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

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ABSTRACT

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Trifolium subterraneum (subterranean clover) inoculated with subterranean clover red leaf virus and control uninoculated plants were grown for 60 days at day/night temperatures of 25/20 C and with a 12-hr photoperiod of three light intensities: photon flux density 200, 400, and 600 μmol m^{-2} s^{-1}. High light intensity promoted the development of red leaves in infected plants, measured by the percentage of red leaves, as well as growth of infected and control plants, measured by leaf number and fresh weight. At all light intensities the fresh weights of tops and roots of infected plants were significantly less than those of control plants. At the highest light intensity the weight of tops of control plants was 66-fold greater than that of infected plants. More mortalities occurred at low light intensity than high and in infected plants than controls. Virus yield per plant, but not virus concentration in plant sap, measured by enzyme-linked immunosorbent assay was greater at the highest than those at the two lower light intensities. At all three light intensities the virus concentration in plant sap was about fivefold greater in tops than that in roots.

Seasonal factors such as temperature and light intensity are well known to have pronounced effects on disease symptom expression (3). Because these two variables usually fluctuate together it can be difficult to assess which is responsible for an observed effect. Rochow and Duffus (10) reported that sugar beet, lettuce, and spinach infected with luteoviruses often remained green in shaded areas, whereas unshaded plants developed typical golden yellow symptoms. They suggested that oats infected with barley yellow dwarf virus were even more likely to develop red leaf symptoms under high rather than low light intensity and under cold rather than warm temperatures. Recently, we reported that the red leaf symptoms on plants of *Trifolium subterraneum* L. (subterranean clover) infected with subterranean clover red leaf virus (SCRLV) (closely related serologically to soybean dwarf virus [1]), and grown under natural light conditions were promoted, not by relatively high or low temperatures, but by temperatures that favor growth of the host plant.

The present paper reports a study of the effects of three different light intensities on development of symptoms, fresh weight, and virus content in plant sap and on total virus yield per plant in plants of subterranean clover infected with SCRLV and grown under temperature conditions that favor development of red leaves. Also, the virus concentrations in sap from roots and tops of the plants are compared.

MATERIALS AND METHODS

Plant growth, virus inoculation, and symptom assessment. Seedlings of *T. subterraneum* 'Mt. Barker' were grown under controlled environmental conditions, inoculated with SCRLV (NSW-K) (6), by means of the aphid *Aulacorthum solani* Kaltenberger, and then transplanted individually into 10-cm pots in the Canberra phytotron as described previously (7).

There were two experiments. In Experiment A (seeds sown 8 February 1982), 30 inoculated plants and 30 uninoculated control plants were placed in two of four trays in each of three LB cabinets (9) maintained at day/night temperatures of 25/20 C, the optimum temperature for development of red leaves (7), and with a 12-hr photoperiod (fluorescent and incandescent light) having three irradiance levels (light intensities) with photon flux densities at pot level of 200, 400, and 600 μmol m^{-2} s^{-1} (300–700 nm). Henceforth these light treatments will be referred to as 200, 400, and 600 μmol.

In Experiment B (seeds sown 22 February 1982), which was of similar design to Experiment A, inoculated and control plants were placed in the remaining two trays in each of the three cabinets.

The temperature of each cabinet was monitored continuously, and the light intensity was monitored each week. The positions of the 60 plants within each experiment in each cabinet were randomized each week.

Records of plant growth in Experiment A and B were made twice weekly as from 11 and 8 days, respectively, after inoculation and plants were harvested at 61 and 58 days, respectively, after inoculation. For convenience, the time of harvest for both experiments will be cited as at 60 days after inoculation. Fresh weights of the tops and roots of each plant were recorded separately. The tops and roots of each infected plant and a bulk sample of tops and roots from control plants from each light intensity and experiment were frozen and stored at −20 C. Enzyme-linked immunosorbent assay (ELISA) was used to measure the virus concentration (mean relative virus concentration in plant sap (ng/ml); this could include subunits as well as intact virus particles [11]) in the inoculated plants.

ELISA was done on sap extracts as described previously (7). For combined tops and roots of individual plants, sap extracts (in the ratio of 0.2 g of tissue: 1 ml of buffer) were prepared as described previously (7). For separated tops and roots, another method (*unpublished data*), which resulted in higher ELISA A_{405nm} absorbance readings for mean relative virus concentration in sap extracts, was used: The samples were frozen in liquid nitrogen before being thoroughly ground in extraction buffer; these were stored at −80 C for 24 hr, thawed, and centrifuged for 10 min at 5,000 rpm, and the supernatant fluids were used for ELISA.

For most plant samples there was sufficient supernatant for ELISA determination to be made in six replicate wells, but for some samples fewer wells could be filled. Because of the large number of samples, 18 ELISA plates were required for the samples from combined tops and roots and 10 ELISA plates for those from separated tops and roots. A randomized block design was used on each plate, as described previously (7).

Analysis of data for disease development and growth. Analyses of variance were performed on numbers of leaves and percentages of red leaves at each time of observation and on fresh weights of plants at the time of harvest to determine differences among light intensities and between infected and control plants.

For percentages of red leaves only data from infected plants were analyzed, and for fresh weights separate analyses were performed for infected and control plants because the difference between them was large. In all analyses, data from both experiments were included and residuals were based on between plant variation within 'treatment' combinations. To normalize the residual variation the following transformations were used: numbers of leaves—square root, percentages of red leaves—arcsine, and fresh weights—log.

As there was only one LB cabinet for each light intensity, differences among intensities are statistically confounded with differences among cabinets, and residual terms in the analyses of variance may be lower than if light intensities had been replicated. However, cabinet temperatures and light intensities were monitored throughout the experiments, and the trends in numbers of leaves and fresh weights were pronounced and consistent with those expected for the large differences in light intensities used. Thus it is unlikely that having only one LB cabinet for each light intensity invalidated our conclusions.

Analyses of ELISA data. For each ELISA plate (18 for tests on combined tops and roots and 10 for tests on separated tops and roots), analyses of variance were made for A_{405nm} absorbance readings 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 combined tops and roots, analyses of variance of infected plants from both experiments were made on adjusted readings at 120 min, virus concentrations (ng/ml), and total virus per plant, and each variable was log transformed before analysis. For control plants, analyses of variance were made similarly but with no transformation.

For separated tops and roots, analyses of variance of plants from both experiments were made on adjusted readings at 90 min and on virus concentrations (ng/ml), using a log transformation; for control plants, only the adjusted readings at 90 min were analyzed, because readings at 120 min for some of the shoots exceeded the range of the colorimeter, and no transformation was used.

RESULTS

Development of red leaves. Red pigmentation developed in leaves of infected plants but not in leaves of control plants at all three light intensities, but the pigmentation was brighter at the two higher light intensities than that of the lowest.

Number of leaves on control and infected plants. Time course data for growth of infected and control plants in relation to light intensity as measured by mean numbers of leaves per plant are shown in Fig. 1A and B. At days 11 and 15, for both infected and control plants, the number of leaves at light intensities of 600 μmol was greater than that at 400 or 200, but the difference between 400 and 200 μmol was not significant. With two exceptions, from day 18 to the day of harvest for both infected and control plants the number of leaves at 600 μmol was greater than that at 400, and this in turn was greater than that at 200 ($P<0.001$). The differences between control plants grown at light intensities of 400 and 600

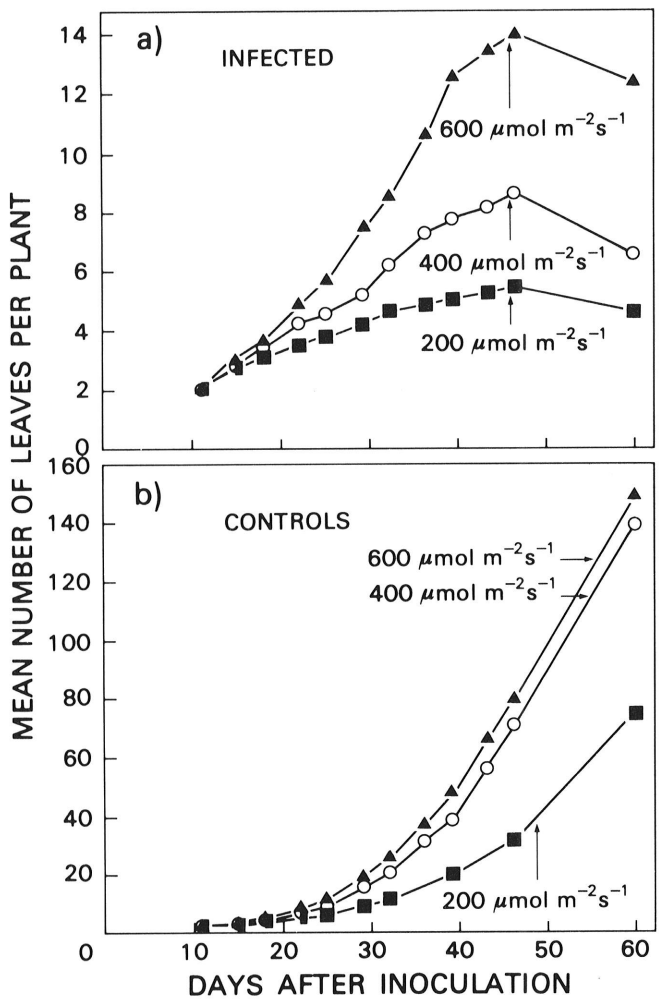

Fig. 1. Time-course changes in numbers of living expanded leaves on **(A)** plants of *Trifolium subterraneum* infected with subterranean clover red leaf virus, and **(B)** uninfected control plants, grown under three different light intensities. For infected plants, all three intensities were significantly different from day 18 onwards; for control plants all three intensities were significantly different for days 18–43, and 200 μmol was significantly lower than the other intensities at days 46 and 60.

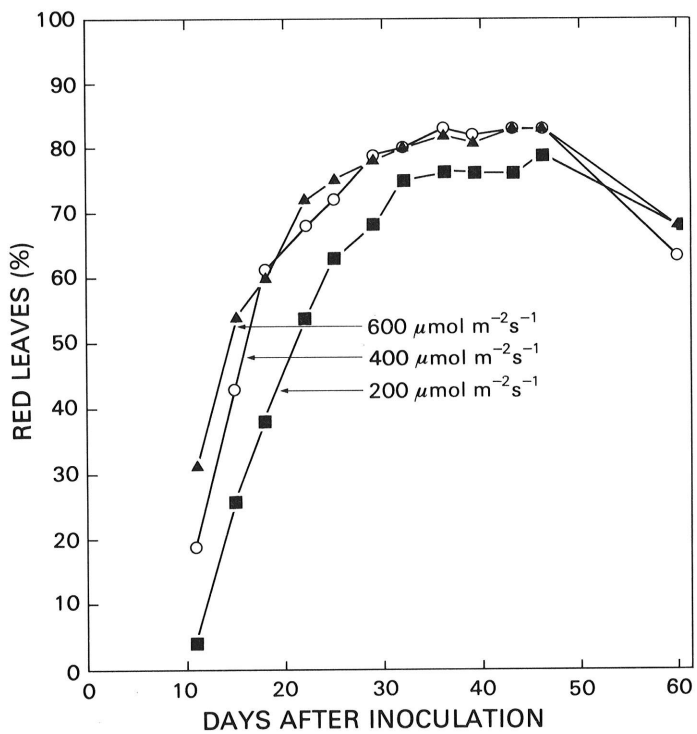

Fig. 2. Time-course changes in percentages of live red leaves for plants of *Trifolium subterraneum* infected with subterranean clover red leaf virus and grown under three different light intensities. Percentages for 200 μmol were significantly lower than those for 400 and 600 μmol for days 11–46, while percentages for 400 and 600 μmol only differed at days 11 and 15.

μmol were the exceptions and were not significant at the last two times of measurement.

The mean numbers of leaves for control plants were greater ($P < 0.001$) than for infected plants at all three light intensities at all times of observation. Differences between control and infected plants increased as the experiment progressed.

The time-course response patterns for infected plants differed from those of control plants in that they reached a maximum at or after 46 days and then decreased. This decrease was associated with loss of older red-brown pigmented leaves. The decrease was greater at the two higher light intensities than at the lowest.

Percentage of red leaves on infected plants. At days 11 and 15 after inoculation the percentage of red leaves was greater ($P < 0.001$) for plants grown at 600 μmol than for those grown at 400 μmol, and this in turn was greater ($P < 0.001$) than for those grown at 200 μmol (Fig. 2). Subsequently, there were no differences in the percentages of red leaves between plants exposed to 600 and 400 μmol. However, the percentage of red leaves was higher for these intensities than for 200 μmol for all observation times up to day 46, when the percentages had risen to 83% for 600 and 400 μmol and 79% for 200 μmol. The percentage of red leaves was lower at 60 days after inoculation than at 46 days, due to loss of red leaves, probably associated with plant aging and/or shading from control plants (7). Shading was more pronounced at light intensities of 400 or 600 than at 200 μmol.

Fresh weight of tops and roots. Combined data for the two experiments indicate that the mean fresh weights of both tops and roots for infected and control plants at 600 μmol were greater ($P < 0.001$) than those at 400 μmol, and these in turn were greater ($P < 0.001$) than those at 200 μmol (Fig. 3A and B).

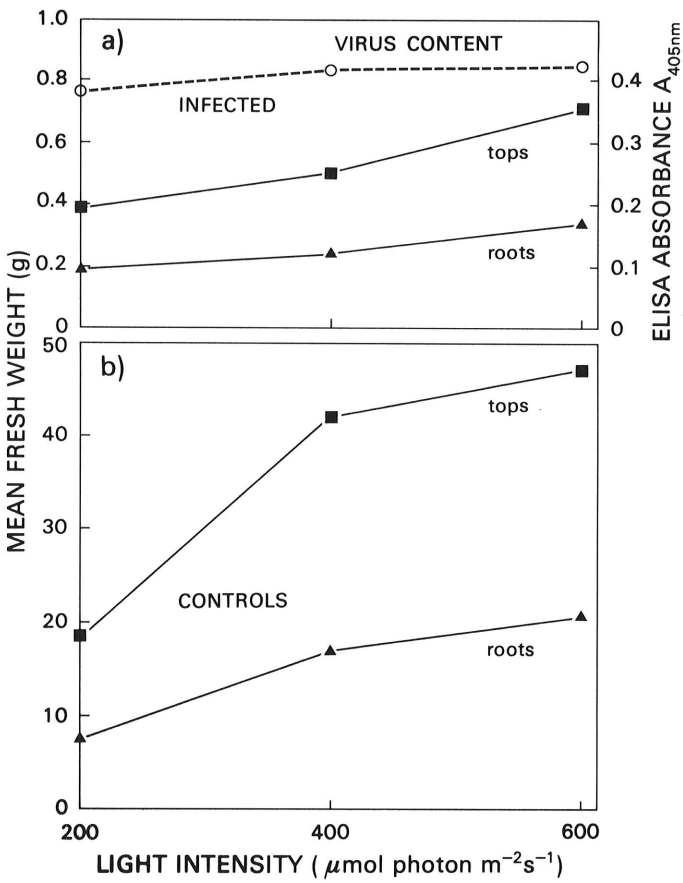

Fig. 3. Effects of three different light intensities on **(A)** fresh weight and relative virus content of sap samples from plants of *Trifolium subterraneum* infected for 60 days with subterranean clover red leaf virus, and **(B)** fresh weight of control plants after 60 days. Fresh weights for all three intensities were significantly different in all cases, whereas relative virus contents of infected plants were not significantly different.

At all three light intensities the weight of both tops and roots of control plants was greater than those of infected plants. For example at 600 μmol the mean fresh weights of tops of control and infected plants were 46.9 and 0.71 g, respectively, i.e., 66-fold greater for control plants.

Numbers of mortalities. For the combined Experiments A and B the number of mortalities at 200, 400, and 600 μmol were 10, 8, and 4, respectively, for SCRLV-infected plants and 2, 1, and 0 for control plants. The data show that there were more mortalities under low light than high and more for infected plants than for controls.

ELISA absorbance readings. Absorbance readings at A_{405nm} for each plate made at 30, 60, 90, and 120 min were highly correlated. Because data obtained at 120 min generally had lower coefficients of variation and because substrate was not limiting at this time, these were used in all analyses for whole inoculated plants. For separate tops and roots, 90-min readings were used because the 120-min readings exceeded the range of the colorimeter for a number of tops.

Table 1 summarizes the data obtained from the ELISA analyses of combined tops and roots. For all light intensities the A_{405nm} absorbance readings for sap samples of virus infected plants were higher than those for control plants ($P < 0.001$). Mean A_{405nm} readings (and hence the calculated mean relative virus concentration per sap samples [ng/ml]) at the three light intensities, were not significantly different (Table 1 and Fig. 3). However, the total virus yield per plant exposed to 600 μmol was greater than that for plants exposed to 400 or 200 μmol. This

TABLE 1. Quantitative assessment of subterranean clover red leaf virus content by enzyme-linked immunosorbent assay (ELISA) for sap samples from plants (combined tops and roots) of *Trifolium subterraneum* 'Mt. Barker' grown under three different light intensities and harvested 60 days after inoculation[x]

Light intensity (μmol m^{-2} s^{-1})	Mean ELISA readings (infected plants) (A_{405nm})	Virus concentration (ng/ml)[y]	Total virus yield per plant (ng)[z]	Mean ELISA readings (control plants) (A_{405nm})
200	0.382 a	33.3 a	102 b	0.118 a
400	0.415 a	37.9 a	134 b	0.108 a
600	0.423 a	39.6 a	199 a	0.113 a

[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 (0.2 g of tissue/ml of buffer) from calibrations of A_{405nm} readings for standard virus concentrations.
[z] Total virus yield per plant (ng) = concentration in sap sample (ng/ml) × 5 × fresh weight (g).

TABLE 2. Quantitative assessment of subterranean clover red leaf virus content by enzyme-linked immunosorbent assay (ELISA) for sap samples from tops and roots of plants of *Trifolium subterraneum* 'Mt. Barker' grown under three different light intensities and harvested 60 days after inoculation[x]

Light intensity (μmol m^{-2} s^{-1})	Mean ELISA readings (infected plants) (A_{405nm})		Virus concentration (ng/ml)[y]		Mean ELISA readings (control plants) (A_{405nm})	
	Tops	Roots	Tops	Roots	Tops	Roots
200	1.002	0.268	378	70	0.077	0.048
400	1.031	0.289	376	77	0.083	0.051
600	1.244	0.303	421	82	0.078	0.050

[x] For all three light intensities ELISA readings and virus concentrations were higher in tops than in roots ($P < 0.001$ for infected plants and $P < 0.01$ for control plants). Data were not different among light intensities.
[y] Virus concentration = mean relative virus concentration per sap sample (0.2 g of tissue/ml of buffer) from calibrations of A_{405nm} readings for standard virus concentrations.

reflected the greater fresh weight of plants grown at the higher light intensity.

Table 2 summarizes the mean ELISA A_{405nm} absorbance readings for separated tops and roots of infected and control plant material and provides calculated virus concentrations for infected plant sap. These values were greater ($P<0.001$) for tops than roots of infected plants at all three light intensities. The calculated virus concentrations were about fivefold greater for tops than roots. As in the analyses for combined tops and roots (Table 1), the mean A_{405nm} readings for sap of virus infected plants and, hence, the calculated virus concentrations in sap (ng/ml) at the three light intensities were not significantly different. A_{405nm} absorbance readings were greater for control tops than roots ($P<0.01$), but these values were considered to be too low to affect the observed differences between tops and roots of infected plants.

DISCUSSION

High light intensity promoted development of red leaves (Fig. 2) as well as growth of infected and control plants measured by numbers of leaves per plant (Fig. 1) and fresh weight (Fig. 3). High light intensity increased total virus yield per plant but had no effect on virus concentration in sap samples (ng/ml). The promotion of red leaf symptoms by high light intensity is in agreement with field observations of Rochow and Duffus (10) that high light intensities favor development of symptoms of luteovirus infections of a number of plants including the red leaf symptoms of oats infected with barley yellow dwarf virus. Symptoms of another luteovirus, beet western yellows, also were more severe and appeared more rapidly at high light intensity and lower temperature (8).

The higher values obtained for virus concentration (ng/ml) in plant sap from separated tops and roots (Table 2) as compared with those for combined tops and roots (Table 1) were due at least in part to differences in methodology. Virus concentration in plant sap for combined tops and roots in Table 1 ranged from 33 to 40 ng/ml. This is slightly above the virus concentration obtained in a previous experiment (7) in which plants were grown at the same temperature in glass cabinets exposed to natural light.

The virus concentration for SCRLV-infected plants of subterranean clover grown at three different light intensities as measured by ELISA was about fivefold greater in tops than in roots. Also using ELISA, Casper (4) reported that the content of potato leaf roll virus in potato and *Physalis floridana* L. was higher for roots than stems, leaf lamina, petioles, and midribs, but, in leaves of *Physalis*, the titer increased gradually over time to a level as high as in roots. These results contrast with those obtained for two other luteoviruses in which yield of virus was measured by UV absorption after extraction and purification. For barley yellow dwarf virus in oats, the yield from roots was about fourfold that from tops (5), and for pea leaf roll virus (synonym bean leaf roll virus [1]) infected pea plants, more virus also was extracted from roots than tops (2).

The present data and those reported elsewhere (7) show that yield of luteoviruses can vary with extraction method, plant part, and environmental factors.

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