Subterranean Clover Red Leaf Virus Disease: Effects of Temperature on Plant Symptoms, Growth, and Virus Content

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ABSTRACT

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Subterranean clover (*Trifolium subterraneum*) plants inoculated with subterranean clover red leaf virus and uninoculated control plants were grown under natural light at five different day/night temperature regimes. Development of red pigment and time for development of red pigment were a function of temperature. At 20/15, 25/20, and 30/25 C older leaves of inoculated plants developed bright red pigment, whereas at 15/10 and 33/28 C they developed mottled reddish-brown or brown pigment. These red-pigmented leaves developed earlier at 20/15, 25/20, and 30/25 C than at 15/10 or 33/28 C. The maximum percentage of red leaves was higher (80%) at 20/15 and 25/20 C than at other temperatures. At harvest (60 days after inoculation), numbers of leaves per plant and fresh weights of both tops and roots of inoculated plants were lower than those of control plants at all temperatures. The temperature response pattern in relation to number of leaves per plant and fresh weight differed for inoculated and control plants. For inoculated plants, the maximum number of leaves per plant and fresh weight of both tops and roots was at 33/28 C. For control plants, the maximum number of leaves per plant and fresh weight of both tops and roots was at 20/15 and 25/20 C. The temperature response pattern for relative virus concentration as measured by enzyme-linked immunosorbent assay (ELISA) was inversely related to fresh weight. At 15/10, 20/15, and 25/20 C, relative virus concentrations were greater than that at 30/25 C and this in turn was greater than that at 33/28 C. The assessed yield of virus was approximately 400 to 700 ng/g fresh weight for plants grown at 15/10, 20/15, and 25/20 C.

Subterranean clover red leaf virus (SCRLV) is a member of the luteovirus group (2,12,13). Subterranean clover (*Trifolium subterraneum* L.) plants infected with SCRLV usually have definitive symptoms under glasshouse conditions. They include red mottling of laminae of relatively young leaves and reddening of those of older leaves, generally from the margin inwards. The laminae of leaflets of isolates that produce severe symptoms are relatively small in size with short, thin petioles (1,4,5). However, SCRLV-infected plants are not readily recognized in the field because reddening of leaves can result from infections by several viruses or viruslike agents as well as (in some plants) from stresses caused by fungal infection and by some nutritional disorders (*personal communications*).

Rochow and Duffus (11) reported that symptoms of plants infected with luteoviruses are less useful for diagnostic purposes than those of some other viruses since they vary with plant species, cultivar, age, and physiological condition. They also reported observations that suggested symptoms are influenced by temperature and light. For example, oats infected with barley yellow dwarf virus were considered more likely to develop characteristic red leaf symptoms when cold, rather than warm, temperatures prevail and under high, rather than low, light intensities. Similar environmental conditions also were noted as requirements for the development of symptoms of beet western yellows (6).

We report here a study on the effects of five different temperature regimes on symptom development, yield, and virus concentration in plants of subterranean clover inoculated with SCRLV in the seedling stage. The purposes of the work were to aid identification of SCRLV-infected subterranean clover plants in the field at different times of the year and in different regions by finding out if development of red pigment in leaves is influenced by temperature, to examine the effect of the disease on plant growth, and to examine the relationship between symptom expression and virus concentration. In a subsequent paper, we will report a study on the effects of light intensity on the disease caused by SCRLV.

MATERIALS AND METHODS

Plant growth, virus inoculation, and symptom assessment. Seeds of *T. subterraneum* 'Mt. Barker' were germinated in vermiculite and grown for 10 days at 20 C under fluorescent light with a 16-hr photoperiod. Then seedlings were transplanted into vermiculite in 29 × 38 × 12-cm plastic boxes. Four days later, aphids (*Aulacorthum solani* Kaltenberg) that had fed for 3 days on

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SCRLV (NSW-K)-infected plants (4) were placed on half of the seedlings (five aphids per plant). After 3 days, the aphids were killed with a pyrethrum insecticide and the seedlings were taken to the Canberra phytotron and transplanted individually into 10-cm-diameter pots containing a mixture of vermiculite and perlite (1:1, v/v).

There were two experiments. In Experiment A (seeds sown 28 December 1981) the pots with inoculated seedlings were placed immediately into five C-type cabinets (9) that received 12 hr of natural light and were regulated at day/night temperatures of 15/10, 20/15, 25/20, 30/25, and 33/28 C, respectively. In the following text these temperature regimes will be referred to by the day temperature only. There were 21 inoculated and 21 uninoculated (control) plants at each temperature (ie, 7 inoculated and 7 control plants) in one half of each of the three units of the C-type cabinet. Since temperature in each unit of a C-type cabinet is controlled independently, the three units constitute three true replicates. Plants were watered with Hoagland's nutrient solution in the morning and water in the afternoon.

In Experiment B (seeds sown 11 January 1982), which was of similar design to Experiment A, inoculated and control plants were placed in the remaining half of each unit occupied by plants of Experiment A. In an attempt to improve plant establishment as compared with that in Experiment A, newly potted plants were maintained at 22 C under low light for 18 hr before being placed under the five different temperature regimes; then they were covered with brown paper for 24 hr before being exposed directly to natural light.

In Experiments A and B, records of plant growth were made from 22 and 8 days, respectively, after inoculation. Twice weekly, numbers of live expanded green leaves and live expanded leaves with red, reddish-brown or brown pigment (red leaves) were recorded on each plant. Dead plants were noted. Once each week plants were randomized within each unit and experiment. Inoculated and control plants were harvested 60 days after inoculation. Fresh weights of the tops and roots of each plant were recorded separately. Then the tops and roots of each infected plant and a bulk sample of tops and roots from control plants from each temperature regime and experiment were frozen at −20 C. About 1 yr later, enzyme-linked immunosorbent assay (ELISA) was used to measure the virus levels in the inoculated plants.

ELISA. The frozen plant samples (combined tops and roots) were ground by four operators using mortars and pestles. The tissue was triturated thoroughly both before and after adding cold buffer (0.01 M sodium-phosphate buffer, pH 7.4, containing 0.8% [w/v] NaCl, 0.05% [v/v] Tween-20, and 2% [w/v] polyvinyl pyrrolidone in the ratio 1 g of tissue:5 ml of buffer) (extraction buffer). When inoculated plant samples weighed more than 0.4 g, subsamples of about this weight were ground separately and pooled. There were two control samples from each temperature in each experiment. Each was obtained by grinding and pooling four 0.4-g subsamples (each consisting of equal weights of roots and shoots) from the control plants which were pooled at the time of harvest. The samples were incubated for 24 hr at 4 C, centrifuged for 10 min at 5,000 rpm and the supernatant fluids were tested by ELISA.

ELISA was done essentially as described by Clark and Adams (3). Twenty-five, flat-bottomed, Immulon II plates (Dynatech Corp., Alexandria, VA) were coated with 1 μg/ml of antibody globulin at the same time and then frozen at −20 C until they were used. The antibody-alkaline phosphatase conjugate was used at a dilution of 1/1,000. After addition of *p*-nitrophenyl phosphate the plates were incubated at 4 C and $A_{405\ nm}$ readings made after 30, 60, 90, and 120 min by using a dual wavelength eight-channel colorimeter (Titerteck Multiskan MC, Flow Laboratories Ltd., North Ryde, N.S.W. 2113 Australia) set at a reference wavelenth of 690 nm.

In a preliminary experiment, the range of ELISA absorbance readings for inoculated plants in each of the five temperature regimes was assessed in relation to four levels of purified virus diluted in extraction buffer. One plant of medium fresh weight was used for each temperature regime. Purified virus was obtained essentially by the method of Takanami and Kubo (14). Three levels of virus (40, 20, and 10 ng/ml), which gave readings within the range of those for samples of the test plants, were selected as the standards for use in subsequent assays.

Assays were made on the first three days in each of two successive weeks. Fresh stock virus (0.25 or 0.50 mg/ml) was made up in 0.006 M Sörenson's phosphate buffer at the beginning of each week from virus newly passed through a sucrose gradient and measured spectrophotometrically. This stock was diluted in Sörenson's buffer on each of the three successive days to form the three standards. The residual standard solutions were stored at 4 C and at the end of each week they were calibrated against nine dilutions of SCRLV, freshly prepared as above. The data showed that $A_{405\ nm}$ absorbance readings for standards decreased about 20% per day during the assay period of each week. These results are supported by earlier data (*unpublished*) which indicate that $A_{405\ nm}$ absorbance readings for purified virus stored in Sörenson's buffer at 4 C decrease with time of virus storage. Therefore, linear calibrations between ELISA readings and known concentrations of the standards (ng/ml) were developed with data obtained only on the first day of each week of assay, when virus standards were made up with virus newly passed through sucrose gradients and measured spectrophotometrically. From these calibrations, virus concentrations (ng/ml) in each sap sample were obtained. Since 0.4 g of tissue was ground in 2.0 ml of extraction buffer, each 1.0-ml sap sample represents 0.2 g tissue. Therefore, the total virus (ng) per infected plant = concentration in sap sample (ng/ml) × 5 × fresh weight (g).

For most plant samples there was sufficient supernatant to enable ELISA determinations to be made in six replicate wells; when insufficient supernatant was available fewer wells were filled. The large number of samples that were measured required 19 ELISA plates. A randomized block design was used on each plate, each block being 3 × 3 wells. Six blocks were fitted onto each plate (8 × 12 wells) by omitting rows 1 and 8 and columns 1, 11, and 12 of the ELISA plate. In each block of each plate, six wells contained supernatant and three contained the standards. For a given plant sample, six replicates, when available, were distributed (one per block) in the same plate. When less than six replicates were available, these were distributed (one per block) at random. Samples for control and inoculated plants were allocated to the 19 plates so that each plate contained inoculated samples from a range of temperatures and at least one control sample. This ensured that possible differences between plates (8) would have a minimum effect.

Analysis of data for disease development and plant growth. As there was differential plant mortality between temperature treatments, resulting in different numbers of plants at each temperature, a regression technique generalized linear models (7,10), was used for numbers of leaves, percentages of red leaves, and fresh weights of tops and roots. Data from the two experiments were combined. The error structures used were: number of leaves–Poisson error, log link; percent red leaves–binomial error, logit link; and fresh weights–normal error, identity link (on fourth root-transformed fresh weights). These analyses enabled the identification of times after inoculation when differences between control and inoculated plants and differences between temperature regimes became significant.

Plants were defined as dead when they were missing on day 53. Counts were made of numbers of dead plants for both control and inoculated plants in each unit of each cabinet. Because the methods of establishment and mortality patterns differed (as described earlier), mortality data from each experiment were analyzed separately by using generalized linear models, as above, with binomial error and logit link.

Analyses of ELISA data. For each of the 19 plates, analyses of variance were made for $A_{405\ nm}$ absorbance readings taken at 30, 60, 90, and 120 min. Only samples and standards that were represented in all six blocks of the randomized block design on the plate were included in these analyses. Block differences were recognized, and data from samples present in less than six blocks were adjusted for

block differences. This provided adjusted mean readings for all samples and standards.

For inoculated plants, analyses of variance were made, using combined data from the two experiments, on adjusted readings, concentrations (nanograms per milliliter) and total virus per plant (nanograms). Each value was log transformed before analysis.

For control plants, analyses of variance were made on adjusted readings only, as the calibrations used for inoculated plants were inappropriate.

RESULTS

Development of red leaves. Development of red leaves occurred only on SCRLV-inoculated plants and was markedly affected by temperature. At 20, 25, and 30 C, relatively young and older leaves developed bright red pigmentation, whereas at 15 and 33 C these leaves developed mottled reddish-brown or brown pigment.

Numbers of leaves on control and inoculated plants. Growth of control and inoculated plants in response to the temperature regimes, as measured by mean numbers of leaves per plant, is shown in Fig. 1a and b, respectively. For control plants, there were differences ($P < 0.05$) between temperatures in numbers of leaves as from day 8. Numbers of leaves produced at 20, 25, and 30 C were greater ($P < 0.001$) than at 15 or 33 C as from day 29. As from day

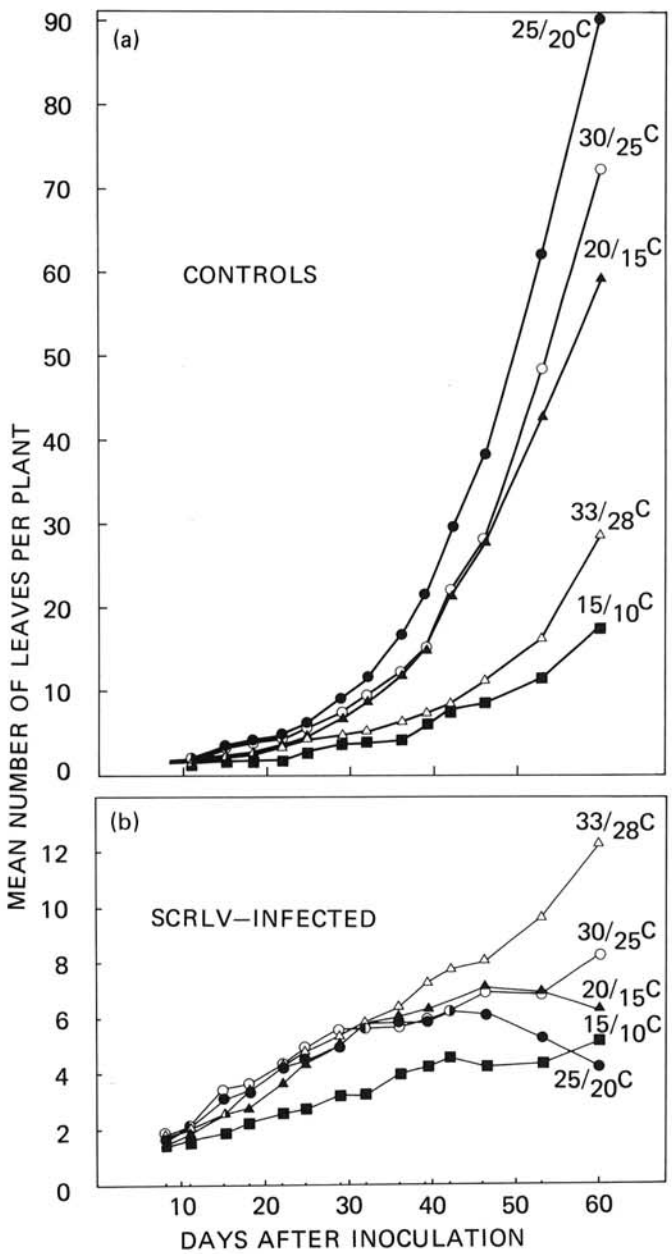

Fig. 1. Time-course changes in numbers of living expanded leaves on plants of *Trifolium subterraneum* infected with subterranean clover red leaf virus (SCRLV) and control plants grown under five different day/night temperature regimes.

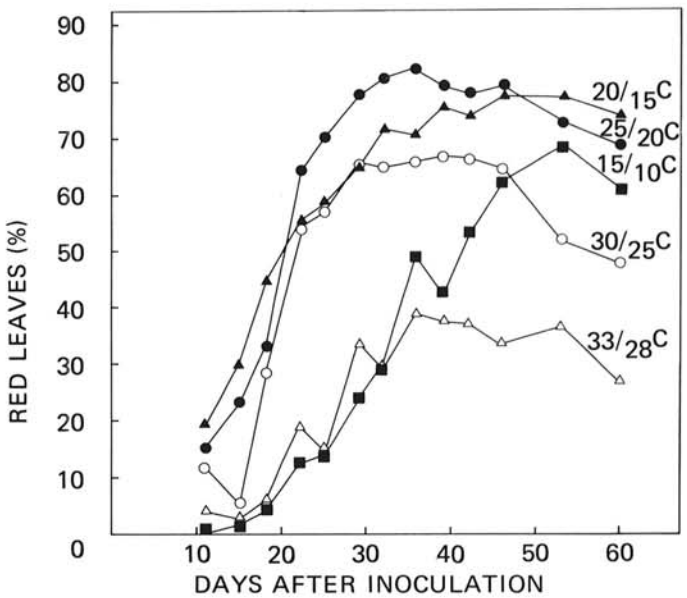

Fig. 2. Time-course changes in percentages of live red leaves (leaves at 20, 25, and 30 C were bright red, those at 15 and 33 C were reddish-brown or brown) in plants inoculated with subterranean clover red leaf virus and grown under five different day/night temperature regimes.

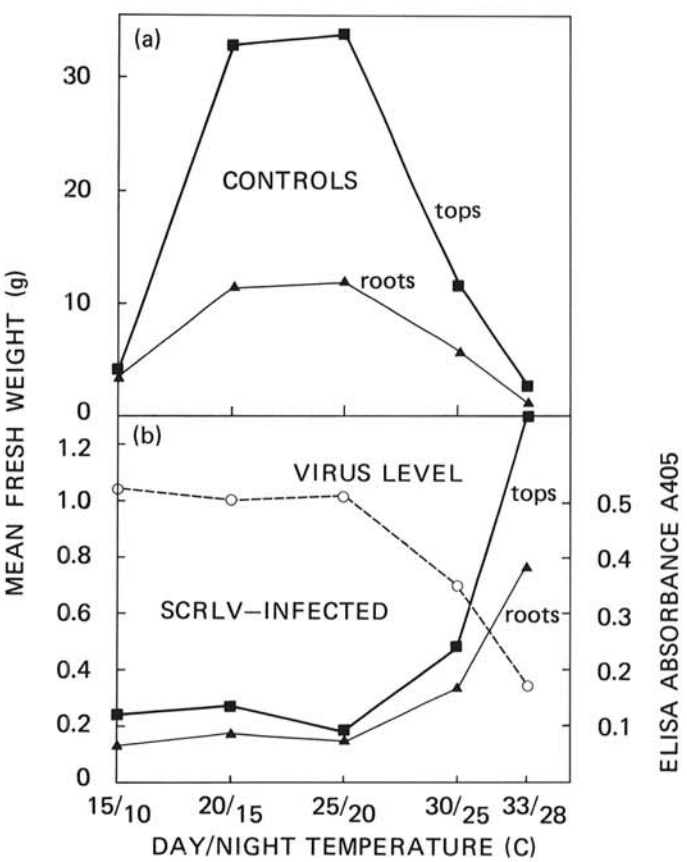

Fig. 3. Effects of five different temperature regimes on yield in terms of fresh weight and relative virus concentration of *Trifolium subterraneum* inoculated with subterranean clover red leaf virus (SCRLV). Control and inoculated plants were grown for 60 days before harvest. Relative virus concentrations were assessed by ELISA.

46, plants growing at 20, 25, and 30 C had reached a stage of rapid growth. The mean numbers of leaves for control plants were greater ($P < 0.05$) than those for inoculated plants at day 25 for 25 C, day 29 for 20 and 30 C, day 42 for 15 C, and day 53 for 33 C. Differences between control and inoculated plants became greater as the experiment progressed, and on day 60 at 25 C the mean number of leaves for control plants was more than 20-fold that of inoculated plants.

The time-course temperature response patterns for inoculated plants differed from those of control plants and were associated with occasional loss of older red or red-brown pigmented leaves. The numbers of leaves on inoculated plants grown at 33 C increased steadily throughout the experiment, so that by day 53 they were greater ($P < 0.05$) than at all other temperatures. For inoculated plants grown at 30 C, numbers of leaves increased continuously but at a slower rate after day 29. Numbers of leaves on inoculated plants grown at 15 C also increased steadily but at a low rate, so that numbers of leaves between days 15 and 42 were lower ($P < 0.05$) than those at all other temperatures. For inoculated plants grown at 20 and 25 C, numbers of leaves increased steadily to a maximum at days 42 or 46 and then decreased; this was due to increased loss of older red leaves. At this stage, inoculated plants at these temperatures were partly or almost completely covered by the swardlike growth of control plants.

Percentage of red leaves. For inoculated plants grown at 20, 25, and 30 C the percentage of red leaves increased rapidly, reaching a maximum of between 65 and 80% at days 20 to 46 and then decreasing (Fig. 2). For those grown at 15 and 33 C, the percentage of red leaves increased relatively slowly, reaching a maximum of about 70% on day 53 for plants grown at 15 C and a maximum of about 40% at day 36 for plants grown at 33 C (Fig. 2). The regression analysis showed that there was a temperature effect at day 11 ($P < 0.01$) and thereafter ($P < 0.001$). Percentages for 20, 25, and 30 C were greater ($P < 0.001$) than those for 33 C from day 18 onwards, and greater ($P < 0.001$) than those for 15 C for days 18–39.

Fresh weight of tops and roots. Weights of both tops and roots of control plants at 20 and 25 C were greater ($P < 0.001$) than those at 30 C and these in turn were greater ($P < 0.001$) than those at 15 and 33 C (Fig. 3). In contrast, for inoculated plants, weights of both tops and roots at 15, 20, and 25 C were lower ($P < 0.05$) than those at 30 C and these in turn were lower ($P < 0.001$) than those at 33 C.

At all temperature regimes the weights of both tops and roots for control plants were greater than those of inoculated plants ($P < 0.001$ for 15, 20, 25, and 30 C; $P < 0.05$ for 33 C). For control plants grown at 20 and 25 C, the most favorable temperatures for growth of these plants, the weights of tops were 120- and 190-fold greater, respectively, than those of inoculated plants, whereas the weights of roots were 60- and 80-fold greater, respectively, than those of inoculated plants.

Percent mortality. Most mortalities in both Experiments A and B occurred during the first 2 wk after seedlings were transplanted and placed under the five different temperature regimes. The lower mortality in Experiment B than Experiment A was attributed to the more favorable environmental conditions seedlings received during the first 2 days after they were transplanted. There were no significant differences in mortality between control and inoculated plants; however, there were differences between temperature regimes ($P < 0.001$, Table 1). In Experiment A the percent mortality at 20 C was lower than at 25 C, which in turn was lower than at 15, 30, and 33 C. In Experiment B the percent mortality at 20, 25, 30, and 33 C was lower than at 15 C. Clearly, seedlings grown initially and inoculated at 20 C were not readily established at either relatively low or high temperature regimes.

ELISA absorbance readings. $A_{405\ nm}$ absorbance readings for each plate made at 30, 60, 90, and 120 min were highly correlated. Since those obtained at 120 min generally had lower coefficients of variation and since substrate was not limiting at this time, these were used in all analyses. Table 2 summarizes the data obtained from the ELISA analyses. For all temperature conditions the $A_{405\,nm}$ absorbance readings for virus-infected plant samples were higher than those for control plant samples ($P < 0.001$). Mean $A_{405\,nm}$ readings (and hence the calculated mean relative virus concentrations per inoculated plant [ng/ml]) at 15, 20, and 25 C were greater than those at 30 C, and these in turn were greater than those at 33 C. Essentially, the temperature response pattern is the inverse of that for the mean fresh weight of infected plants (Fig. 3).

There were no significant differences in total virus per plant between temperature treatments. These differences failed to achieve significance for two reasons: first, the association of high levels of virus with low fresh weight and low levels of virus with high fresh weight; second, the high variability of both fresh weight and virus concentration.

Data from two separate experiments (*unpublished*) show that the methods of grinding and incubation of plant material in the present experiments resulted in ELISA absorbance readings that were three and five times (respectively) lower than those for plant material prepared by freezing in liquid nitrogen immediately before grinding, grinding in phosphate buffer, and storing at −80 C for 24 hr prior to centrifugation. Therefore, the values in Table 2 for virus concentration and total virus per plant would be more realistic if they were increased between threefold and fivefold. Thus, for plant tissue at 15 C with an absorbance value of 0.520, the virus concentration would range between $27.8 \times 5 \times 3 = 417$ and $27.8 \times 5 \times 5 = 695$ ng/g.

Readings for control plants at 25 C were higher ($P < 0.05$) than those for other temperature regimes (Table 2).

TABLE 1. Mortality of *Trifolium subterraneum* grown under five day/night temperature regimes[a]

Experiment	Mortality (% ± S.E.) 15/10 C	20/15 C	25/20 C	30/25 C	33/28 C	Significance of temperature differences
A	76 ±6.6	5 ±3.3	19 ±6.1	57 ±7.6	61 ±7.5	$P < 0.001$
B	64 ±7.4	14 ±5.4	10 ±4.5	2 ±2.4	21 ±6.3	$P < 0.001$

[a] Plants were harvested 60 days after inoculation. Mean data for control plants and plants inoculated with subterranean clover red leaf were not significantly different and were combined.

TABLE 2. Quantitative assessment of subterranean clover red leaf virus content by enzyme-linked immunosorbent assay (ELISA) in whole infected plants (tops and roots) of *Trifolium subterraneum* 'Mt. Barker' grown under five different temperature regimes and harvested 60 days after inoculation[x]

Day/night temperature (C)	Plants tested (no.)	Mean ELISA absorbance reading ($A_{405\ nm}$)	Virus concentration in sap sample (ng/ml)[y]	Total virus per plant[z] (ng)	Mean control ELISA absorbance reading ($A_{405\ nm}$)
15/10	10	0.520 a	27.8 a	39.3 a	0.113 b
20/15	32	0.502 a	26.3 a	75.0 a	0.108 b
25/20	28	0.510 a	26.5 a	53.4 a	0.161 a
30/25	29	0.350 b	17.2 b	71.8 a	0.123 b
33/28	18	0.170 c	5.6 c	61.6 a	0.112 b

[x] Combined data for experiments A and B. Within each column values followed by the same letter are not significantly different ($P < 0.05$).

[y] Virus concentration = mean relative virus concentration per sap sample calculated from calibrations of $A_{405\ nm}$ readings for standard virus concentrations.

[z] Total virus per plant (ng) = concentration in sap sample (ng/ml) × 5 × fresh weight (g).

DISCUSSION

Temperature markedly affected the development of red leaves of SCRLV-infected plants. Since bright red leaves developed in plants grown at intermediate temperatures but not at 15 or 33 C, field-infected plants grown under relatively warm conditions may not develop the bright red pigment. As subterranean clover germinates in the autumn and sets seeds in the summer, one could

expect that the bright red leaf symptom in field infected plants would be most pronounced in early winter, spring, and early summer.

The swardlike growth that prevailed at temperatures of 20, 25, and 30 C during the last few weeks prior to harvest, as a consequence of vigorous growth of control plants, caused shading of many inoculated plants and probably precipitated the loss of older red leaves. Thus, shading may have affected some differences between treatments. For example, between days 15 and 42, the number of leaves on infected plants grown at 15 C was lower than for plants grown at 20 and 25 C (Fig. 1b) and this difference might have continued during days 42 to 60 had shading not occurred. Shading is unlikely to have been responsible for the lower number of leaves on day 60 for infected plants grown at 25 C as compared with 30 C since this difference already was apparent at day 42 (Fig. 1b). If loss of leaves of infected plants also occurs under swardlike conditions in the field, early death of plants could follow. However, plants infected with isolates of SCRLV that cause less severe symptoms than isolate NSW-K (4) may be more competitive.

The pattern of fresh weight response to temperature was markedly different for SCRLV-infected and control plants. Whereas the maximum fresh weight of control plants was at 20 and 25 C, that for infected plants was at 33 C, a temperature unfavorable for growth of control plants (Fig. 3). At all temperatures, the fresh weight of infected plants was lower than that of control plants, the greatest differences being at 20 and 25 C where SCRLV reduced yield by 120- and 190-fold, respectively. Ten (15) detected no differences between the fresh weights of SCRLV-infected and control plants in an experiment run in New Zealand. Probably, this was due to plants being harvested as early as 21 days after inoculation. Differences between cultivars or the isolates of SCRLV used also may have contributed to the different results.

The present study appears to be the first report of the use of ELISA in a quantitative study of virus in plants grown under a range of environmental conditions. Virus concentrations in plants grown at 15, 20, and 25 C were greater than those in plants grown at 30 and 33 C (Table 2 and Fig. 3); ie, virus concentrations at the time of harvest were greater in plants that were most severely stunted. This temperature response pattern is essentially the inverse of that for fresh weights of infected plants (Fig. 3). High concentrations of virus and the production of bright red leaves were not correlated closely. The highest concentrations of virus were at 15, 20, and 25 C, temperatures at which most stunting occurred, whereas the greatest development of bright red pigmented leaves was at 20, 25, and 30 C.

The ELISA absorbance readings for test plants in Table 2 reflect the relative concentrations of virus in plants grown under the five different temperature regimes. Calculated virus concentrations ranged from 400 to 700 ng/g. The latter is within the range of the yield of virus particles from the present SCRLV isolate (*unpublished*) and from a New Zealand isolate (2) when purified from cultivar Puget peas grown at 15 C. The amount of virus recorded in SCRLV-infected plants may have been greater than the above in plants harvested earlier, or in plant material assayed immediately after harvest rather than after storage at −20 C for almost 1 yr prior to assay.

It is of interest that ELISA absorbance readings for control plants grown at 25 C were higher than those for control plants grown at other temperatures and this suggests that environmental conditions can affect these readings. Consequently, when ELISA is used to measure virus concentrations in plants grown under a range of environmental conditions, control samples should be obtained for each condition.

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