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Covariate Measurements for Increasing the Precision of Plant Response to O_3 and SO_2

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Abstract. **Lettuce (*Lactuca sativa* L. cv. Grand Rapids) and radish (*Raphanus sativus* L. cv. Cherry Belle) plants growing at baseline environmental conditions were exposed to charcoal-filtered air, 0.40 ppm (v/v) ozone, and 0.80 ppm sulfur dioxide alone or in combination for 6 hours at 14 days from seeding. Analysis of covariance was used to account for significant within-treatment variation in plant growth. Covariates used were: planar leaf area (PLA) at 14 days for leaf area, fresh weight, and dry weight at harvest; plastochron index (PI) at 14 days for PI at harvest; and hypocotyl diameter for hypocotyl weights of radish roots at harvest. The covariates reduced the variability (standard geometric errors) of the response variables and increased the precision of statistical tests substantially for lettuce but much less for radish. For lettuce, the effect of the gas mixture on plant growth and foliar injury was less severe than that of the single gases. Radish plants, in contrast, exhibited no response to SO_2 and the effects of O_3 and the mixture on foliar injury and plant growth were similar.**

Air pollution effects on plant growth may be more subtle and difficult to detect than foliar injury, especially when there is large plant-to-plant variation as observed with lettuce (3). Analysis of covariance, in this situation, may be an efficient means of accounting for within-treatment variation (9). Nondestructive measures of plant growth are well-suited for analysis of covariance and should increase the precision of the harvest data from air pollution studies.

Lettuce has been used as a test plant in standardized growth comparisons in controlled-environment chambers among various laboratories (3). In these studies, 'Grand Rapids' lettuce displayed substantial variation within populations, even though mean growth rates were comparable among several laboratories. Preliminary studies in our controlled-environment chambers yielded essentially the same growth curve for 'Grand Rapids' lettuce as published in the baseline study (3), with significant variation within the population.

Information available concerning the sensitivity of lettuce to air pollutants is limited. Cultivar differences in O_3-induced foliar injury have been reported (8), but there are no accounts of differential effects on growth and development. Ozone reduced the growth of a head lettuce cultivar about 50% at a mean O_3 concentration of 0.106 ppm, in field studies, based on 7 hr day^{-1} over the growing season (5). The responses of many species to mixtures of SO_2 and O_3 have been studied (6), but there is no information on lettuce. In contrast, radish growth responses to mixtures of O_3 and SO_2 have been studied extensively (7, 10, 11). 'Cherry Belle' radish is sensitive to O_3 (8) and has been the most widely used cultivar in pollutant mixture studies.

The objectives of this research were: 1) to evaluate the usefulness of analysis of covariance to improve detection of subtle air-pollution-induced changes in plant growth; and 2) to study the effects of O_3 and SO_2 on the growth and foliar injury of lettuce and radish.

'Grand Rapids' lettuce and 'Cherry Belle' radish were grown from seed in 10-cm-diameter pots containing a peat, vermiculite, perlite mixture (Pro-Mix BX) and maintained in Conviron Model E15 growth chambers with similar conditions to baseline studies (3): day/night temperatures, 25°/20° ± 1°C; relative humidity, 72 ± 5%; photosynthetic photon flux density, 325 ± 10 $\mu mol\ s^{-1}\ m^{-2}$ at the top of the plant canopy for 16 hr per day with 75% and 25% input wattage from cool-white fluorescent and incandescent lamps, respectively. Seedlings were thinned to one per pot at 7 days. The plants were watered daily with North Carolina State University Phytotron Nutrient Solution (1). The plants were exposed in modified growth-chamber exposure chambers (4) with 2 exposure chambers in each of 2 growth chambers (Sherer Model CEL 37-14) and maintained at similar environmental conditions to those used in the plant culture chambers. The exposure chamber modifications included increased air flow (100 cfm) to reduce the leaf boundary layer resistance to gas exchange, an upper plenum with a perforated barrier for better mixing and uniformity of concentration in the chamber, a partial recirculating air system, and slightly taller chamber size. Ozone was generated by a shielded ultraviolet lamp in the lower plenum of each chamber and monitored with a Dasibi Model 1003 AH analyzer. Sulfur dioxide was metered from a tank containing 1% SO_2 in N_2 and monitored with a fluorescent SO_2 analyzer (ThermoElectron Series 43 or Monitor Labs Model 8850). The O_3 analyzers were calibrated with a Dasibi Model 1008 PC UV standard source and the SO_2 analyzers were calibrated with a Bendix Model 8861 DATM field calibration system using tank SO_2 traceable to the National Bureau of Standards.

Plants (14 days old) were exposed to one of four SO_2 and/or O_3 combinations for 6 hrs: pollutant-free air (control), 0.4 ppm O_3, 0.8 ppm SO_2, or 0.4 ppm O_3 + 0.8 ppm SO_2. This experimental design was replicated 4 times with 'Grand Rapids' lettuce and twice

Table 1. Examples of within-population variation among control lettuce and radish plants.

	Lettuce				Radish			
Response variable	Mean$_g$[z]	SGD[y]	Max	Min	Mean$_g$[z]	SGD[y]	Max	Min
Leaf area (cm^2)	139	1.31	203	85	126	1.42	192	76
Leaf fresh wt (g)	4.17	1.37	5.88	2.21	5.09	1.47	8.21	2.78
Leaf dry wt (g)	0.243	1.39	0.371	0.123	0.435	1.55	0.731	0.230
Plastochron index	5.46	1.13	6.47	4.42	5.09	1.14	6.70	4.29
Hypocotyl fresh wt (g)	---	---	---	---	10.1	1.75	18.3	4.53
Hypocotyl dry wt (g)	---	---	---	---	0.574	1.77	1.21	0.253

[z] Geometric means are based on 4 replicates with 3 plants per replicate for lettuce and 2 replicates with 6 plants per replicate for radish. Means were not adjusted by analysis of covariance.
[y] Pooled standard geometric deviations (SGD) were calculated based on variation within replicates.

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with 'Cherry Belle' radish, with the assignment of treatments to exposure chambers changed for each replication. Just prior to pollutant exposure, PLA and PI (2) were measured on each plant for use as covariates in subsequent statistical analyses. Planar leaf area was determined by gently placing a transparent, 10-mm grid sheet over the plant and counting the number of grid intersections directly over leaf tissue. Hypocotyl diameter before exposure was used as a covariate for radish hypocotyl measurements at harvest. Leaf area, fresh weight, dry weight, and PI were measured on each plant 3 days after exposure. Weight was separated into leaf and hypocotyl components on radish plants. Foliar injury was measured as both percentage of leaves injured and percentage of leaf area injured for each plant. Because the relationship between the means and SD of the growth variables indicated they followed a lognormal statistical distribution, the different variables were transformed to their natural logarithms before analyses of variance and covariance were performed (9). Geometric means and standard geometric deviations were obtained by computing the antilog of means and SD of the transformed data. Transformation did not improve the variance stability for foliar injury estimates; therefore, the data were not transformed.

There were relatively large variations among lettuce and radish plants within the treatments (Table 1). Using standard geometric deviations as a measure of within-population variability, radish exhibited a higher variability than lettuce. The standard geometric deviations of lettuce were about 30% to 40% of the mean, while those of radish were about 40% to 80%. The natural variations among plants may be greater than the effects of pollutants, making it difficult to detect these effects. Initial studies indicated that the covariates ln PLA and ln PI were both highly correlated ($r > 0.98$) with the logarithms of leaf area, leaf fresh weight, leaf dry weight, and PI so that ln PLA was used as a covariate for leaf area, fresh weight, and dry weight, and ln PI before exposure was used as a covariate for ln PI at the time of harvest. Before performing the analysis of covariance for the 'Grand Rapids' lettuce and 'Cherry Belle' radish experiments, the assumption was tested that the slopes of the covariates did not change from treatment to treatment. There were no significant differences in slopes; therefore, the analysis of covariance was appropriate for all growth variables (F tests for equal slopes, $\alpha = 5\%$).

The use of PLA and PI as covariates significantly improved the precision of estimates of pollutant effects on ln leaf area and ln leaf fresh and dry weights for lettuce and radish (analysis of covariance, $P < 0.1\%$) (Table 2). The SE for ln lettuce leaf parameters were reduced about 70% using ln PLA as a covariate and for radish about 13% (Table 2). The use of ln PI as a covariate reduced the SE of the ln PI for lettuce and radish about 70% and 30%, respectively. In contrast, radish ln hypocotyl diameter at exposure was not an effective covariate for ln hypocotyl growth variables, as there were no significant improvements in the SE of those variables by the use of the covariate.

The analysis of variance for lettuce indicated no pollutant effects on leaf area or leaf fresh weight, while analysis of covariance detected a significant interaction of effects of SO_2 and O_3 on both leaf area and leaf fresh weight (Table 3). Both analyses detected significant effects on dry weight; neither detected effects on PI. When there were significant pollutant effects, the effect of the interaction of SO_2 and O_3 on the foliar growth parameters was less severe than that of either pollutant alone, an antagonistic effect. The individual gases at the concentrations tested were sufficient to induce significant foliar injury. The effect of the pollutant mixture,

Table 2. Reduction in among-plant variation with the use of covariates for lettuce and radish.

Response variable	Lettuce standard geometric error[z]		Reduction[y] (%)	Radish standard geometric error[z]		Reduction[y] (%)
	ANOVA	ANOCOVA		ANOVA	ANOCOVA	
Leaf area	1.112	1.035	67.6	1.124	1.105	14.6
Leaf fresh wt	1.119	1.034	70.3	1.138	1.120	12.3
Leaf dry wt	1.127	1.031	74.5	1.135	1.117	12.6
Plastochron index	1.031	1.009	70.7	1.052	1.036	30.2
Hypocotyl diam	---	---	---	1.074	1.072	2.6
Hypocotyl fresh wt	---	---	---	1.181	1.184	− 1.5
Hypocotyl dry wt	---	---	---	1.201	1.205	− 1.8

[z] Standard geometric errors were calculated from analyses of variance (ANOVA) or covariance (ANOCOVA) based on 11 lettuce plants or 12 radish plants per treatment.

[y] The percentage of reduction in SE by the use of covariance analysis. Calculations were performed in the logarithmic scale.

Table 3. Response of 'Grand Rapids' lettuce to SO_2 and O_3.

Response variable[z]	Covariate	SO_2/O_3 combination (ppm)				Significant factors[y]	
		0.0	0.80/0	0/0.40	0.80/ 0.40	ANOVA	ANOCOVA
Leaf area (cm^2 plant^{-1})	PLA	130	114	113	121	None	$SO_2 \times O_3$
Fresh wt (g plant^{-1})	PLA	3.87	3.30	3.30	3.59	None	$SO_2 \times O_3$
Dry wt (g plant^{-1})	PLA	0.224	0.181	0.193	0.208	$SO_2 \times O_3$	$SO_2 \times O_3$
Plastochron index	PI	5.29	5.28	5.37	5.40	None	None
No. leaves injured (%)	None	3	43	71	48	$SO_2 \times O_3$	---
Area injured per leaf (%)	None	0.28	11	24	4.1	$SO_2 \times O_3$	---

[z] For leaf area, fresh wt, dry wt, and plastochron index, each entry is the geometric mean of 11 plants after adjustment for pretreatment differences by use of covariates. The foliar injury entries are arithmetic means.

[y] Results of analysis of variance and analysis of covariance. Factors tested (F test, $\alpha = 5\%$) were O_3 effect, SO_2 effect, and $O_3 \times SO_2$ interaction. If a significant interaction occurred, the main effects were not tested.

Table 4. Response of 'Cherry Belle' radish to SO_2 and O_3.

Response variable[z]	Covariate	SO_2/O_3 combination (ppm)				Significant factors[y]	
		0.0	0.80/0	0/0.40	0.80/0.40	ANOVA	ANOCOVA
Leaf area (cm^2 plant^{-1})	PLA	124	128	111	96	None	None
Leaf fresh wt (g plant^{-1})	PLA	5.02	5.28	4.26	4.15	None	None
Leaf dry wt (g plant^{-1})	PLA	0.428	0.412	0.400	0.393	None	None
Plastochron index	PI	5.20	5.47	5.60	5.41	None	$SO_2 \times O_3$
Hypocotyl diam (mm)	HD	21	19	17	15	None	O_3
Hypocotyl fresh wt (g plant^{-1})	HD	10.10	9.25	5.49	4.64	O_3	O_3
Hypocotyl dry wt (g plant^{-1})	HD	0.573	0.482	0.287	0.253	O_3	O_3
No. leaves injured (%)	None	0	0	89	87	O_3	---
Area injured per leaf (%)	None	0	0	53	57	O_3	---

[z] The entries for the growth variables are geometric means of 12 plants after adjustment for pretreatment differences by use of covariates. The foliar injury entries are arithmetic means of 12 plants.

[y] Results of analysis of variance (ANOVA) and analysis of covariance (ANOCOVA). Factors tested (F test, $\alpha = 5\%$) were O_3 effect, SO_2 effect, and $O_3 \times SO_2$ interaction. If a significant interaction occurred, the main effects were not tested.

however, was less severe than the effects of O_3 alone and similar to or less severe than the effects of SO_2 alone for both measures of foliar injury. For lettuce, the general effect of the pollutant mixture was to reduce the effects of the individual gases.

No significant effects were found on any foliar growth response parameters using either statistical analysis with radish (Table 4). Ozone reduced hypocotyl fresh and dry weights, and SO_2 had no significant effect. Analysis of covariance indicated an O_3 effect in decreasing hypocotyl diameter and an interactive effect on PI in addition to the effects detected by analysis of variance. The rate of leaf development in terms of PI was increased by O_3 singly but not in mixture. The use of the analysis of covariance with radish detected fewer significant effects than with lettuce. This probably resulted from the greater, inherent variability in radish than in lettuce, even following analysis of covariance (Table 2). As a result, reductions of about 20% in leaf area and leaf fresh weight in radish exposed to O_3 were not declared statistically significant, while smaller growth reductions in lettuce were found to be statistically significant. This indicates either that additional observations should be included in the radish studies to detect significant effects or that other covariates should be selected to detect effects of the magnitude observed in this study. Radish responses were dominated typically by response to O_3 alone, in contrast to lettuce, which tended to show a significant interaction between O_3 and SO_2.

The use of nondestructive measures at the beginning of an exposure to an environmental stress appears to be a practical means of increasing the precision of statistical tests of plant responses. Analysis of covariance significantly reduced the variability and increased the precision, especially for lettuce. Subtle changes induced by environmental stresses are readily detectable with a careful selection of covariates, thus this procedure has particular value for plant researchers.

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Vegetative Propagation of Winged Bean (*Psophocarpus tetragonolobus*)

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Worldwide interest in the winged bean [*Psophocarpus tetragonolobus* (L.) D.C.] as a food legume has increased in the past decade (6). The crop has the potential for greatly improving human nutrition needs in the tropics due to the relatively high protein content of the seeds (30–42%) and tuberous roots (8–20%, dry weight basis) and the fact that all plant parts except the stem are edible (4). It also is grown as a cover crop or green manure crop and is useful for livestock feed (5).

It frequently is desirable in a genetic or breeding program to obtain crosses and/or additional seeds from a particular plant after the normal period of anthesis is over. Herklots (1) stated that the winged bean plant dies after fruiting in the tropics, and new shoots are formed from tuberous roots the following season. Vegetative propagation of the winged bean by stem cuttings (2) and tissue culture techniques (3) have been reported. The purpose of this study was to investigate various treatments of winged bean roots in the greenhouse that might enhance plant production from tuberous roots after the initial seed crop had been produced.

Two greenhouse experiments were conducted during 1981 and 1982. Seeds of WB 10-9, a winged bean line obtained from Puerto Rico, were planted in 4-liter pots on January 20, 1981, in the first experiment. Seeds were harvested May 19, 1981; the root system [crown (1)] of each plant was washed in a 3 g/liter benomyl fungicide solution and then drained and weighed. Crowns were paired by weight and assigned at random into one of 2 groups. Crowns were intact in the first group and subdivided into 3 to 6 pieces each in the 2nd group. Both groups were further divided and either 1) not cured before replanting; 2) cured at 30°C for 4 days before replanting; or 3) cured at 30°C for 8 days before replanting. Roots were replanted about 2 cm deep in a steam-pasteurized medium of commercial potting mix (shredded peatmoss and vermiculite) and sand (3:1, by volume). Six replications of plots consisting of a single intact or separated crown were arranged in a split-plot design with root separation as main plots and curing treatments as subplots.

The first plants (slips) derived from adventitious buds on the crowns or root pieces emerged 17 days after replanting (Fig. 1). Soil was carefully removed from around the rooted stem when the slips had developed 2 or 3 leaves. Slips were detached from the crown and transplanted into 4-liter pots in the medium previously described. Slip production continued in this study for about 3 months.

Seeds were sown in pots in the greenhouse on December 14, 1981, and plants were grown to maturity in the 2nd experiment. One week before and 2 weeks after seed harvest, watering was halted on half the pots while the other half received normal watering to maintain the medium at near field capacity. The seeds were harvested on March 17, 1982, and half the

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