Response of Two White Clover Clones to Peanut Stunt Virus and Ozone

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ABSTRACT

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Effects of ozone (O_3) and peanut stunt virus (PSV) on two clones of white clover (*Trifolium repens*) were measured in open-top field chambers. An O_3-resistant clone (NC-R) and an O_3-sensitive clone (NC-S), with and without PSV infection, were exposed to O_3 for 12-h day^{-1} for 111 days. The exposures were proportional to ambient O_3 and resulted in 12-h day^{-1} mean concentrations of 26, 45, 64, and 76 nL L^{-1} for the 111 days of exposure. Plant shoots were harvested five times to measure effects of O_3 and PSV on foliar injury, foliar chlorophyll, and shoot dry weight. Infection by PSV caused foliar chlorosis, which tended to be more severe on NC-S than on NC-R. PSV infection suppressed shoot dry weight accumulation of NC-R by 23% and of NC-S by 18%. O_3 also caused foliar chlorosis and suppressed shoot dry weight accumulation, and the severity of the effects increased with increased O_3 dose. Seasonal shoot weight of NC-S plants exposed in nonfiltered air chambers to ambient concentrations of O_3 (45 nL L^{-1}) was 20% less than for NC-S plants in charcoal-filtered air chambers (26 nL L^{-1}). Shoot weight of NC-R was not significantly affected by any of the O_3 treatments. The clone × O_3 interaction was significant for all measures for each harvest except for the first harvest. Although the O_3 concentrations remained relatively constant, the differences between NC-S and NC-R shoot weight became greater as the season progressed. There were no significant interactions between O_3 and PSV for any of the response measures.

White clover (*Trifolium repens* L.) and tall fescue (*Festuca arundinacea* Schreb.) are commonly grown together in the southeastern United States to provide high quality forage for livestock. However, the clover usually persists for only a few years. Microorganisms, insects, poor management practices, plant competition, poor drought tolerance, and tropospheric ozone (O_3) have been suggested as causes for white clover decline (3,5).

Tropospheric O_3 causes foliar injury and suppresses yield of many crops (11,16), and white clover is among the most sensitive (2,3,15,21). In a 2-yr field study with 'Tillman' white clover grown with fescue at Raleigh, NC, ambient O_3 suppressed shoot weight production of the clover by 22% each year (3). In a subsequent field study with 'Regal' white clover grown with fescue at Raleigh, ambient O_3 suppressed shoot weight production of the clover by 8% the first year and by 44% in the second (15,24). Clover plants that survived that study were propagated clonally to determine whether selection for resistance to O_3 had occurred. More individuals from the population of plants that survived exposure to high O_3 levels were resistant to foliar injury induced by short-term O_3 exposure than were individuals from the population that survived exposure to low O_3 levels (13). Whether the observed selection for resistance of foliage to injury from short-term (acute) O_3 exposures is related to resistance to growth effects caused by long-term (chronic) O_3 exposure is not known.

Peanut stunt virus (PSV) is one of the most prevalent viruses of white clover in the southeastern United States (1,20). One field study with two clover clones, propagated vegetatively from PSV-infected plants, showed that PSV caused from 49 to 91% loss in shoot weight accumulation, depending on the year and clone (23). In another field study, in which seedlings were inoculated with PSV before placement in the field, PSV caused a 28% suppression in shoot weight production, and the level of reduction was greater with increased duration of infection (8). Results from growth chamber studies with PSV (10) were similar to those reported for seedlings (8).

Virus infection often causes some protection from O_3 injury, and the type and degree of protection depends on the specific host and virus (4,6,7,22,26,27). However, there are exceptions to this generality. O_3 caused more injury on tobacco infected with tobacco streak virus than it did on uninfected tobacco (25). Three burley tobacco cultivars infected with tobacco etch virus tended to show less O_3-induced growth suppression than uninfected plants, but tobacco vein mottling virus tended to cause the opposite effect (26). For both viruses, the response to O_3 was dependent on the cultivar (26). The mechanisms for virus-induced changes in plant response to pollutants are unknown, although virus titer, plant age, and season of the year are important factors. There have been no studies to determine whether clover viruses affect clover response to O_3 or vice versa.

This study was done to determine: the differences in growth response to long-term O_3 exposure for two clover clones known to be sensitive or resistant to injury from short-term O_3 exposure; the relative importance of tropospheric O_3 and PSV in causing growth decline of white clover; and whether PSV infection affects clover response to O_3 or vice versa.

MATERIALS AND METHODS

White clover plants that survived a 2-yr field study to determine effects of chronic O_3 exposure (15,24) were propagated and screened for relative sensitivity to O_3. One clone survived 2 yr of exposure to high O_3 levels and subsequently was shown to be resistant to O_3, whereas the other had been exposed to low levels of O_3 and was very sensitive (13). The resistant clone (NC-

R) and the sensitive clone (NC-S) were subsequently freed of viruses by shoot-tip meristem culture (13).

On 6 February 1989, cuttings of each clone were placed in pots containing 0.22 L of a 2:1:1 mixture of sandy loam topsoil/sand/Metro Mix 220 (W. R. Grace Co. Cambridge, MA) in a greenhouse. Half of the plants (84 of each clone) were mechanically inoculated with PSV on each of 3 days (13, 14, and 15 March 1989). On each date, upper leaf surfaces were rubbed with expressed sap from PSV-infected white clover leaves in 0.03 M sodium phosphate buffer at pH 7.4, containing 0.02 M 2-mercaptoethanol and 600 mesh Carborundum. On 23 March, all plants were individually transplanted to pots containing 14 L of the 2:1:1 medium and were next inoculated with *Rhizobium*. Plants were cut to a height of 5 cm on 24 April.

The experimental design was a randomized complete block with four blocks of four O_3 treatments in open-top chambers (12) with subplots of two clones (NC-S and NC-R) and two virus treatments (plants with and without PSV inoculation). Each of the 16 chambers contained 16 pots (four pots each of the NC-S and NC-R clones, with and without inoculation with PSV). Plants were transferred to open-top field chambers on 3 May and were watered as needed to prevent moisture stress throughout the season. To decrease the chances of spreading PSV to noninoculated plants, all inoculated plants were randomly assigned to one side of each chamber (east or west). This arrangement allowed a minimum of 30 cm between inoculated and noninoculated plants. The two clones were arranged in two randomized 2 × 2 latin squares on each side of the chamber.

An enzyme-linked immunosorbent assay (ELISA) (19) done on 22 May for all PSV-inoculated plants showed that 60 of the 64 NC-S plants were infected, but that only 33 of the 64 NC-R plants were infected. However, there were at least two NC-R plants that tested positive for PSV in all but three chambers and four NC-S plants with PSV in all but four chambers. Inoculated plants that tested negative for PSV were not included in any data analyses or interpretation of results but were retained to maintain plot uniformity.

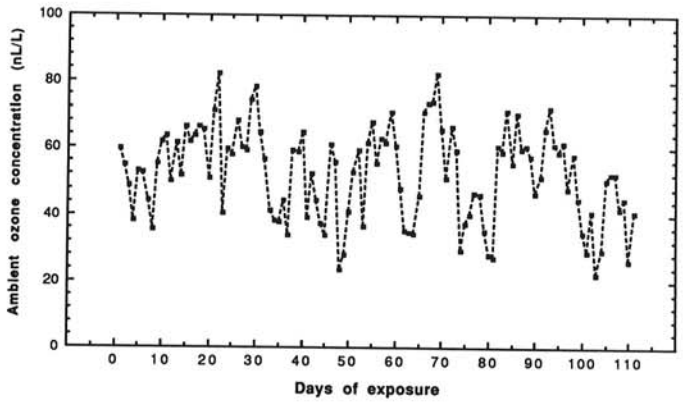

Fig. 1. Daily 12-h per day (0800–2000 h EST) ozone (O_3) concentrations in ambient air 8 km south of Raleigh, NC, during studies to measure effects of O_3 and peanut stunt virus on two ladino clover clones. The figure shows ambient O_3 concentrations for the 111 days from 4 May to 23 August 1989.

O_3 dispensing and monitoring techniques have been described previously (14). The O_3 treatments, which began on 4 May, were charcoal-filtered air, nonfiltered air, and two nonfiltered air treatments to which O_3 was added for 12-h day^{-1} (0800–2000 h EST) in amounts proportional to ambient O_3 concentrations. The seasonal (4 May to 23 August) 12-h day^{-1} mean O_3 concentrations in ambient air and in the charcoal-filtered air, nonfiltered air, and two O_3-added treatments were 51, 26, 45, 64, and 76 nL L^{-1}, respectively. The chamber fans were turned off from 2100 to 0500 h EST daily.

Plants were cut to a height of approximately 7 cm above the soil level on five dates during the experiment: 11 May; 5–6 June; 28–29 June; 25–26 July; and 23–24 August. Stolons growing outside of the perimeter of each pot were also cut. At each harvest, the shoots (leaves, petioles and/or stolons and flowers) were placed in paper bags, dried for 2 days at 55 C, and weighed.

Estimates of foliar injury and foliar chlorophyll analyses were performed one day before the second, fourth, and fifth harvests using five adjacent leaves on one stolon, starting with the youngest fully expanded leaf. Visible foliar injury was estimated for each leaf as the percentage of chlorosis and necrosis in 5% increments (0–100%). The same leaves were used for chlorophyll analyses as described by Knudsen et al (18). Leaves were placed in approximately 70 ml of ethyl alcohol (one brown glass container per five-leaf sample) and placed in the dark. After 3 days, the volume of alcohol for each container was increased to 100 ml, and the amounts of chlorophyll a and b were measured spectrophotometrically.

Starting on 19 May, all plants were sprayed at 2- to 3-wk intervals with Capture (bifenthrin), 3.2 EC, 3.1 mL L^{-1} to prevent infestation of aphids and decrease the potential for spread of viruses. ELISA tests were done on 7 August to determine whether plants not inoculated in March had become infected with PSV, alfalfa mosaic virus, clover yellow vein virus, red clover vein mosaic virus, or white clover mosaic virus. The results were negative for all but five plants: two NC-R and two NC-S plants (three separate plots) were positive for PSV, and one NC-R plant was positive for clover yellow vein virus. Therefore, data from these plants were discarded.

Data from inoculated plants that tested negative for PSV on 22 May were not used in statistical analyses, so the latin square design was incomplete. Therefore, the design was reduced to a split-split plot with unequal samples in the subplots. Analyses of variance were done for shoot weight, chlorophyll content, and foliar injury for each harvest separately, and for total seasonal shoot weight using SAS software (SAS Institute, Cary, NC). Mild heterogeneity of variance was found for the last two harvests, but data transformations were not considered to be advantageous.

TABLE 1. Mean squares from analyses of variance for effects of ozone on shoot (leaves, petioles, and/or stolons and flowers) dry weight (grams per pot), total chlorophyll (μg/ml), and foliar injury (mean percentage per leaf) for two white clover clones, with and without infection by peanut stunt virus[a]

		Harvest 1	Harvest 2			Harvest 3	Harvest 4			Harvest 5			Total
Source	df	Shoot dry wt	Shoot dry wt	Total chlorophyll	Foliar injury[b]	Shoot dry wt	Shoot dry wt	Total chlorophyll	Foliar injury	Shoot dry wt	Total chlorophyll	Foliar injury	Shoot dry wt
Block (B)	3	47*	787**	215	7	200*	50	1	14	52	77	7	1,650*
Ozone (O)	3	8	599**	188	117**	2,575**	6,021**	739**	438*	5,557**	693**	357**	50,462**
Virus (V)	1	206**	2,598**	197**	59**	4,919**	1,719**	78**	39**	3,225**	188**	43**	54,111**
V × O	3	3	40	61	1	74	162	37	0	248	76	10	985
Clone (C)	1	184**	4,081**	486**	194**	6,917**	20,132**	403**	204**	11,466**	229**	185**	170,344**
C × O	3	4	1,058**	378**	84**	3,035**	3,929**	230**	108**	1,450**	211**	50**	35,160**
C × V	1	16**	388**	75*	0	195**	94*	19	1	1,243**	0	3	6,650**
C × O × V	3	2	32	10	2	88	120	35	5	84	15	4	683

[a] * and ** = significant at the 0.05 and 0.01 level of confidence, respectively.
[b] Values for foliar injury have been multiplied by 0.001.

Regression analyses were done using SAS or Cricket Software (Cricket Software, Malvern, PA) with shoot weight and chlorophyll content as the dependent variables and mean 12-h day^{-1} O_3 concentrations (for individual growth periods and for the total season) as the independent variable.

RESULTS

The daily O_3 fluctuations (Fig. 1) and seasonal mean O_3 concentrations in ambient air during this experiment were similar to previous seasons at the site (15). The weather during the study was somewhat cooler and wetter than normal with daily mean maximum temperatures of 20, 28, 30, 31, and 30 C and rainfall of 6, 4, 17, 12, and 19 cm for growth periods 1–5, respectively.

Virus effects. Infection by PSV caused approximately 5–10% foliar injury (chlorosis) on both the O_3-sensitive clone (NC-S) and O_3-resistant clone (NC-R) at each harvest (Tables 1,2). The response of chlorophylls a and b to PSV and the O_3 treatments were similar, so chlorophyll responses will be presented in terms of total chlorophyll. Total chlorophyll content of PSV-infected NC-S plants (mean across all treatments) was 21 and 23% less than for uninfected NC-S plants for harvests 2 and 4, respectively (Table 2). The comparable numbers for NC-R were 5 and 8%, respectively. The clone × PSV interaction for chlorophyll was significant at harvest 2 (Table 1), but the PSV effect was similar for both clones at harvest 5.

Fewer NC-R than NC-S plants became infected by PSV from mechanical inoculation, and PSV generally caused smaller decreases of NC-R chlorophyll than of NC-S chlorophyll. The clone × PSV interaction was significant at harvest 2 (Table 1). However, NC-R was more sensitive to growth effects of PSV than was NC-S. For all chamber treatments combined, infection by PSV suppressed seasonal shoot weight production of NC-R by 23% and of NC-S by 18% (Table 3; Fig. 2), and the clone × PSV interaction for shoot weight was significant at each harvest (Table 1). There were no significant PSV × O_3 or three-way interactions for shoot weight. The differences in shoot weight response of the two clones to PSV at the different O_3 levels at the individual harvests (Table 3) were similar to those shown for seasonal shoot weight production (Table 3; Fig. 2).

O_3 effects. O_3 exposure caused foliar injury (chlorosis and necrosis) and decreased foliar chlorophyll content (Tables 1,2). The effects were significant except for chlorophyll at harvest 2. The effects of O_3 were much greater on the O_3-sensitive NC-S than on the O_3-resistant NC-R (Table 2) and caused the significant clone × O_3 interaction for all harvests for both measures (Table 1).

Except for harvest 1 (after 7 days of O_3 treatment), the effect of O_3 on shoot weight was significant at all harvests (Table 1). O_3 suppressed seasonal shoot production of NC-S more than that of NC-R (Table 3; Fig. 2), and the O_3 × clone interaction was significant at all harvests. The difference between shoot weight of plants grown in charcoal-filtered air and shoot weight of plants grown at higher O_3 concentrations increased as the season progressed. For example, shoot weight of NC-S in the nonfiltered air treatment (45 nL L^{-1} of O_3) was 88, 88, 79, and 56% of that in the charcoal-filtered air treatment (26 nL L^{-1} of O_3) for harvests 2, 3, 4, and 5, respectively. Likewise, shoot weight of NC-R in the highest O_3 treatment (76 nL L^{-1}) was 104, 102, 95, and 77% of that at 26 nL L^{-1} for harvests 2, 3, 4, and 5, respectively. The standardized slopes (standardized to a maximum of 1 by dividing the slope of each regression model by its intercept) of the shoot weight response increased for NC-S across all harvests and increased for NC-R between harvests 4 and 5 (Table 3). The same trends for increased response with successive exposure occurred for PSV-infected plants of both clones, and there were no PSV × O_3 interactions.

DISCUSSION

The present study showed that NC-S, which was more sensitive to foliar injury from acute O_3 exposure than NC-R, was also more sensitive than NC-R to growth effects caused by chronic O_3 exposure. These results agree with previous studies showing the relationship between O_3 doses and forage production of white clover (3,15,21). The present study also showed that the amount of yield loss caused by PSV and ambient O_3 was similar for NC-S and corroborated a previous report of differences in sensitivity to PSV between clones of white clover (23). Because cultivars of white clover are extremely heterozygous, cultivars probably contain genotypes with a wide range in sensitivity to both stresses, so the relative importance of O_3 and PSV for a given cultivar will presumably depend on the degree of sensitivity to both stresses among the genotypes.

The results suggest that chronic exposure to O_3 caused plants to become more sensitive to effects of subsequent exposure. However, the differences in response could have been caused by other factors, including the influence of weather patterns or physiological effects related to onset of flowering. We can only surmise as to the relative importance of these factors, because none was specifically studied. There were no obvious relationships between temperature or rainfall and the change in response to O_3; temperatures were relatively uniform, and plants were irrigated to prevent moisture stress. Flowering of the two clones began at different times (near the beginning of growth period 1 for NC-R and near

TABLE 2. Effects of chronic exposure to different levels of ozone on foliar injury and chlorophyll content of an ozone-resistant (NC-R) and an ozone sensitive (NC-S) white clover clone with and without infection by peanut stunt virus (V)

Growth period[b]	Number of exposure days	Ozone treatment[c]	12-h mean ozone concentration (nL L^{-1})	Percentage of foliar injury[a]				Total chlorophyll (μg/ml)[a]			
				NC-R	NC-S	NC-RV	NC-SV	NC-R	NC-S	NC-RV	NC-SV
2	25	CF	32	1	4	10	12	29.1	29.7	30.4	25.9
		NF	56	2	18	14	24	29.1	31.7	29.6	26.9
		NF-1	78	1	32	16	44	32.7	26.1	29.1	20.9
		NF-2	97	5	41	14	60	33.0	23.1	28.4	14.2
4	27	CF	25	0	1	17	5	19.5	22.8	18.4	17.1
		NF	43	2	31	10	38	20.8	16.7	22.7	15.4
		NF-1	63	24	50	33	64	18.0	11.9	16.2	7.6
		NF-2	72	24	65	33	74	18.0	7.9	13.0	5.5
5	29	CF	23	2	5	19	22	20.6	23.3	14.8	17.9
		NF	43	7	41	27	48	18.5	15.0	13.9	12.1
		NF-1	63	29	56	35	65	13.8	8.1	13.5	6.7
		NF-2	73	30	66	46	64	16.2	6.9	10.2	5.9

[a] Each value is the mean injury per leaf or mean chlorophyll per five leaves for 20 leaves (five leaves on one plant in four blocks).
[b] Growth period 2 = from 11 May to 4 June; growth period 4 = from 28 June to 24 July; growth period 5 = from 25 July to 22 August.
[c] Plants were exposed for 12 h per day in open-top field chambers to charcoal-filtered air (CF), nonfiltered air (NF), or to NF with different proportions of ambient ozone added.

TABLE 3. Dry weight of shoots (leaves, petioles, and/or stolons and flowers) of an ozone-resistant (NC-R) and an ozone-sensitive (NC-S) clone of white clover with and without infection by peanut stunt virus (V) after exposure to different levels of ozone[a]

Growth period	Days of growth	Ozone treatment[a]	12-h mean ozone concentration (nL L^{-1})	Ozone dose (nL L^{-1} h/100)	Shoot dry weight per plant (g)[b]			
					NC-R	NC-S	NC-RV	NC-SV
1	7	CF	31	26	10.0	7.9	7.3	6.4
		NF	48	40	10.1	7.3	7.2	5.7
		NF-1	57	48	10.5	7.5	7.8	6.1
		NF-2	65	55	10.1	7.7	8.0	6.4
2	25	CF	32	96	35.4	28.9	26.3	25.7
		NF	56	168	36.8	25.5	26.2	21.8
		NF-1	78	234	37.1	22.9	26.0	17.1
		NF-2	97	291	36.9	19.6	28.1	14.0
				Standardized slope[c]	+0.65	−4.19	+0.90	−5.78
3	23	CF	22	61	59.9	56.3	47.9	45.9
		NF	38	105	59.1	49.3	46.5	41.9
		NF-1	55	152	57.7	40.7	49.5	33.1
		NF-2	65	179	61.2	35.5	47.7	27.8
				Standardized slope	+0.15	−7.21	+0.43	−7.59
4	27	CF	25	81	50.4	41.9	44.4	34.9
		NF	43	139	50.5	33.4	42.0	27.0
		NF-1	63	204	48.7	18.7	45.0	15.9
		NF-2	72	233	48.1	15.0	38.4	12.1
				Standardized slope	−1.02	−10.37	−1.65	−10.40
5	29	CF	23	80	38.7	28.9	23.9	19.6
		NF	43	150	38.3	16.3	23.8	13.6
		NF-1	63	219	34.8	7.6	23.0	8.1
		NF-2	73	254	29.9	5.0	16.9	3.5
				Standardized slope	−3.78	−12.42	−4.12	−11.58
1–5	111	CF	26	344	194	164	150	133
		NF	45	602	195	132	146	110
		NF-1	64	857	189	97	151	80
		NF-2	76	1,012	186	83	139	64
				Standardized slope	−0.86	−8.02	−0.96	−8.21

[a] Plants were exposed for 12-h day^{-1} in open-top field chambers to charcoal-filtered air (CF), nonfiltered air (NF), and to NF with different proportions of ambient O_3 added (NF-1 and NF-2). Growth periods 1 = 4–10 May; 2 = 11 May–4 June; 3 = 5–27 June; 4 = 28 June–24 July; 5 = 25 July–22 August.

[b] Each value for NC-R and NC-S is the mean of 16 plants (four pots, four blocks [chambers]) except for two NC-S and three NC-R plants that were discarded because of virus infection; each value for NC-RV is the mean of six to nine plants (one to three pots, four blocks); each value for NC-SV is the mean of 13–16 plants (two to four pots, four blocks).

[c] Defined as the slope of the linear response model adjusted by dividing the slope by the intercept. Models were estimated using Cricket Software with the independent variable as O_3 in μL L^{-1}.

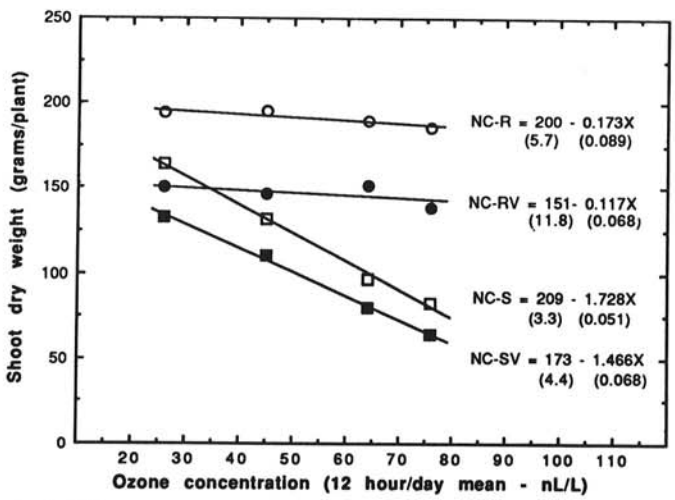

Fig. 2. Effects of chronic exposure to ozone (O_3) on seasonal shoot dry weight production by two clover clones (NC-R and NC-S) with and without infection by the peanut stunt virus (V). The regression models show the relationships between seasonal production of shoot dry weight per plant (grams) and seasonal 12-h per day O_3 concentration (X) in nL L^{-1}. Standard errors are shown in parenthesis. The models for NC-S and NC-SV were statistically significant (slope different from 0 as shown by an F test), but the models for NC-R and NC-RV were not significant.

the end of growth period 3 for NC-S). Both clones produced flowers until the end of the experiment. The greatest changes in response to O_3 occurred between growth periods 3 and 4 for both clones. Thus, the onset of flowering occurred near the time of a large change in O_3 response for NC-S but not for NC-R. The most plausible explanation for the change in response to a given O_3 dose is that the observed decrease in foliar chlorophyll concentration (Table 2) was accompanied by a decrease in photosynthesis and, therefore, decreased energy reserves in stolons and roots. O_3 has been shown to decrease white clover root-shoot ratios (20) and to decrease levels of starches in white clover roots (23). A gradual decrease in energy reserves probably would be accompanied by decreased capacity for detoxification or repair.

Because the effects of a given O_3 dose increased with successive growth periods, a cumulative O_3 dose metric would probably be more appropriate as the independent variable in regression analyses than a growth period mean. A cumulative O_3 dose, differentially weighted for successive growth periods, might be suitable. Further research is required to clarify the role of weather conditions and the level of cumulative effects for each clone.

The effect of PSV on shoot growth was variable over the season. For NC-R in charcoal-filtered air, PSV decreased shoot weight by 27, 26, 20, 12, and 38%, respectively, for the five consecutive harvests. The comparable values for NC-S were 19, 12, 18, 17, and 32%. No gradual trend for increased effects of PSV occurred with increased duration of infection. The large increase in PSV-

induced loss for both clones at harvest 5 may have been due to increased duration of infection combined with our practice of harvesting stolons that grew outside of the pots. Infection with PSV is known to reduce the root system in white clover (10), and harvesting stolons that grew outside of the pots decreased the establishment of secondary root systems.

The response of NC-S and NC-R, expressed as a ratio, could be a useful indicator of ambient O_3 levels and of the O_3 effects on other crop species. The usefulness for indicating ambient O_3 will depend on how much the two clones vary in response to other factors that affect growth. Measurements of shoot weight production indicated little or no effect of differences in weather conditions on the relative growth of NC-S and NC-R in charcoal-filtered air; the percentage of the total seasonal shoot weight produced during each growth period was almost identical for both clones. Further development of these clones as an O_3 indicator will require more data on their relative response to variation in weather conditions, edaphic factors, biotic diseases, as well as other atmospheric factors such as carbon dioxide and sulfur dioxide. Using the clover system to indicate the effects of O_3 on other crops will require knowledge of how given factors affect clover response to O_3 relative to how the same factors affect response of other crops to O_3. In other words, this will require information on how the clover clones and other crops respond to O_3 over a wide range of conditions.

Although PSV-resistant germ plasms that are adapted to the southeastern United States (9,17) are available, their reaction to O_3 is not known. Thus, characterization of the overall level of resistance to O_3 and PSV in white clover strains might be worthwhile as part of the development of strains with improved persistence in the Southeast.

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