Development of clover buds and grass tillers

The total number of buds and terminal buds of white clover changed only little during winter (Figure 4), increasing slightly in monocultures. In mixtures with perennial ryegrass, AberHerald retained nearly all of its buds, while the total number of Huia's buds decreased.

In general, Huia had a higher number of total buds and terminal buds than AberHerald. Monocultures had more terminal and axillary buds than mixtures
(Figure 4). The total number of buds and terminal buds increased from year to year. The number of axillary buds of both varieties decreased similarly from November to April in each year. The percentage of axillary buds compared to the total number of buds was highest in the first winter (52% for AberHerald and 44% for Huia) than in the second and third winters (28% for AberHerald and 23% for Huia).

The number of grass tillers (Figure 4) decreased from the first winter to the second winter (87% in the AberHerald plots and 48% in the Huia plots) and increased at the end of the third winter to a level comparable to the first winter. The number of grass tillers was always higher in April than in the preceding November.

During the first winter, the total number of clover buds relative to tiller number of ryegrass was comparable for both varieties (1:2). It was highest during the second winter (9:1 for AberHerald and 2.5:1 for Huia) and dropped during the third winter to 1.7:1 for both varieties after planting ryegrass in the preceding growing season.
Figure 4: Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on the number of terminal (■) and axillary (□) buds of white clover and on the number of ryegrass tillers (—) during three winters. Mean values of four replicates; error bars = standard error of mean.
4.3 Development of the stolons and their carbohydrate concentrations

During the first and second winter a decrease (approximately 15%) in stolon length per area (Figure 5 A) was observed from November to March for both varieties (monoculture or in mixture). During the third winter, stolon length per area remained constant. While varieties did not differ in stolon length per area, plants in monoculture had longer stolons than plants in mixture. Stolon length per area was lowest in the first, highest in the second and intermediate in the third winter.

Specific stolon weight is calculated by dividing stolon weight by stolon length and is a measure of the thickness of the stolon. In all three winters, a continuous drop in specific stolon weight was determined from November until March for all treatments (Figure 5 B). Specific stolon weight showed very clear differences between varieties during the whole observation period. The specific stolon weight of AberHerald was approximately 1.5 times higher compared to Huia. Stolons in monocultures had significantly higher specific stolon weights than in mixtures. Specific stolon weight was lowest in the first and highest in the third winter.

In general, AberHerald had more stolon dry mass per area than Huia and monocultures higher stolon dry mass than mixtures (Figure 5 C). The stolon dry mass decreased at least from December to February. The percentage from the maximum value of the stolon dry mass decreased from 25 to 50% in 1992/93, depending on the treatment, and was 35% in 1993/94 and 1994/95. This loss was due mainly to the reduction in the specific stolon weight, while stolon length changed only slightly, as described above.
Figure 5 Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on stolon length (A), specific stolon weight (B), stolon dry mass (C) and TNC concentration in stolon dry mass (D) of white clover during three winters. Mean values of four replicates; error bars=standard error of mean.
The concentration of carbohydrates in the stolons showed clear and consistent differences between the varieties. AberHerald had higher total non-structural carbohydrate (TNC) concentrations than Huia (Figure 5 D). Competition with perennial ryegrass reduced carbohydrate concentration. Almost 20 mg TNC/100 mg stolon dry mass of both varieties were lost during the first and second winter. During the third winter, TNC loss was greater for AberHerald than for Huia (14 and 11 mg TNC/100 mg stolon dry mass respectively). These losses correspond to 50% of the initial value in November for AberHerald and 60% for Huia during the first and second winter. During the third winter, the initial TNC concentration of AberHerald and Huia decreased by about 33%.

4.4 Development of the leaves
AberHerald had 20 to 50% larger leaves in April than Huia (Figure 6 A), while differences in leaf size in winter were not significant. In general, the smallest leaves were found in February and the largest in April. The increase (%) in individual leaf size from February to April varied from 300 to 450%, depending on the year and treatment. AberHerald had higher rates of leaf enlargement than Huia from February to April in all three years (+10%, +24% and +7% higher rates in the first, second and third spring respectively). While the increase in leaf size started in March of the second winter, it did not begin until April in the first and third winter.

In monocultures, varieties did not differ in leaf number per terminal bud (Figure 6 B). In mixtures, however, AberHerald tended to have more leaves per terminal bud than Huia. In the first and third winters, the number of leaves per terminal bud was lowest in February (between 0.5 and 1.5), while in the second winter the minimum leaf number was already reached in November (from 0.2 to 0.4). In all three winters, the highest number of leaves per terminal bud was reached in April after regrowth (between 1.5 and 2.5).
Figure 6 Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on leaf area of individual leaves (A), number of leaves per terminal bud (B), leaf area per terminal bud (C) and leaf area index (D) during three winters. Mean values of four replicates; error bars=standard error of mean.
Leaf area per terminal bud was calculated as the size of the individual leaf multiplied by the leaf number per terminal bud. Because of its larger leaves, AberHerald had a greater leaf area per terminal bud than Huia in spring. Leaf area per terminal bud was highest in the third winter and in spring (Figure 6 C). The smallest leaf area per terminal bud in all three winters was found in February due to the small number of leaves per terminal bud and due to the small leaves (Figure 6 A).

Leaf area index is the product of the number of terminal buds per square meter multiplied by leaf area per terminal bud. Leaf area index is a measure of ground cover. Leaf area index of white clover was lowest in February (0.1, 0.3 and 0.7 in the first, second and third years respectively) and highest in April (1.7, 2.4 and 2.6 in the first, second and third years respectively) (Figure 6 D). Leaf area index tended to be higher for AberHerald than for Huia; this was especially pronounced in mixtures. In mixture, AberHerald had greater leaf area indices (30, 55 and 10%) in April than Huia.
5 Discussion

The annual yield of AberHerald was higher in both growing seasons; in the second growing season the spring yield was also higher. AberHerald was much more competitive with perennial ryegrass than was Huia. The yield advantage of AberHerald may be based on its larger individual leaves in spring compared to Huia and/or its higher carbohydrate concentration in the stolons in early spring when regrowth started. Winter damage to stolons or buds was very low for both varieties in all three winters. Thus, winterhardiness of both varieties was high enough for successful overwintering and for high yields in the following season.

5.1 Development of white clover yield and proportion in spring and summer

In both years, AberHerald produced a higher annual yield than Huia; its spring yield was also higher in the second year (Figures 2 and 3). The yield difference between AberHerald and Huia was greater when grown in mixture (+27%) with perennial ryegrass as compared to monocultures (+10%). Throughout the experiment, the proportion of clover in the mixtures was unusually high for Huia (65%) but even more so for AberHerald (85%). AberHerald suppressed ryegrass tillering dramatically and even planting ryegrass plants in spring 1994 could not stop the steady decrease in ryegrass yield and proportion during the experiment. Both, higher yield and a greater proportion of AberHerald, show that AberHerald's main advantage over Huia was a greater competitive ability with perennial ryegrass.

In general, plants compete mainly for light, and yield depends largely on the amount of radiation that can be absorbed by the leaf area of a species (Schwank et al., 1986; Menzi et al., 1991; Carlen, 1994). We suggest that faster regrowth and the greater leaf area of AberHerald in spring are key factors in its better competitive ability as compared to Huia (Figure 6 A). Fothergill (personal communication) also observed a faster leaf area production of AberHerald after each cut. Casal et al. (1987) showed the sensitivity of grasses to changes in
light quality. A reduction in tillering, with small decreases in the R/FR ratio at the base of the stem, was found. Thus, we suggest that the increased leaf area index of AberHerald (Figure 6 D) early in spring led to: a decrease in the R/FR ratio, stronger shading of the perennial ryegrass plants compared to grass plants grown with Huia and, thus, to a suppression of tillering (Davidson and Robson, 1986, Simon, 1989). This negative effect of increased growth of white clover on tiller density was also observed by Hebeisen et al. (1997) and Clark et al. (1997). The competitive advantage of AberHerald over Huia may be based partly on the higher leaf area index and partly on the thicker stolons of AberHerald (Figure 5 B). In our experiment, the stolons in several layers were very dense (up to 200 m/m²; compare Figure 5 A), meaning that thicker stolons may have shaded the base of the stems of the perennial ryegrass which, in turn, suppressed tillering.

Stolon length in all our treatments was more than 100 m per m² in the spring. AberHerald had a similar number of stolons and buds which survived the winter, but its yields were higher than those of Huia. Huia had more buds than AberHerald and a higher ratio of terminal to axillary buds throughout the experiment (Figure 4). Thus, the higher spring and annual yields were not related to the number of stolons and buds which survived the winter, in contrast to other experiments (Harris et al., 1983; Collins et al., 1991). Rhodes (1991) found a linear relationship between stolon length in spring and annual clover yield over a range of stolon densities from approximately 20 m per m² to 100 m per m². Above 100 m per m² stolon length, the response of yield becomes rapidly weaker and differences between varieties are often related to other characters such as leaf size. In fact, in early spring, AberHerald had much larger leaves than Huia (Figure 6 A) which were probably responsible for the yield advantage of AberHerald. Ollerenshaw and Haycock (1984) found a correlation between maximum leaflet length in early spring and maximum leaflet length in late spring. This supports our hypothesis that the larger leaves of AberHerald are a key factor in its competitive advantage over Huia.

Results of our experiment show that the higher yields of AberHerald in summer (both growing seasons) and spring (1994) were achieved at the expense of ryegrass, because the total annual mixture yield (clover and ryegrass) was
similar for both varieties (Figure 2). This is in contrast to the other sites of the European multi-site experiment where the total annual mixture yield was higher for the plots with AberHerald than for those with Huia due to generally higher spring and annual yields of AberHerald compared to Huia and a similar yield of perennial ryegrass in both treatments (Collins et al., 1994; written communication). Perennial ryegrass was probably less affected or not affected by AberHerald at the other sites, though the leaf area index was higher for AberHerald than for Huia, as in our experiment. One explanation may be the much lower clover density at the other sites compared to our experiment. While stolon length in spring was much higher than 100 m per m² in our experiments (Figure 5 A), it varied at the Scandinavian sites and in Nancy from 20 m per m² to 100 m per m² and from 100 m per m² to 180 m per m² in Aberystwyth.

5.2 Regrowth and the implication of carbohydrate reserves

Our results demonstrate that AberHerald produced more leaf area in spring than Huia due to its larger leaves and a similar number of leaves per growing point (Figures 6 A and B). This advantage was confirmed at the other sites of the European multi-site experiment (Collins et al., 1994; written communication).

When regrowth started in spring, the concentration of total non-structural carbohydrate in the stolons was higher for AberHerald than for Huia, especially in the first two years (Figure 5 D). At the same time, AberHerald had a greater leaf area per terminal bud during regrowth due to its larger leaves (Figures 6 D and A). This suggests that the increased regrowth of AberHerald may be related to the greater amount of available carbohydrate reserves. Such a positive relation was shown by Lüscher (1989) in an experiment conducted in a controlled environment. White clover plants with high concentrations of carbohydrates produced more leaf area per bud during regrowth after a severe defoliation but had similar relative growth rates due to a lower number of buds. In a field experiment, where all the leaves died during winter, the spring yield of white clover was very positively correlated to the TNC levels of the plants in autumn but especially so to the levels at the beginning of regrowth (Frankow-Lindberg et al., 1995). A positive effect of higher TNC concentrations in stolons
on the regrowth of severely defoliated white clover plants was also shown by Baur-Höch et al. (1990). However, severely defoliated plants (Lüscher, 1989; Baur-Höch et al., 1990) had only 0.3 leaf stages (Carlson, 1966) left per bud after defoliation, while in our experiment plants started regrowth in spring with at least 0.7 leaf stages (Carlson, 1966) per terminal bud (Figure 6 C). These 0.7 leaf stages (Carlson, 1966) correspond to slightly defoliated plants which were shown to depend to a lesser extent on TNC (Baur-Höch et al., 1990). Thus, we suppose that regrowth in our experiment was less dependent on carbohydrate reserves than was shown for severely defoliated plants and that considerable amounts of carbohydrates might have been available from current photosynthesis of the remaining leaf area.

In addition to the carbohydrate reserves, N reserves may also affect regrowth (Ourry et al., 1990; Kim et al., 1991; Volenec et al., 1996).

A discussion of leaf development during winter and spring and its significance for overwintering and spring growth follows in Part V. While leaf death was similar for both varieties, leaf production was higher for AberHerald due to higher meristematic activity. Plants without leaves were smaller and regrew slower than plants with leaves (Part V).

5.3 Winter survival influenced by climate, variety and competition with perennial ryegrass and the implication of carbohydrate reserves

Climate
Stolon length was reduced by 0 (1994/95) to 20% (1993/94); winter damage to buds was also very low for both varieties. The number of terminal buds even increased during most of the winters, and the number of axillary buds decreased slightly. The development of axillary and terminal buds during winter will be discussed in Part V.

The slight damage in winter can be explained by the rather mild winters. The mean temperatures from November until the end of February in the first (1.8°C) and third (3°C) winters were milder compared with the long-term average (1.4°C) (Table 1). In the second winter, the mean temperatures (0.9°C) were lower than average due to unusually low temperatures in November. Apart from
these low temperatures in the November of the second winter, the lowest minimal temperatures (below -5°C) occurred in January and February and were usually accompanied by snow. Snow-cover protects against freezing temperature; the temperature of the stolons under snow-cover was -1°C when the air temperature (weekly mean) was -10°C (Lüscher, 1989). The LD50 values (lethal temperature for 50% of the plants) were -6.22°C for AberHerald and -4.27°C for Huia in field-grown populations in Aberystwyth, Wales (Collins and Rhodes, 1995), thus supporting low levels of damage because of the mild winters.

Eagles and Othman (1981), Harris et al. (1983) and Collins et al. (1991) found an important decrease of the stolon length (30 to 100%) during the winter. It is suggested that winter damage in these experiments in Aberystwyth was caused by a coincidence of low temperature and strong dehydrating winds without snow-cover (Rhodes et al., 1994). Differences in winter damage were also due to the different varieties of white clover which were tested.

**Variety**

In our experiment both varieties had similar losses; thus, under the climatic conditions of the experimental years, winter-hardiness of both varieties was apparently sufficient for the plants to survive the winter. In the experiments of Eagles and Othman (1981, 1988), varieties of Mediterranean origin were more susceptible to low temperature stress. In the experiment of Ollerenshaw and Haycock (1984), 45 ecotypes of white clover were collected from sites differing in latitude and altitude in Great Britain and Norway and tested for frost tolerance. Unexpectedly, frost-sensitive and frost-tolerant genotypes were found at all the latitudes and altitudes studied. Some genotypes from the most northern latitudes within the Arctic Circle were frost-sensitive after a hardening pre-treatment. Differences in frost tolerance were explained by different climatic conditions at the sites of origin. Frost-sensitive genotypes came from sites with prolonged snow-cover which prevented freezing, while frost-tolerant genotypes were exposed to low temperature stress due to cold winds at their sites of origin. AberHerald originally came from the hilly regions of Zurich with prolonged winters, while Huia is a New Zealand variety that is acclimated to
much milder weather conditions, where the minimum temperature at 20 cm above-ground hardly ever drops below 0°C (Brock et al., 1996).

The survival of AberHerald and Huia in winter, was investigated in the European multi-site experiment under very different climatic conditions. Damage to plants in winter was minimal, and stolon length remained more or less constant at all sites during the winter 1993/94 (Collins et al., 1994). During the winter 1994/95, however, the extent of damage to plants differed from site to site (written communication). While the stolon length in Germany, France and UK was, at most, only slightly, whereas in Finland and Sweden the stolon length of Huia (-75%) and AberHerald (-35%) was drastically reduced.

**Competition with perennial ryegrass**

Loss of stolon dry mass varied in all three winters in our experiment (Figures 5 A and C). The reduction in stolon dry mass (-25 to -50%) was associated with a deterioration of the structural dry mass of stolons during winter and to a decrease in TNC content of the stolons (-55%) (Table 3). Loss of structural dry mass corresponds well with the loss of stolon length (Table 3; Figure 5). The loss of structural dry mass of white clover stolons was zero to -10% in all treatments except during the winter 1992/93 for white clover grown in mixture (-30%).

The rather large reduction of white clover grown in mixture during the first winter was probably due to the effects of competition. The tiller density of perennial ryegrass in winter 1992/93 was twice as high as that of white clover and much higher than during the other winters (Figures 4 C and D). This high density may have led to shading of white clover stolons and leaves, because leaves tend to have a lower position in the sward relative to grasses during winter and spring (Woledge et al., 1990). Shading may have prevented photosynthesis of leaves and stolons and may have induced stolon death. Chapman and Robson (1992) examined the physiological role of old stolon material in white clover. The experiment showed that more stolons died when stolons were covered; this was even more pronounced when plants were defoliated. The authors explained the reaction by the lack of direct assimilation by stolon tissue and by an accentuation of the carbohydrate deficit. Carbohydrate was withdrawn from the
old stolon tissue on day 70 and reallocated to the apex. Old stolon tissue subsequently died. Harris et al. (1983) pointed out the significance of stolon photosynthesis during winter in Aberystwyth, and we suggest that, in our experiment in winter 1992/93 the white clover grown in mixture lost more structural dry mass of stolons than white clover grown in monoculture (Table 3), because stolons and leaves were shaded by perennial ryegrass and white clover suffered from a carbon deficit. This hypothesis is supported by the low TNC content of stolons in mixtures compared to stolons in monocultures (Table 3).

Table 3  Effects of white clover variety and competition with perennial ryegrass on structural dry mass of stolons and TNC content of stolons in autumn and its change during three winters.

<table>
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<td>without AberHerald</td>
<td>87</td>
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<td>207</td>
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<td></td>
<td>Huia</td>
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<td>136</td>
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<tr>
<td>with AberHerald</td>
<td>76</td>
<td>-23</td>
<td>183</td>
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<tr>
<td></td>
<td>Huia</td>
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<td>TNC content of stolons</td>
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<td>without AberHerald</td>
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<td>158</td>
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<td></td>
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<td>with AberHerald</td>
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<td>Huia</td>
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Implication of carbohydrate reserves
The varieties in our experiment in Zurich differed in the highest concentrations of TNC in late autumn (35% - 40% TNC for AberHerald and 30% - 35% TNC for Huia); they also differed in lowest concentrations of TNC: 15% for AberHerald and 10% for Huia at the end of the winter (Figure 5 D). Similar observations were made by Collins and Rhodes (1995) who also found higher TNC
concentrations in autumn and comparable concentrations in spring for AberHerald and Huia. At all sites of the European multi-site experiment, AberHerald had higher TNC concentrations in stolons than Huia in autumn and tendencially in spring. In the experiment of Harris et al. (1983), stolons of different varieties contained about 30% TNC in December and 4 to 7% TNC in spring. In the experiment of Winkler (1984), stolons contained about 30% TNC at the end of the growth period and 9 and 16% TNC in spring when grown at high and low altitudes respectively. This demonstrates that, in our experiment, the TNC concentration of both varieties was relatively high, especially in spring. This supports our suggestion that even the lower TNC concentration of Huia was not limiting for winter survival under the climatic conditions of the experiment, since neither of the varieties showed significant winter damage.

At the Scandinavian sites, however, loss of stolon length was considerable (see above) but lower for AberHerald than for Huia. The difference in the carbohydrate content of the two varieties was probably responsible for the fact that AberHerald suffered less damage in winter (Frankow-Lindberg et al., 1995).

In Finland, plants were covered with snow from the beginning of November until the end of April. Since plants had no opportunity to produce carbohydrates under the snow, their carbohydrate reserves were probably too low to meet their energy demands during this period. Sagisaka (1995) showed that the survival of white clover under snow-cover was dependent on the amount of carbohydrate reserves in autumn and on the basal metabolic rate. When plants had levels lower than 80 μmol hexose equivalents g⁻¹ fresh weight, survival and regrowth was no longer ensured. In the experiment of Sagisaka (1995), plants lost 70% of their initial hexose equivalents during the period with snow-cover; this decrease continued a carbohydrate threshold concentration was reached. Both clover varieties in Finland lost more than 80% of their initial stolon carbohydrate content, suggesting that they reached the carbohydrate threshold concentration and died.

In Sweden winter damage to stolons was probably caused by low temperatures (weekly mean of -10°C in February) and the lack of a protective snow-cover. It is assumed that clover plants were not very tolerant to freezing, probably
because of insufficient carbohydrate concentrations (Frankow-Lindberg, personal communication). The physiological basis of the ability to survive adverse conditions in winter may be related to high concentrations of carbohydrate reserves, which may serve to maintain the metabolism (see above) or to promote freezing tolerance. Collins and Rhodes (1995) found that AberHerald had a lower LD50 value and a higher carbohydrate concentration than Huia. It seems, however, that not only TNC content, but also the concentrations of some specific carbohydrate compounds are responsible for freezing tolerance. While starch decreased, sugars increased with decreasing temperatures in winter (Lüscher, 1989; Li et al., 1996). Sucrose is the most abundant compound of the sugars (Lüscher, 1989; Rosnes et al., 1993). However, differences in freezing tolerance between cold-tolerant and cold-sensitive cultivars of alfalfa were closely associated with the accumulation of the oligosaccharides stachyose and raffinose but not with the levels of sucrose (Castonguay et al., 1995). Pinitol is a compound belonging to the myo-inositol sugars. Baur-Höch (1988) found relatively high concentrations of pinitol in young stolons. Rosnes et al. (1993) examined the development of cold tolerance in white clover in relation to carbohydrate content and suggested that one of the unidentified inositols in the samples may have been pinitol. The role and significance of pinitol is not yet clear.

In addition to the carbohydrate reserves, N reserves may also affect winter-hardiness (Guinchard, 1995, Volonec et al., 1996).

Based on the results of our experiment we consider that the leaves of white clover, especially leaf size, are important for overwintering and spring growth as well as for the competitive ability in spring. Winterhardiness was less important under the climatic conditions of the experimental years.
V LEAF DEVELOPMENT AND ITS SIGNIFICANCE FOR THE PERFORMANCE OF WHITE CLOVER DURING WINTER AND SPRING - EFFECTS OF TEMPERATURE, VARIETY, COMPETITION AND DEFOLIATION

1 Summary

In experiments in the field and in a controlled environment, the leaf development of individual plants of two white clover varieties (AberHerald and Huia), grown without and with competition from perennial ryegrass \((Lolium perenne \text{ L.})\), was examined. Our aim was to test the temperature-dependence of leaf development and the leaf turnover during winter and spring and to determine whether the higher yielding variety exhibits enhanced leaf development at low temperature. In addition, we continuously defoliated half of the plants during winter to test the hypothesis that leaves are important for successful overwintering and spring growth. Our results are:

1) Leaf emergence was found to be very temperature-dependent and continued, to a small extent, throughout the winter. AberHerald produced 20% more leaves on the main stolon during winter than Huia, probably due to higher meristematic activity (tested in a controlled environment). Competition with perennial ryegrass and a lack of leaves reduced leaf emergence by about 30%.

2) Leaf number on the main stolon was at least 30% higher for AberHerald than for Huia, and plants grown without competition had about 40% more leaves than plants grown with perennial ryegrass.

3) The leaves of AberHerald were about 40% bigger than those of Huia in early and late spring. Plants grown without competition had bigger leaves in early (40%) and late (15%) spring. The defoliation treatment considerably reduced leaf size. Undefoliated plants had leaves 350% larger than defoliated plants.
4) Defoliated plants lost 90% of their carbohydrate reserves during winter, while undefoliated plants even showed an increase (+50%) in carbohydrate reserves.

5) Defoliation induced the death of buds and nodes, while undefoliated plants retained their buds and nodes.

We clearly demonstrated the significance of the leaf area during winter and early spring. Thus, we conclude that the increased leaf area of Aber-Herald was its main advantage over Huia.
2 Introduction

One of the major problems related to the management of grass-clover mixtures is the fluctuating yield of white clover (*Trifolium repens* L.) during and between growing seasons. A low proportion of white clover is observed especially in spring. It is suggested that processes during winter and spring, such as death of stolons and buds as well as slow regrowth at cool temperatures, explain, in part, the poor performance of white clover in spring. White clover seems to be at a competitive disadvantage, because the grasses have a lower threshold temperature for growth (Mitchell and Lucanus, 1960). An improvement in growth at low temperature may, thus, increase the competitive ability of white clover against the companion grass (Davidson et al., 1986).

Based on the results of the European multi-site experiment (COST 814), we considered factors which influence the development of leaves, in particular leaf size, to be important for overwintering and spring growth of white clover under the climatic conditions of the experimental years. However, the turnover and function of leaves during winter and spring are not fully understood.

High rates of leaf death, probably related to insufficient cold acclimation, occurred at the beginning of the winter, when leaves are exposed to frost during the night (Guinchard, 1995; Guinchard et al., 1997). Substantial losses of leaves of a wide range of natural genotypes were also observed in late spring (Ollerenshaw and Haycock, 1984). But in winter, leaves lived longest: they survived up to four months (Chapman et al., 1984).

Leaf production of white clover is very temperature-dependent, being lowest at low temperatures (Sackville Hamilton and Harper, 1989). Davies and Evans (1982) found that 0.02 leaves emerged per week in Aberystwyth, near the coast (mean winter temperature: 6°C). In Nancy, with a mean temperature of 3.5°C during winter, 0.01 leaves emerged per week (Guinchard, 1995). Winters in Zurich are even colder; nevertheless, our results of the second winter in the European multi-site experiment demonstrated that leaf production did occur. In November nearly all the leaves were killed by frost; leaves then increased in number until February (the mean temperature from December to February was
1°C). Under controlled conditions, Haycock (1981) found the threshold temperature for leaf emergence to be 2.6°C.

The growth of genotypes of white clover at low temperature varies widely (Ollerenshaw and Haycock, 1984). The combination of fast growth at low temperature and winterhardiness is important for maintaining white clover plants throughout winter and spring. The variety AberHerald, tested in Wales, seemed to possess this combination under a wide range of climatic conditions (Collins and Rhodes, 1989; Collins et al. 1991; European multi-site experiment). In the European multi-site experiment, AberHerald survived the winter well and had high spring yields, which may have been due to larger individual leaves in early spring. Thus, growth at low temperature, especially leaf enlargement, seemed to be very important for the competitive ability of AberHerald. Unfortunately, an increase in this character was not observed until rather late in spring (beginning of April) when the temperature exceeded 9°C (Lüscher, 1989). Under controlled conditions, Haycock (1981) found the threshold temperature for an increase in leaf size to be 6.3°C.

The main function of leaves is photosynthesis. Woledge and Dennis (1982) and Woledge et al. (1989) showed that white clover leaves also photosynthesise at low temperatures and during winter, though only at a low rate due to the low irradiance in winter. These were short-term measurements, but we suggest that plants with leaves meet some of their carbohydrate requirements during winter through photosynthesis if they are not covered by snow. Under the climatic conditions of the experimental years, leaves may be able to photosynthesise during the winter. The white clover plants in the European multi-site experiment retained some of their leaves, and buds always had more than one leaf except sometimes during the second winter.

Newton and Hay (1996) showed that leaves guaranteed the viability of axillary buds in the meristem. When the main stolons had less than one leaf for a period of five weeks, the viability of the axillary buds in the meristem was affected. During harsh winters, when the number of leaves is low due to leaf death and slow leaf production, we expect damage to the axillary buds and, hence, a long-term decrease in the branching rate. However, detailed
measurements of the turnover of buds and the significance of leaves for the
development of these buds and for the carbohydrate budget of the whole plant
during winter have not been made. In addition, the turnover of leaves and leaf
development during winter and spring have not been investigated.

In the experiments described below, the leaf development of individual plants of
two white clover varieties, grown without and with competition from perennial
ryegrass (*Lolium perenne* L.), was examined at low temperature in winter/spring
and in a controlled environment. We wanted to test the temperature-
dependence of leaf development and leaf turnover during winter and spring and
to determine whether the higher yielding variety exhibits enhanced leaf
development and whether the latter is due to higher meristematic activity. In the
field, we continuously defoliated half of the plants during winter to test the
hypothesis that leaves are important for successful overwintering and early
spring growth.

Leaf emergence was found to be very temperature-dependent, and leaf
development was faster for AberHerald than for Huia over the whole range of
low temperatures. Leaf size and leaf area per bud were larger for AberHerald
than for Huia during winter and early spring. We suggest that this was the key
advantage of AberHerald. Plants with leaves showed lower death rates and
higher emergence rates of buds and nodes compared to plants without leaves,
probably due to the increased availability of carbohydrates.
3 Material and Methods

3.1 Experiments in the field

The site and soil of the field experiments (winter 1992/93, 1993/94 and 1994/95), were as described in Part IV. Air temperature was monitored 20 cm above the ground, using the same sensors as described in Part IV. Daily mean values were used to calculate the mean temperature between observation dates.

In 1992/93 and 1993/94, the factorial experiments included four treatments and twelve or ten replicates respectively. The four treatments resulted from the combination of the two white clover varieties, AberHerald and Huia, grown without or with competition from perennial ryegrass (*L. perenne*). In 1994/95, eight treatments and ten replicates were established. The treatments resulted from the combination of the two varieties, grown without and with competition and defoliation. Defoliation was carried out by cutting off all leaves larger than 0.5 Carlson stages (Carlson, 1966) seven times throughout the winter (Table 4).

In 1992/93, 36 plots, 4.2 m long and 1.8 m wide, including a border of 0.6 m on each side, with five plants per plot, were established. In 1993/94, 40 plots had one plant each, and in 1994/95 the same plots had five plants each. The plots measured 1 m x 1 m and had a border of 0.2 m on each side in both years.

In 1992/93, white clover plants were sown in 'quick pots' at the end of May 1992. In 1993/94 and 1994/95, white clover plants were grown from cuttings which were sampled in the plots of the European multi-site experiment (Part IV) in mid-August. After propagating for one month in the greenhouse (18/13°C day/night temperatures and a 16 h photoperiod), white clover plants had two unfolded leaves. The plants were then transplanted to the plots without vegetation (without competition) or to plots with established perennial ryegrass swards (with competition) at a distance of 0.6 m x 0.6 m from each other. After stolon elongation and nodal rooting had taken place at the end of August in 1992, one rooted stolon with ten nodes was cut from each parent plant and left in the plots. Parent plants were removed.
Perennial ryegrass swards were sown with 20 kg (ha)\(^{-1}\) ryegrass (cv. Préférence) at the end of June 1992 and 1993 respectively. In 1994/95, plots with perennial ryegrass were the same as in 1993/94. The ryegrass was cut at a height of 10 cm three times during the summers of 1992 and 1993 and four times during the summer of 1994.

Plots without competition were kept free of other plants by treating them with 1.2% Roundup two weeks before the white clover plants were planted and by weeding throughout the experimental period.

Plots were fertilized in spring with 90 kg P\(_2\)O\(_5\) (ha)\(^{-1}\) and 180 kg K\(_2\)O (ha)\(^{-1}\) applied as Foskal (15% P / 30% K) before starting the experiment. Nitrogen fertilizer was applied as ammonium-nitrate (27.5% N). After sowing the grass and at the beginning of each regrowth period, nitrogen was applied at a rate of 25 kg N ha\(^{-1}\) in summer and at a rate of 60 kg N ha\(^{-1}\) in the early spring of the following year.

### 3.2 The experiment in a controlled environment

The objective of the experiment in a controlled environment was to compare temperature-sensitivity of two white clover varieties. The experiment comprised four treatments and 12 replicates. The four treatments were the combination of the two white clover varieties, AberHerald and Huia, and two temperature levels: 5/3°C and 10/3°C (day/night). The temperature 1 cm below the surface of the sand during the day was 5 °C higher than the air temperature in the growth chamber (heating of the lamps).

Cuttings of *T. repens* (AberHerald and Huia) were taken from cloned stock plants and propagated in sand in a controlled environment (PGV 33 Conviron Instruments Co., Winnipeg, Canada): 18/13°C (day/night temperatures), 16 h photoperiod and increasing photonradiation (\(E_P\)) (from 100 to 350 \(\mu\)mol m\(^{-2}\)s\(^{-1}\)). After two weeks the plants were transplanted to the final pots (40 cm x 17.5 cm x 12.5 cm) and the photoperiod was reduced to 12 h. The 72 pots contained two plants each.

After beginning the temperature treatment, the experiment lasted 39 days at a photonradiation of 465 \(\mu\)mol m\(^{-2}\)s\(^{-1}\).
Nutrients were supplied to all the plants during the first two weeks three times a day in the form of 0.5 l nutrient solution (Hammer et al., 1978) with Sequestrene (NaFe 13 %) and 3.75 mM nitrate and thereafter, twice a day, with 5 l nutrient solution containing 7.5 mM nitrate.

3.3 Data collection

3.3.1 Sampling of the whole plant in the field

Four times during the winter of 1992/93, once in spring 1994 and three times during the winter of 1994/95 (Table 4), the white clover plants were carefully removed from the soil and washed. In 1992/93 and 1993/94, the plants were divided into main and lateral stolons and in 1994/95 into main, primary and secondary stolons. The number of nodes, axillary and terminal buds were recorded separately. The definition of axillary and terminal buds was the same as described for the European multi-site experiment in Part IV. In 1992/93 and 1994/95, we counted the number of clover laminae (in Carlson stages; Carlson, 1966) and measured the lamina area of white clover with the area meter (Li-3000, Li-Cor, Lincoln, Nebraska, USA). The individual leaf area was calculated by dividing the leaf area of the plant by the number of leaves. In addition, the dry mass of the stolons was recorded after drying in an oven at 60°C for 48 h during the third year.

Table 4 Dates of sampling and observation during the winter and spring of 1992/93, 1993/94 and 1994/95 and dates of defoliation during winter 1994/95.

<table>
<thead>
<tr>
<th>1992/93</th>
<th>1993/94</th>
<th>1994/95</th>
</tr>
</thead>
<tbody>
<tr>
<td>sampling</td>
<td>observation</td>
<td>sampling</td>
</tr>
<tr>
<td>19 Oct.</td>
<td>8 Oct.</td>
<td>18 Apr.</td>
</tr>
<tr>
<td>29 Mar.</td>
<td>16 Nov.</td>
<td></td>
</tr>
<tr>
<td>21 Jan.</td>
<td></td>
<td>15 Mar.</td>
</tr>
<tr>
<td>12 Mar.</td>
<td></td>
<td>7 Apr.</td>
</tr>
<tr>
<td>19 Mar.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Apr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 Apr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 Apr.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.2 Observations during winter and spring

At the beginning of each winter one plant per treatment was selected at random for the observations. Eleven observations were made in 1992/93, seven in 1993/94 and three in 1994/95 (Table 4). In 1992/93 and 1993/94, we monitored the death or the developmental stage of each leaf on the main stolon according to the Carlson stages (Carlson, 1966). In addition, leaf development in 1993/94 was monitored on two lateral stolons with two or three nodes in autumn in the same manner as on main stolons. In 1992/93 and 1993/94, leaf production between two observation periods was calculated by adding the grown Carlson stages (Carlson, 1966) of each leaf. In 1994/95, observations were made on the whole plants and we monitored the emergence, death and branching of each node.

In spring 1994, leaflet length and width of the first fully expanded leaf on the main and primary stolons were monitored. Leaf size was calculated by multiplying the formula that estimates the area of an ellipse (0.5 length x 0.5 width * \( \pi \)) by three (for three leaflets).

Leaf emergence between two observation dates was calculated by adding the increase in Carlson stages (Carlson, 1966) of the youngest leaf and, if present, the Carlson stage of a new leaf. Rates of leaf emergence were calculated per week and related to the mean temperature of the corresponding period.

3.3.3 Observations in a controlled environment

Twelve observations were made during the experimental period (39 days). The nodes were counted, and branching of the main stolon was noted. Buds present at the beginning of the experiment, which had not develop by the end, were termed dormant. Average leaf emergence was determined over a period of 39 days.

3.3.4 Dissection of the stolon tip and staining of condensed chromosomes

We counted the leaf primordia and the dividing meristematic cells on six main apices at four harvests. The presented results are means of all four harvests. For the dissection, all visible leaves at Carlson stages 0.1 until 1 (Carlson,
1966) were cut off. Then we carefully removed and counted all trifoliate leaf primordia, one by one, until the apex became visible, as illustrated by Thomas (1980) and Denne (1966).

Dissected stolon tips with an intact apex were cut to a length of 2 mm and placed in an ethanol-ice-acid solution (3:1) for 12 to 24 hours for fixation. Apices were then cut off the other plant tissue and put into Orcein-acetic-acid for 12 hours to stain the condensed chromosomes in the dividing cells. Staining was improved by heating the apices to 60 °C for 5 minutes. Cells with stained chromosomes (prophase, metaphase and anaphase) were counted in 45% acetic acid with the aid of a microscope (Zeiss Axioplan, Ocular PI 10x / 25, Object Plan-Neofluar 10x / 0.75) at 400 fold magnification.

3.3.5 Determination of carbohydrate reserves in the stolons

Carbohydrates were analysed for all three destructive harvests in 1994/95. Oven-dried stolon fractions were mixed, ground to powder and analysed according to the method described in Part IV.

3.3.6 Statistics

The statistical analyses were carried out with the computer program SAS (Statistical Analysis System, SAS Institute, Cary, North Carolina, USA). Analyses of variance were carried out according to the General Linear Model procedure. T tests were used to estimate significant differences among treatment means.

Linear regression analyses were carried out to calculate the relationship between temperature and the rate of leaf emergence for each treatment. Slopes of the regressions were compared using the Student’s t Test, as described by Zar (1984).
4 Results

4.1 Development of the number of clover buds, the number of nodes and the percentage of branched nodes

In the field experiments, the number of axillary and terminal buds of the varieties in autumn did not differ, but white clover plants grown without competition had 180% more buds (total) than plants grown with competition (Figure 7). Significant changes in the number of buds were found in all three field experiments and in all treatments until early and late spring (Figure 7). Net increases in the number of axillary buds of 50% and as high as 300% in the number of terminal buds were observed in all three experiments; half of the increase had occurred by early spring and half by late spring. AberHerald (+80%) showed a higher net increase in the number of axillary buds compared to Huia (+20%), while the net increase in the number of terminal buds was similar for both varieties (+300%). Plants grown without competition showed a clear net increase of 130% in the number of axillary buds and of 350% in the number of terminal buds, while plants grown with competition showed a net decrease in the number of axillary buds of 30% and a net increase in the number of terminal buds of 250%. Defoliated plants (-30%) showed a higher net decrease in the number of axillary buds compared to plants without defoliation (-10%). The net increase in the number of terminal buds was much smaller for defoliated plants (+160%) than for undefoliated plants (+440%). The differences between treatments in the net changes of the buds were due to different rates of bud emergence and bud death in 1994/95. The rate of bud emergence as a percentage of the number of buds present in autumn tended to be higher for AberHerald (73%) than for Huia (64%) (Figure 7). Plants grown without competition (84%) showed a significantly higher rate of bud emergence than plants grown with competition (54%). Defoliated plants (45%) had a much lower rate of bud emergence than undefoliated plants (93%) (Figure 7). The rate of bud death as a percentage of the number of buds present in autumn tended to be higher for Huia (31%) than for AberHerald (22%) and similar for plants grown without and with competition. Defoliated plants (48%) suffered a much higher rate of bud death than undefoliated plants (5%).
Figure 7 Effects of white clover variety, competition with perennial ryegrass and continuous defoliation on the number of axillary (■) and terminal (▲) buds and on cumulative bud emergence (▲) and bud death (■) of individual plants during three winters. Mean values of 12 (1992/93) and 10 (1993/94, 1994/95) replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th>Year</th>
<th>SE / BUDS</th>
<th>axillary</th>
<th>terminal</th>
<th>emergence</th>
<th>death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992/93</td>
<td>4.6</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1993/94</td>
<td>1.1</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1994/95</td>
<td>0.5</td>
<td>1.3</td>
<td>1.7</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>
The proportion of axillary buds to terminal buds was lower from autumn (1.17) to spring (0.48) in all three experiments (Figure 7). In spring, this proportion was higher for plants grown without competition (0.52) than for plants grown with competition (0.27). The proportion of defoliated plants (0.13) to undefoliated plants (0.17) was lower in spring.

In autumn, the total number of nodes of field-grown plants did not differ between varieties, but white clover plants grown without competition had a higher number of nodes (+50%) than plants grown with competition (Figure 8). The number of nodes showed a net increase of 290% from autumn to late spring in all three field experiments (Figure 8). While in 1992/93 the main net increase in the number of nodes occurred from early to late spring, half the net increase in 1994/95 occurred by early spring and the rest by late spring. Both varieties showed a similar net increase in the number of nodes, but plants grown without competition (380%) increased the number of nodes to a greater extent than plants grown with competition (200%). Defoliated plants (110%) showed a smaller net increase in the number of nodes than undefoliated plants (310%).

In 1994/95 the rate of node emergence as a percentage of the number of nodes present in autumn was similar for both varieties, but plants grown without competition (171%) showed a higher rate of node emergence than plants grown with competition (99%) (Figure 8). Defoliated plants (83%) showed a much lower rate of node emergence than undefoliated plants (187%). The rate of node death as a percentage of the number of nodes present in autumn tended to be higher (p=0.06) for Huia (31%) than for AberHerald (17%) as well as for plants grown without competition (31%) compared to plants grown with competition (17%). Defoliated plants (44%) had much higher rates of node death than undefoliated plants (4%).
Figure 8  Effects of white clover variety, competition with perennial ryegrass and continuous defoliation on the number of nodes (total [ ], on the main [ ], primary [ ] and secondary [ ] stolons) and on cumulative node emergence ( [ ] ) and node death ( [ ] ) of individual plants in winter 1992/93, 1993/94 and 1994/95. Mean values of 12 (1992/93) and 10 (1993/94, 1994/95) replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th></th>
<th>SE / NODES</th>
<th>total</th>
<th>emergence</th>
<th>death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992/93</td>
<td>22.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1993/94</td>
<td>6.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1994/95</td>
<td>6.7</td>
<td>7.9</td>
<td></td>
<td>4.2</td>
</tr>
</tbody>
</table>
The branching rate (Table 5) decreased in all three field experiments during winter by 50% in 1992/93, 23% in 1993/94 and 34% in 1994/95. While varieties had the same percentage of branched nodes, plants grown without competition (35%) had a higher percentage of branched nodes than plants grown with competition (23%).

Table 5  Effects of white clover variety, competition with perennial ryegrass and continuous defoliation on the percentage of branched nodes of individual plants in winter 1992/93, 1993/94 and 1994/95. Mean values of 12 (1992/93) and 10 (1993/94;1994/95) replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th>Date of harvest</th>
<th>AberHerald without competition</th>
<th>Huia without competition</th>
<th>AberHerald with competition</th>
<th>Huia with competition</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992/93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 Oct.</td>
<td>55</td>
<td>74</td>
<td>51</td>
<td>48</td>
<td>7</td>
</tr>
<tr>
<td>9 Mar.</td>
<td>60</td>
<td>66</td>
<td>43</td>
<td>46</td>
<td>6</td>
</tr>
<tr>
<td>20 Apr.</td>
<td>37</td>
<td>40</td>
<td>18</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>1993/94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 Oct.</td>
<td>35</td>
<td>35</td>
<td>18</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>18 Apr.</td>
<td>26</td>
<td>25</td>
<td>16</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>1994/95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Nov.</td>
<td>30</td>
<td>30</td>
<td>16</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>13 Mar.</td>
<td>25</td>
<td>24</td>
<td>17</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>20 Apr.</td>
<td>20</td>
<td>20</td>
<td>14</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>1994/95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Nov.</td>
<td>30</td>
<td>30</td>
<td>16</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>13 Mar.</td>
<td>23</td>
<td>23</td>
<td>10</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>20 Apr.</td>
<td>17</td>
<td>14</td>
<td>17</td>
<td>11</td>
<td>2</td>
</tr>
</tbody>
</table>

Experiment in a controlled environment

In the experiment in a controlled environment, the number of buds and the percentage of branched nodes were similar at both temperatures for both varieties, as observed in the field experiments. However, 30% of the buds of AberHerald and only 5% of the buds of Huia were dormant. Excluding these buds, AberHerald had a smaller percentage of branched nodes than Huia (-30%) (Table 6).
Table 6  Effects of white clover variety and low temperature on the number of buds and on the percentage of branched nodes of individual plants in a controlled environment. Mean values of 12 replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th>temperature [°C]</th>
<th>variety</th>
<th>Number of buds</th>
<th>Percentage of branched nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no./plant</td>
<td>total</td>
</tr>
<tr>
<td>10/3</td>
<td>AberHerald</td>
<td>13.6</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>11.1</td>
<td>0.1</td>
</tr>
<tr>
<td>5/3</td>
<td>AberHerald</td>
<td>11.4</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>10.0</td>
<td>0.8</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>0.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

4.2 Development of the stolons and their carbohydrate concentrations

While undefoliated plants increased stolon dry mass (Table 7) by 250%, defoliated plants decreased stolon dry mass by -30% during the winter. At the end of the experiment defoliated plants had only 20% of the stolon dry mass of undefoliated plants. This is a very considerable effect, because the stolon dry mass of the two defoliation treatments was the same in November.

Table 7  Effects of white clover variety, competition with perennial ryegrass and continuous defoliation on stolon dry mass of individual white clover plants in winter and spring 1994/95. Mean values of ten replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th>Stolon dry mass [g/plant]</th>
<th>3 Nov. 94</th>
<th>13 Mar. 95</th>
<th>20 Apr. 95</th>
</tr>
</thead>
<tbody>
<tr>
<td>competition   variety</td>
<td>without</td>
<td>with</td>
<td>without</td>
</tr>
<tr>
<td>without        AberHerald</td>
<td>0.223</td>
<td>0.174</td>
<td>1.007</td>
</tr>
<tr>
<td>Huia</td>
<td>0.135</td>
<td>0.125</td>
<td>0.765</td>
</tr>
<tr>
<td>with           AberHerald</td>
<td>0.177</td>
<td>0.106</td>
<td>0.302</td>
</tr>
<tr>
<td>Huia</td>
<td>0.102</td>
<td>0.065</td>
<td>0.237</td>
</tr>
<tr>
<td>SE</td>
<td>0.010</td>
<td>0.020</td>
<td>0.048</td>
</tr>
</tbody>
</table>
AberHerald had a consistently higher stolon dry mass than Huia. At the beginning of the experiment, the stolon dry mass of AberHerald was 50% higher than Huia's; at the end of the experiment this value was 30%. The treatment with competition had a large effect on stolon dry mass: at the end of the experiment plants grown with competition had 65% less stolon dry mass compared to plants grown without competition.

TNC concentration of stolons decreased from autumn to spring (Figure 9). AberHerald had a higher TNC concentration than Huia at every harvest. Plants grown with competition had a higher TNC concentration at the beginning of the experiment but a lower TNC concentration at the end of the experiment than plants grown without competition. The difference in the TNC concentration between undefoliated plants and defoliated plants was 15 and 8 mg TNC/100 mg stolon dry mass respectively in the middle of March and April.

![Figure 9](image)

**Figure 9** Effects of white clover variety, competition with perennial ryegrass and continuous defoliation (without (A) and with (B)) on TNC concentration of the stolons of individual plants in winter 1994/95. Mean values of ten replicates; error bars=standard error of mean.
The calculation of absolute values of mg TNC in stolons per plant showed that undefoliated plants grown without competition increased their TNC concentration during the experiment (in percent of autumn values): +50% by the middle of March and +230% for AberHerald and +140% Huia by 20 April. Undefoliated plants grown with competition showed a reduction of 45% in TNC content. Defoliated plants showed up to a 90% reduction in the TNC content compared to the beginning of the experiment.

4.3 Development of leaves
4.3.1 Main stolon

During the experimental periods in 1992/93 and 1993/94 AberHerald had more leaves on the main stolon than Huia (Figure 10). When the number of leaves was smallest in January 1993 and in December 1993, AberHerald had 30% more leaves than Huia. Plants grown without competition had 40% more leaves in autumn than plants grown with competition, 55% more leaves in winter when the leaf number was lowest and 35% more leaves in spring.

In the experiments in winter 1992/1993 and 1993/1994, the varieties and competition treatments had a significant effect on the cumulative number of emerged leaves on the main stolon. AberHerald produced about 20% more leaves than Huia, and plants grown without competition produced about 28% more leaves than those grown with competition. In the experiment in winter 1994/1995, node production on the main stolon showed comparable results (data not shown). During the winters 1992/93 and 1993/94, the main stolon of white clover plants produced fewer leaves at the beginning of December 1992 and November 1993, increasing leaf production in mid-March in 1993 and at the end of February in 1994 (Figure 10).
Figure 10 Effects of white clover variety and competition with perennial ryegrass on the number of leaves (— ), the cumulative number of emerged (— ) and of dead (···) leaves on the main stolon in winter 1992/93 and 1993/94. Mean values of 12 (1992/93) and 10 (1993/94) replicates; error bars=standard error of mean.
High rates of leaf death usually occurred until the end of January in winter 1992/93 and until the end of December in winter 1993/94 (Figure 10). Calculated as a percentage of the leaves in autumn, the rate of leaf death was higher in winter 1992/93 (147%) than in winter 1993/1994 (127%). Values above 100% indicate leaves which were produced after autumn died.

The accumulated number of dead leaves tended to be higher for AberHerald than for Huia; calculated as a percentage of the leaves in autumn, the death rate of the varieties was similar (137%). Plants grown without competition lost 35% more leaves than those grown with competition; calculated as percentage of the leaves in autumn, the death rate was higher for plants grown with competition (145%) than for plants grown without competition.

### 4.3.2 Lateral stolons

In winter 1993/94 (Figure 11) and 1994/95 (Table 8), the varieties had a similar number of leaves on the lateral stolons. However, plants grown with competition had 25% fewer leaves per lateral stolon in spring than plants grown without competition.

**Table 8** Effects of white clover variety, competition with perennial ryegrass and continuous defoliation of individual plants on the number of leaves per lateral stolon in winter 1994/95.

<table>
<thead>
<tr>
<th>competition</th>
<th>variety</th>
<th>Number of leaves [no./lateral stolon]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 Nov. 94</td>
</tr>
<tr>
<td>without</td>
<td>AberHerald</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>2.58</td>
</tr>
<tr>
<td>with</td>
<td>AberHerald</td>
<td>3.98</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>2.84</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>0.36</td>
</tr>
</tbody>
</table>
We observed a significant variety x competition interaction in leaf production on lateral stolons in winter 1993/94. Compared to AberHerald, Huia produced 4% more leaves when grown without competition and 16% fewer leaves when grown with competition (Figure 11).

![Figure 11](image)

**Figure 11** Effects of white clover variety and competition with perennial ryegrass on the number of leaves (—), the cumulative number of emerged (——) and dead (-----) leaves on a lateral stolon in winter 1993/94. Mean values of ten replicates; error bars=standard error of mean.

Node emergence (Table 9) per lateral stolon in winter 1994/95 was similar for both varieties. Plants grown without competition produced 14% more nodes than plants grown with competition. Defoliated plants produced 30% fewer nodes than undefoliated plants. We observed a significant variety x defoliation interaction in node emergence from 2 December to 3 February. Huia produced 34% more nodes when undefoliated and 15% fewer nodes when defoliated than AberHerald.
Table 9  Effects of white clover variety, competition with perennial ryegrass and continuous defoliation of individual plants on node emergence on lateral stolons in winter 1994/95. Mean values of ten replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>without</td>
<td>with</td>
</tr>
<tr>
<td>without</td>
<td>AberHerald</td>
<td>0.76</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>1.04</td>
<td>0.59</td>
</tr>
<tr>
<td>with</td>
<td>AberHerald</td>
<td>0.68</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>0.89</td>
<td>0.49</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

4.3.3 Development of leaf size in spring
The individual leaf area was smallest in March in all three experiments. In general, AberHerald had larger leaves than Huia in early and late spring. In 1993/94, the variety difference was 55% in early spring and 70% in late spring, determined by measuring the youngest fully unfolded leaf of the main and of the lateral stolons. In 1992/93 and 1994/95, the individual leaf area was 20% larger for AberHerald than for Huia in early and late spring (mean of all leaves). Plants grown without competition had 60% larger leaves than plants grown with competition in 1993/94 and 25% larger leaves in 1992/93 and 1994/95 in early spring (Table 10). In late spring this difference was reduced to 25% in 1993/94 and to 7% in 1992/93 and 1994/95. The defoliation treatment considerably reduced the individual leaf area. Undefoliated plants had 350% larger leaves than defoliated plants.
Differences in leaf size between years were due to different measuring techniques which included all leaves in spring 1993 and 1995, but only the youngest fully unfolded leaves in spring 1994. Treatment effects were not affected by the different measuring techniques.
Table 10  Effects of white clover variety, competition with perennial ryegrass and continuous defoliation of individual plants on the individual leaf area in spring 1993 and 1995 (mean of all leaves (mlf)) and in spring 1994 (youngest unfolded leaf (ylf)).

Mean values of 12 (1992/93) and 10 (1993/94) replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th>Date of harvest</th>
<th>AberHerald</th>
<th>Huia</th>
<th>AberHerald</th>
<th>Huia</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1993 (mlf)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Mar.</td>
<td>1.22</td>
<td>1.09</td>
<td>0.92</td>
<td>0.79</td>
<td>0.09</td>
</tr>
<tr>
<td>29 Mar.</td>
<td>1.81</td>
<td>1.37</td>
<td>1.45</td>
<td>1.07</td>
<td>0.12</td>
</tr>
<tr>
<td>20 Apr.</td>
<td>3.44</td>
<td>3.12</td>
<td>2.87</td>
<td>2.41</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>1994 (ylf) (main stolon)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 Mar.</td>
<td>6.00</td>
<td>3.64</td>
<td>3.41</td>
<td>2.33</td>
<td>0.77</td>
</tr>
<tr>
<td>7 Apr.</td>
<td>12.82</td>
<td>7.65</td>
<td>8.43</td>
<td>4.60</td>
<td>1.07</td>
</tr>
<tr>
<td><strong>1994 (ylf) (lateral stolon)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 Mar.</td>
<td>5.25</td>
<td>3.70</td>
<td>3.50</td>
<td>1.91</td>
<td>0.50</td>
</tr>
<tr>
<td>7 Apr.</td>
<td>12.09</td>
<td>7.55</td>
<td>10.12</td>
<td>5.76</td>
<td>1.69</td>
</tr>
<tr>
<td><strong>1995 (mlf)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 Mar.</td>
<td>1.20</td>
<td>0.91</td>
<td>0.94</td>
<td>1.34</td>
<td>0.07</td>
</tr>
<tr>
<td>20 Apr.</td>
<td>2.99</td>
<td>2.50</td>
<td>2.21</td>
<td>2.45</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>1995 (mlf)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 Mar.</td>
<td>0.41</td>
<td>0.28</td>
<td>0.32</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>20 Apr.</td>
<td>0.59</td>
<td>0.27</td>
<td>1.02</td>
<td>0.13</td>
<td>0.14</td>
</tr>
</tbody>
</table>

4.3.4 Rate of leaf emergence in relation to temperature

The rate of leaf emergence depended strongly on temperature (Figure 12; Table 11, R^2>0.92). Over the whole range of temperatures, AberHerald had 20% and plants grown without competition 30% higher rates of leaf emergence on the main stolon than Huia and plants grown with competition. In a controlled environment at 12.5°C day temperature, the rates of leaf emergence on the main stolon were 0.79 and 0.58 leaves per week for AberHerald and Huia respectively and 0.70 and 0.60 leaves per week on lateral stolons.
In contrast, the rate of leaf emergence on lateral stolons in the field was similar for both varieties (data not shown). However, plants grown without competition had 30% higher rates of leaf emergence on lateral stolons than plants grown with competition. Comparing the regression coefficients of the curves, AberHerald had higher rates of leaf emergence than Huia. This effect was significant when the plants were grown with competition and was still evident when plants were grown without competition but was not significant. The increase in the rate of leaf emergence per temperature increment was significantly higher for plants grown without competition than for plants grown with competition. The mean threshold temperature for leaf emergence was 2.9°C (2.3°C - 3.3°C) for all treatments.

Figure 12 Effects of white clover variety and competition with perennial ryegrass on the temperature dependence of leaf emergence on the main stolon in winter 1992/93 and 1993/94. Mean values of 12 (1992/93) and 10 (1993/94) replicates; error bars=standard error of mean.
Table 11  Effects of white clover variety and competition with perennial ryegrass on regression equations and correlation coefficients of the relation of leaf emergence and temperature on the main stolon in winter 1992/93 and 1993/94. Linear regression; \( y = \text{leaf emergence [leaves/week]} \); \( x = \text{temperature (mean day temperature at 20 cm height [°C])} \); \( n = 7 \).

<table>
<thead>
<tr>
<th>competition</th>
<th>variety</th>
<th>equation for the fitted curve</th>
<th>( r^2 )</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>without</td>
<td>AberHerald</td>
<td>( y = 0.092 \pm 0.006 x - 0.266 \pm 0.066 )</td>
<td>0.98</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>( y = 0.076 \pm 0.005 x - 0.249 \pm 0.058 )</td>
<td>0.98</td>
<td>0.0001</td>
</tr>
<tr>
<td>with</td>
<td>AberHerald</td>
<td>( y = 0.065 \pm 0.007 x - 0.208 \pm 0.076 )</td>
<td>0.95</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>( y = 0.044 \pm 0.006 x - 0.099 \pm 0.065 )</td>
<td>0.92</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

4.4 Number of leaf primordia and of dividing cells in the meristem

Table 12  Effects of white clover variety and temperature on the number of leaf primordia and the number of dividing cells in a controlled environment. Means of 4 harvests and 12 replicates; SE=standard error of mean.

<table>
<thead>
<tr>
<th>temperature [°C]</th>
<th>variety</th>
<th>Leaf primordia [no./main stolon]</th>
<th>dividing cells in the meristematic zone [no./main stolon]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/3</td>
<td>AberHerald</td>
<td>7.1</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>5.7</td>
<td>76</td>
</tr>
<tr>
<td>5/3</td>
<td>AberHerald</td>
<td>6.6</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>5.5</td>
<td>55</td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td>0.1</td>
<td>8</td>
</tr>
</tbody>
</table>

The number of leaf primordia was 20% higher for AberHerald than for Huia on the main stolon (Table 12). AberHerald also had 20% more dividing cells in the meristematic zone of the apex than Huia. Plants grown at the higher temperature had 5% more leaf primordia and 25% more dividing cells than plants grown at low temperature. Results are the mean values of four harvests which did not show significant differences.
5 Discussion

In the field experiments, the rates of leaf emergence, very temperature-dependent, were higher for AberHerald than for Huia. This was confirmed by results of a controlled environment experiment and seemed to be due to more intense meristematic activity. AberHerald had bigger leaves than Huia, especially pronounced when grown in competition with perennial ryegrass. The higher number of leaves and the larger individual leaves resulted in a higher leaf area per bud for AberHerald throughout the winter and in early spring. We conclude that the increased leaf area per bud is an important advantage for AberHerald for overwintering and spring growth. The crucial role of leaf area during winter is demonstrated by the fact that the lack of leaves brought a reduction in (I) the rate of node and bud emergence, (II) the individual leaf area of leaves that had emerged in spring and (III) the TNC reserves in stolons and brought about an increase in (IV) death rates of nodes and buds.

5.1 Development of leaves during winter

5.1.1 Leaf turnover

Leaf production was 20% higher for AberHerald than for Huia (Figure 10). AberHerald had a higher rate of leaf emergence on the main stolon than Huia, not only in the field experiments (Figure 12), but also in the experiment in a controlled environment (Chapter 4.3.3). Comparing these experiments with other experiments in a controlled environment and in the field in spring, AberHerald was superior to other varieties as far as leaf emergence at temperatures of 5°C and higher was concerned. AberHerald (+0.3 leaves per week) and Huia (+0.1 leaves per week) had higher rates of leaf emergence than S184 in the experiment conducted by Haycock (1981). The threshold temperature for leaf emergence was 2.9°C for both varieties in the experiments reported here, which is very close to the results of measurements in a controlled environment (2.6°C) found by Haycock (1981). A New Zealand variety examined by Mitchell and Lucanus (1960) had rates of leaf emergence between those of AberHerald and Huia in a temperature range of 7 to 11°C. Compared to the experiment of Eagles and Othman (1988), AberHerald had
higher and Huia equal rates of leaf emergence compared to the tested varieties in spring (April/May with mean temperatures of 8 to 10°C).

While leaf production on the main stolon was higher for AberHerald than for Huia (Figure 10), we observed a variety x competition interaction on primary stolons which means that the rate of leaf production was lower for AberHerald than for Huia when grown without competition and higher when grown with competition (Figure 11). We suggest that the higher number of leaves and, thus, a higher leaf area may account for the higher rate of leaf production of AberHerald compared to Huia (on the main and primary stolons when grown in competition) (Figures 10 and 11). Sackville Hamilton and Harper (1989) attributed 3 to 15% of the variation in leaf emergence to the number of leaves supporting the apex.

Apart from the dependence on genotype, leaf emergence was found to be very temperature-dependent (92 to 98%), both in the field during winter as well as in a controlled environment (Figure 12, Table 11 and chapter 4.3.3). Our findings agree with those of Sackville Hamilton and Harper (1989): the rate of leaf production throughout the year is determined by temperature (82 to 85%) and, to some extent, by the genotype (11 to 15%). In our experiments, the relationship of temperature and leaf emergence was stronger than in the experiment of Sackville Hamilton and Harper (1989). Temperature may have a stronger effect on plant growth during winter and spring than during the growing season, because temperature is the main limiting factor for growth in winter. Davies and Jones (1992) also found a close relationship between temperature at 10 cm soil depth and leaf emergence. In our experiments, day temperatures at 20 cm height showed the best relationship with leaf emergence. The difference in these temperatures and in day/night temperatures, measured at 2 m height, was between 2 and 5°C in the temperature range of 7.5 to 20°C (i.e., day/night temperatures were between 5.5°C and 15°C). Day/night temperatures were used in the comparisons of leaf emergence in the above-mentioned experiments.

Leaf emergence and leaf initiation are equal and constant under unchanging conditions (Thomas, 1987; Amott and Ryle, 1982). However, if conditions (light or temperature) change, the rate of leaf emergence reacts faster than leaf
initiation, resulting in a change in the number of leaf primordia. Thomas (1987) found an increase in the number of leaf primordia as temperature decreased. In our experiment in a controlled environment, no such change in the number of leaf primordia was found, even though conditions during propagation and the duration of the experiment were different. Temperature was lower and light intensity higher (Chapter 3.2). The reaction of the plant may have been counterbalanced or the experiment may have been too short to observe significant changes in the number of leaf primordia. However, the number of leaf primordia was higher for AberHerald than for Huia throughout the experiment (Table 12). Thus, we assume that the higher rates of leaf emergence were due to the higher rate of leaf initiation. The rate of leaf emergence (Boller and Nösberger, 1983) and the rate of leaf initiation (Beinhart, 1963) reflect the meristematic activity. We investigated meristematic activity by staining chromosomes of dividing cells in the apical meristem. Higher meristematic activity (more dividing cells) may be due to higher proportion of dividing cells or to larger meristems (more cells but the same proportion of dividing cells). However, AberHerald and the higher temperature treatment had higher meristematic activity, as reflected in leaf emergence, number of leaf primordia and number of dividing cells (Chapter 4.3.3 and Table 12).

The rate of leaf death was highest until January in the experiment in 1992/93 and until December in the experiment in 1993/94 (Figure 10). The remaining leaves were those that were produced after November and October respectively and may have adapted to freezing temperatures, because these leaves lived for more than three months. This corresponds well with a leaf life of 15 to 17 weeks for leaves produced in early winter (Chapman et al., 1984).

5.1.2 Number of leaves
As a result of higher rates of leaf emergence and similar rates of leaf death, AberHerald always had more leaves than Huia during winter and spring (Figure 10). This is in contrast to the findings of the European multi-site experiment (Part IV) in which both varieties had a similar number of leaves per bud (Figure 6 B). Moreover, these buds had considerably fewer leaves in
autumn than in the experiments reported here (Part V). Differences in the number of leaves (Parts IV and V) in autumn can be explained by the different cutting management. Plots in the European multi-site experiment (Part IV) were cut at the end of October, thus, at the first sampling date in November, only leaves produced from the end of October until November were present. On the other hand, plants in these experiments (Part V) were uncut and had leaves that were produced from September to November. However, temperature decreased, and a considerable number of leaves died in late autumn (Part V), so that the plants had a comparable number of leaves per bud during winter and spring to those plants described in Part IV.

One reason for the similar number of leaves of varieties during winter and spring (Part IV) may be the different methods of harvesting (Parts IV and V). As described in Part IV, we sampled a random mixture of main and lateral stolons of different length and calculated the mean of number of leaves per bud, while, as described in Part V, we counted the number of leaves only on main stolons only with the same length. Differences among varieties were accentuated on the main stolons (Figures 10 and 11 and Table 8). Thus, the sampling method applied in Part IV may have masked the variety differences found for the main stolons (Part V). Another reason may be that some of the leaves (< 0.6 Carlson (Carlson, 1966)) were not counted in the experiment described in Part IV, while in the experiments reported here (Part V) these leaves were included. AberHerald had more developing leaves than Huia due to the higher rate of leaf emergence.

Plants in the experiment of Davies and Evans (1982) in Wales usually had more or less the same number of leaves as our plants (Part V). Only at the lowest level in mid-winter, did our plants have fewer leaves (0.5 - 2 leaves per bud) than plants in Aberystwyth (2.2 - 2.6 leaves). This may be due to different air temperatures in February in Aberystwyth (4°C) and in Switzerland (0°C).
5.1.3 Leaf size

Leaf area per bud was greater for AberHerald than for Huia, due partly to a greater number of leaves but mainly to considerably larger individual leaves (Table 10). In general, leaf size has a large genetic component (Caradus et al., 1990), clover varieties being classified according to leaf size. AberHerald and Huia both have medium-sized leaves throughout the growing season (Collins and Rhodes, 1995). Thus, the larger leaves of AberHerald at low temperature in spring indicates higher rates of leaf enlargement than Huia. These results confirm the European multi-site experiment (Part IV) in which the individual leaf size as well as the increase in leaf size was greater for AberHerald than for Huia (Figure 6). Larger leaves in spring were shown to be very important in competition (Part IV).

The temperature threshold for leaf enlargement also agrees with the findings of the European multi-site experiment (Part IV). In the experiments reported here (Part V), the leaf began to expand when the mean temperature between harvests was 9.4°C, while at a mean temperature of 7.1°C, leaves did not enlarge. Therefore, the temperature threshold for an increase in leaf size must lie between 7 and 9°C. In the European multi-site experiment (Part IV) an increase in leaf size was observed when the temperature exceeded 7.5 °C. In the experiment of Lüscher (1989), the leaves of white clover started to increase in size at 9°C. In a controlled environment, Haycock (1981) found 6.3 °C to be the temperature threshold for leaf enlargement.

5.2 The significance of leaves for overwintering and spring growth

5.2.1 Changes in carbohydrate reserves

Leaves strongly influenced the carbohydrate budget of plants during winter. Undefoliated plants (290 mg) had twice as much structural dry mass of stolons in early spring than defoliated plants (136 mg) (Table 13). In addition, undefoliated plants, grown without competition, increased their autumn carbohydrate reserves (+50% TNC), while defoliated plants lost reserves (-90% TNC) in winter. We observed an interaction for node production between variety and defoliation in winter. This means that, when defoliated, AberHerald produced more nodes than Huia. Because AberHerald had more carbohydrate
reserves than Huia (Figure 9) suggesting, that carbohydrate reserves are an important source of carbohydrates when plants have only few leaves. In general, a lack of leaves during winter resulted in a low rate of node production (leaf emergence) compared to plants with leaves (Table 9). Thus, we conclude that plants with leaves during winter have a better carbohydrate supply due to photosynthesis than defoliated plants. Photosynthesis can take place at low temperatures and in winter (Woledge and Dennis, 1982; Woledge et al., 1989), though daily totals of net photosynthesis were a tenth of the values found in summer due to short photoperiods and low irradiance. However, the net change in the carbohydrates of the whole plant during winter depends on loss by death or respiration and increase through photosynthesis. In our experiment, the loss of carbohydrates through death (44% of nodes present in autumn died) (Figure 8) and respiration was higher for plants without leaves than was the increase through photosynthesis; in plants with leaves, the increase through photosynthesis exceeded losses due to death (4% of nodes present in autumn died) and respiration. In the experiment of Woledge et al. (1990), losses tended to equal or exceed gains, especially for clover which had a smaller individual leaf area and leaf area per bud than grass.

Table 13 Effects of white clover variety, competition with perennial ryegrass and continuous defoliation of individual plants on structural dry mass and TNC content of stolons in autumn and their change during winter 1994/95.

<table>
<thead>
<tr>
<th>competition</th>
<th>variety</th>
<th>structural DM of stolons [mg]</th>
<th>change [%]</th>
<th>TNC content of stolons [mg]</th>
<th>change [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 Nov. 94</td>
<td>13 Mar. 95</td>
<td>3 Nov. 94</td>
<td>13 Mar. 95</td>
</tr>
<tr>
<td>without</td>
<td>AberHerald</td>
<td>149</td>
<td>+147</td>
<td>+10</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>99</td>
<td>+121</td>
<td>+10</td>
<td>36</td>
</tr>
<tr>
<td>with</td>
<td>AberHerald</td>
<td>93</td>
<td>+82</td>
<td>+7</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Huia</td>
<td>69</td>
<td>+126</td>
<td>-10</td>
<td>33</td>
</tr>
</tbody>
</table>
The effect of leaves on the carbohydrate budget of the plant when grown with competition in winter was still visible, but not to the same extent as for plants grown without competition (Table 13). It is suggested that shading of clover leaves by the perennial ryegrass reduced the photosynthetic capacity of the leaves. Woledge et al. (1990) showed that, during winter, clover was in a less favourable position for intercepting light than was perennial ryegrass.

5.2.2 Regrowth
Leaves in our experiments (Part V) were very important for subsequent regrowth in spring. Plants with at least two leaves and, thus, a greater leaf area per bud showed strong regrowth, while plants without leaves produced only few and small leaves (Tables 8 and 10). This is in agreement with the experiment of Baur-Höch et al. (1990) which showed that the remaining leaf area after a cut is an important factor for regrowth. Sackville Hamilton and Harper (1989) attributed 3 to 15% of the variation in leaf emergence to the number of leaves supporting the apex.

Haycock (1984) also found a greater leaf area and strong regrowth in a set of five varieties of white clover. Three varieties had more leaves and greater leaf areas in February, when the number of leaves was lowest, and were considerably more productive in spring. Lüscher (1989) showed that buds with a greater leaf area in early spring had greater leaf dry mass in late spring. The main stolons of AberHerald and Huia were compared in a controlled environment (Guinchard (1995)) with low temperatures and a subsequent regrowth period. While both varieties each had two leaves, AberHerald had a greater leaf area during the cold and regrowth periods. It also produced more dry mass per plant.

Thus, we suggest that greater leaf area per bud early in the growing season is important for the performance of this bud later in spring. As a consequence, it seems very important to increase the leaf area per bud by leaf production at low temperature in autumn and winter and/or by low rates of leaf death. AberHerald may have a large advantage over Huia with respect to leaf production and enlargement.
5.2.3 Change in plant structure

In general, a net increase in the number of buds and nodes occurred during winter and spring (Figures 7 and 8). This result confirms our observations in the European multi-site experiment (Part IV: Figures 4 and 5). The observed increase must be caused by rather mild winters during the experimental years and/or the ample supply of carbohydrates. The latter is supported by the fact that defoliated white clover plants (+10%) showed a much smaller increase in structural dry mass of stolons than undefoliated plants (+130%) (Table 13). In addition, they showed a much smaller net increase in the number of buds and nodes than undefoliated plants. A lack of leaves during winter drastically reduced emergence rates and increased death rates of buds and nodes. It also reduced the carbohydrate concentrations of stolons to a very low level. Lüscher (1989) made similar observations: plants, which had very few leaves during winter and low levels of carbohydrates in autumn, had fewer buds in spring. The level of carbohydrates was apparently associated with the number of buds and may have influenced their development.

A decrease in the number of axillary buds due to low bud emergence was observed for defoliated plants and plants grown with competition. We suggest that axillary buds in autumn developed to terminal buds and that only few buds emerged during winter. This is supported by the relation of axillary to terminal buds which was sharply reduced during winter (Figure 7). Similar observations were made in the European multi-site experiment for white clover grown with competition (Figure 4) and by Grant et al. (1991), where defoliation early in the growing season resulted in a reduction of axillary buds. The experiments of Newton and Hay (1996), who investigated factors which influence the viability of axillary buds and the outgrowth of a viable bud to form a branch, may provide an explanation. Defoliation was the only factor which affected the viability of buds. Axillary bud viability within the apical meristem was affected when the main stolon had only one leaf (sum of Carlson stages (Carlson, 1966)) for a period longer than five weeks. In our experiments a similarly small number of leaves was found for defoliated white clover plants and for plants grown with competition (Table 8; Figures 10 and 11). Thus, a small number of leaves may have caused the low rate of bud emergence.
While varieties did not differ significantly in the number of buds and nodes, plants grown with competition were significantly smaller than plants grown without competition in autumn and even more so in spring (Figures 7 and 8). Part of the response of white clover to competition may have been due to the low number of leaves (discussed above), while light quantity and quality may also play a role (Lötscher and Nösberger, 1997; Robin et al., 1994a and 1994b). Lötscher and Nösberger (1997) found that light quantity and quality influenced the outgrowth of branches and the percentage of branched nodes. Shading of the whole plant, including leaves, was an important factor in the reduction of branched nodes. In our experiments during winter, white clover plants grown with competition were strongly shaded by the ryegrass, though the level of competition was not measured. The competition reduced the branching rate in autumn and winter (Table 5).

In the experiment in a controlled environment we found dormant buds (Table 6) that were alive but did not develop further during the experimental period. We cannot explain the dormancy of the buds. There was no relationship between morphological features, such as the number or position of roots, and the dormancy of the bud, because nearly all the nodes on the main stolon were rooted. Lötscher and Nösberger (1996) examined rooting and the percentage of branched nodes. They found that the position of the root influenced the initiation of the development of buds. Buds remained dormant longer, the further away they were from the next root.

More dormant buds were observed at low temperatures. Thus, it seems likely that low temperature stress influenced dormancy. If temperatures had risen (as in spring), then the dormant buds may have started to grow again. This is supported by our field experiments where no dormant buds were found. Fluctuations in temperature probably did not initiate dormancy.

Growth was very temperature-dependent, and AberHerald had higher rates of leaf emergence and leaf enlargement than Huia due to its higher meristematic activity. Our results clearly show the importance of leaves for overwintering and growth of white clover. A lack of leaves induced death of stolons and buds and drastically reduced node emergence and leaf size.
VI GENERAL DISCUSSION

Effect of temperature and variety on the development of white clover

The development of clover during winter was mainly influenced by temperature. Low temperature drastically decreased growth rates, but growth continued throughout the whole experimental period. Low temperature mainly affected the size of newly produced leaves, that remained very small during winter and increased in size only when the temperature rose above 7°C in spring. Death due to freezing was a minor factor in the overwintering ability of both varieties, apart from the second winter when all the leaves died early in winter due to heavy frosts at night (temperature <-5°C). Varieties differed in growth rates at low temperatures. Rates of leaf enlargement and leaf emergence were higher for AberHerald than for Huia. Faster growth rates at low temperature were an important advantage for AberHerald, because yield and proportion of AberHerald were higher than those of Huia. We suggest that faster growth rates of AberHerald compared to Huia were due to higher meristematic activity which can be used as an indicator of growth ability at low temperature. Measuring meristematic activity is easier and less time-consuming than measuring growth at low temperature. Thus, measuring meristematic activity using the method described here may help breeders test new varieties. Meristematic activity and freezing resistance can be examined at the same time.

Effect of leaves on the development of white clover

Leaves strongly influenced the carbohydrate budget and the development of white clover. White clover plants with leaves had a higher content of and a higher net increase in carbohydrate reserves than defoliated plants. In addition, growth rates (i.e., of the structural dry mass of stolons and of the number of buds and nodes) were much more pronounced. On the other hand, death of buds and nodes were induced only in defoliated plants. It is assumed that clover without leaves during winter had insufficient assimilates to meet the
demands of metabolism. We suggest that the greater leaf area per bud of AberHerald during winter and spring was its key advantage over Huia. Thus, a breeding target for successful overwintering of white clover should be an enhanced leaf area per bud during winter and spring.

**Effect of competition on the development of white clover**

Competition by perennial ryegrass reduced growth rates in autumn and winter and, thus, the size of all organs of clover plants were smaller in spring compared to plants grown on bare soil. Leaf number and size as well as the number of nodes and buds were negatively affected by competition with perennial ryegrass. We assume that competition for light was the main factor affecting white clover grown with perennial ryegrass. Woledge et al. (1989) showed that, during winter, the rate of photosynthesis per unit leaf area of clover in the sward is lower than that of grass, probably due to the less favourable position of clover plants for intercepting light compared to grass. Apart from its position in the lower layers of the sward, clover had fewer and smaller leaves earlier in spring than grass and, thus, a much smaller leaf area index (0.1 and 0.8 respectively).

In our experiments, we clearly showed the crucial role of leaves for the competitive ability of white clover. AberHerald still had larger leaves than Huia in early and late spring. This effected a higher yield and a greater proportion of AberHerald compared to Huia. In addition, the higher leaf area index may have reduced the tiller density of perennial ryegrass. A lack of leaves induced the death of stolons and buds and drastically reduced node emergence and leaf size. White clover plants without leaves were at a strong disadvantage in competition with perennial ryegrass compared to plants with leaves. Thus, the competitive ability of clover can be supported by management practices which preserve clover leaves and control grass growth during winter. In autumn, the closure date may be rather late to prevent excessive grass regrowth. Control of grass growth during winter may be achieved by letting cattle graze for a short period, weather and soil conditions permitting. Thus, grass competition can be reduced; clover leaves would hardly be affected by such management because of their very small leaves with short petioles.
VII CONCLUSIONS

Overwintering and competitive ability is very important for the persistence of white clover. While white clover can maintain or even increase its proportion relative to grasses during the growing season, it is generally overcome by competition in winter. Furthermore, white clover may be damaged by very low temperatures. Our experiments clearly showed the crucial role of leaves for overwintering and for the competitive ability of white clover. To improve the persistence of white clover, the plants must retain their leaves during winter. Competition by perennial ryegrass affected the development and functioning of leaves. Thus, a reduction in grass competition either by management (short grazing in autumn and winter) or by less aggressive grass varieties (compatibility), may support overwintering and spring growth of white clover. The examined varieties differed in yield and proportion. Thus, use and breeding of appropriate varieties may also improve the persistence of clover. Apart from winterhardiness, an enhanced leaf area per bud in early and late spring may be a key factor for successful overwintering and competitive ability and, thus, an important breeding goal for the future.
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seit 1992  Assistentin und wissenschaftliche Mitarbeiterin am Institut für Pflanzenwissenschaften der ETH Zürich

1992  Heirat mit Richard Posch
### IX ANNEX

**Table A1:** Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on dry mass of leaflets (A) and buds and petioles (B) of white clover [g/m$^2$] during three winters

<table>
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<th></th>
</tr>
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<tr>
<td></td>
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</tr>
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**Table A2:** Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on dry mass of roots (A) of white clover and tillers of perennial ryegrass (B) [g/m²] during two and three winters

<table>
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**Table A3:** Effects of white clover variety and competition with perennial ryegrass (monoculture versus mixture) on starch concentration (A) and water soluble sugar concentration (B) of the stolons of white clover [mg/100 mg] during three winters.

### A

<table>
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### B

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