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Measurement of Ozone Injury by Determination of Leaf Chlorophyll Concentration[1]

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ABSTRACT

A simple, rapid procedure is described for evaluating ozone injury to leaves of *Phaseolus vulgaris* L. cv. Pinto. Leaf chlorophyll is extracted with ethanol and analyzed spectrophotometrically; the concentration is expressed on the basis of leaf dry weight.

The per cent chlorophyll reduction of ozone-injured leaves was highly correlated with the per cent visible necrosis and chlorosis (r = 0.96). The variability in injury estimates with chlorophyll analysis was slightly less than with visual evaluation. An evaluation of chlorophyll *a* and *b* concentrations separately showed that the chlorophyll *a*/*b* ratio decreased with increasing amounts of injury. Chlorophyll determinations made for leaves harvested at intervals after an ozone treatment indicated that maximum chlorophyll reduction had occurred by 4 days.

This procedure for measuring ozone injury should be useful in eliminating the human bias associated with visible estimates of injury.

The most common method of assessing plant injury due to air pollution or pathogens involves visual estimation of the per cent leaf surface area that is necrotic and chlorotic (3). This type of injury measurement contains inherent observer bias. An observer's perspective will likely vary between experiments and among plants within an experiment. One observer will likely make different evalutions from another. Observer bias is reduced by having more than one observer evaluate each sample (8). Another problem associated with visual injury estimates is that results from different laboratories may vary greatly due to the lack of accepted standards for visual injury evaluation.

Several attempts have been made to measure Chl loss by extraction and spectrophotometry for evaluation of ozone injury (1, 4-8). A problem with these procedures is that they do not utilize a suitable unit of measurement for expressing Chl concentration. Measurement of leaf dry wt could provide a suitable basis for expression of Chl concentration; however, in previously reported methods, dry wt determinations were not made because most extraction procedures involved grinding the leaf tissue. Expression of Chl concentration based on a measurement of fresh wt (1, 6) is undesirable because injured tissue becomes desiccated and therefore fresh wt is decreased along with the reduction in Chl. Concentrations of Chl based on content per plant or per leaf (4, 5) are imprecise due to variations in plant and leaf size. Measurement of leaf area as a basis for expressing Chl concentration (8) is difficult because of the distortion of leaves that occurs with severe injury. It is not possible to obtain a true estimate of injury to a whole leaf by sampling segments of leaves (7) because of the uneven distribution of ozone injury on leaves.

We have developed a simple, rapid procedure for determining Chl concentration as Chl per unit dry wt. In this paper we present the procedure and describe studies conducted to evaluate it as a method for assessing injury to ozone-treated leaves of bean plants.

MATERIALS AND METHODS

Chl Determination Procedure. The following Chl extraction and analysis procedure was developed for use with plant leaves. The solution volumes indicated were found suitable for bean unifoliate leaves.

The blades of both unifoliate leaves of each bean plant were excised (excluding the pulvinus). Each leaf was rolled, folded, and placed in a 28-ml jar. Each jar was then filled with 100% ethanol, capped, and stored in the dark.

After ~24 hr in storage, the ethanol-Chl solution in a jar was decanted into a 227-ml jar. The jar containing the leaf was then rinsed by filling it one-half to two-thirds full with ethanol, and this rinse solution was added to the 227-ml jar. The jar with the leaf was then refilled with ethanol, capped, and returned to the dark. After another 24 hr, the decanting and rinsing were repeated and the solutions added to the same 227-ml jar. The procedure was repeated a third time after another 24-hr period. After the final rinse, the leaf was placed in a drying oven at 70 C, dried for 3 days, and then weighed.

The ethanol-Chl solution was decanted into a 200-ml volumetric flask and brought to 200 ml with ethanol. An aliquot, ~10 ml, of this final solution was placed in a test tube and the remainder was discarded.

Absorbances of the Chl extract at 665 and 649 nm were measured spectrophotometrically. To convert these readings to Chl content of the leaf, the following equations were utilized (9):

$$\frac{\mu g \text{ Chl } a}{\text{ml solution}} = (13.70)(A_{665\text{ nm}}) - (5.76)(A_{649\text{ nm}}) \quad (1)$$

$$\frac{\mu g \text{ Chl } b}{\text{ml solution}} = (25.80)(A_{649\text{ nm}}) - (7.60)(A_{665\text{ nm}}) \quad (2)$$

The Chl concentration, expressed as μg Chl/mg dry wt, was then obtained.

Evaluation of Chl Determination Procedure. The completeness of Chl extraction was determined for healthy and ozone-injured leaves. After the final Chl extraction, the leaves were ground and 15 ml of ethanol were added. This suspension was

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centrifuged and the Chl content of the supernatant was determined.

An evalution of Chl extraction with acetone was made for comparison to extraction with ethanol, as most previously reported Chl extraction procedures for evaluating ozone injury utilized acetone as the solvent (1, 5-8). The same extraction procedure was followed, using 80% acetone with healthy leaves. Absorbance readings were taken at 652 nm (2). The completeness of Chl extraction was evaluated in the same manner as described above for ethanol-extracted leaves.

The stability of Chl in ethanol over the 72-hr extraction period was determined under conditions of light and dark, and at room temperature (~20 C) and refrigerator temperature (~5 C). Also, the stability of Chl in ethanol was compared to the stability of Chl in acetone over the 72-hr period in the dark at room temperature.

The stability of the ethanol-Chl solutions was determined for periods of time after extraction with solutions kept in the dark at room temperature.

Ozone Treatments and Injury Measurements. Bean plants (*Phaseolus vulgaris* L. cv. Pinto, Northrup, King and Co. Lot No. 35486-00400) were grown from seed in the Biotron at the University of Wisconsin. A 16-hr photoperiod at 20.2 ± 0.3 C (± SD) air temperature and 69 ± 5% relative humidity was used during this study. The temperature measurements were taken daily with thermocouples and relative humidity was measured once a day with a thermistor psychrometer. Cool white fluorescent and incandescent lamps provided a quantum flux density of 30.42 ± 1.47 nE/sec·cm² (400-700 nm) and IR radiation of 0.100 ± 0.005 w/m²·nm (755-825 nm) at the top of the pots. The light measurements were taken with a Lambda model LI-185 meter, using quantum and IR sensors. Plants were grown in peat-vermiculite, contained in 10-cm plastic pots. From the time of seeding, the pots were supplied with an excess of half-strength modified Hoagland nutrient solution through an automatic watering system four times per day at the rate of 40 ml per pot per watering. The plants were selected for treatment at 11 days.

Plants were exposed to ozone in single, 1-hr fumigations 12 days after seeding. The fumigations were conducted in a 0.3-m³ Plexiglas chamber in an adjacent controlled environment room with conditions maintained within 5% of the conditions in the growing room. The ozone was generated with UV lamps, monitored with a Mast oxidant meter, and recorded continuously. Plants were treated between the 4th and the 7th hr of the 16-hr light period. Following treatment, they were returned to the growing room until harvested. One to three fumigations were conducted each day, with six plants fumigated at each time. Each group of fumigated plants was matched with an equal number of control plants.

The optimum time to harvest leaves following fumigation was determined for plants from six different fumigations at 0.90 μl/l ozone. The unifoliate leaves of plants from each fumigation were harvested for Chl determinations at 2, 4, and 6 days after treatment.

The Chl determination procedure was compared to visual observation for estimating injury by fumigating plants with different ozone concentrations between 0.50 and 0.90 μl/l. Six plants were fumigated in each of 10 separate, 1-hr fumigations, and the leaves were harvested 4 days after treatment. For the visual evaluation of injury, the per cent of a unifoliate leaf's surface area showing necrosis and chlorosis was estimated to the nearest 5% by the senior author. The injury estimate for a leaf was taken as the average of the values obtained for the upper and the lower leaf surfaces. After the visual evaluation of injury, the Chl determination procedure was followed for each leaf. For this measurement, injury was determined by calculating the per cent reduction in Chl concentration of treated leaves compared to the Chl concentration of untreated leaves.

RESULTS AND DISCUSSION

Evaluation of Chl Determination Procedure. The extraction procedure with whole, uncut leaves effectively removes the Chl, as only a small amount of additional Chl was recovered from leaves subjected to grinding and further extraction. The extractions with the whole leaves were found to remove more than 99% of the total Chl obtained from healthy leaves with the combined extraction procedures. Slightly less (96%) was recovered from severely injured leaves.

Acetone was not as effective as ethanol for extracting Chl from whole leaves, as only 92% of the total Chl was recovered from healthy leaves when this solvent was utilized. Acetone was also found to be more difficult to work with because of its higher volatility.

No significant degradation of Chl in ethanol was observed after 72 hr for samples kept in the dark. However, 54% degradation of Chl was observed after 72 hr for samples kept in the light. There was no significant degradation of Chl at either room temperature or refrigerator temperature after 72 hr for samples in the dark.

Analysis of the stability of the ethanol-Chl solutions indicated less than 3% (± SD) degradation of Chl for up to 6 weeks of storage in the dark.

The formulas for determining Chl solution concentrations were developed for Chl extracted with 96% ethanol (9). We found no difference in absorbance readings between Chl extracted from leaves in 96% and in 100% ethanol. We considered it desirable to start with 100% ethanol to avoid measurement errors that might develop from water absorption.

The actual total working time required to carry out the Chl determination procedure was about 15 min/sample.

Ozone Treatments and Injury Measurements. Analysis of Chl reduction in plants harvested at intervals after fumigation indicated that maximum reduction had occurred by 4 days after fumigation. The plants harvested at 2, 4, and 6 days had 58, 69, and 64% reduction in total Chl (Chl *a* + Chl *b*), respectively, compared to the control plants.

Treatment of plants at the different ozone concentrations resulted in injury to individual leaves ranging from none to 88.4% total Chl reduction and from 3 to 93% visible necrosis and chlorosis. In Figure 1, per cent visible necrosis and chlorosis is plotted against per cent Chl reduction for 116 leaves. The data were analyzed by the least squares method. The correlation coefficient, r, for the two methods is 0.9, and the standard deviation, $s_{y \cdot x}$, is 7.20. The line shown in the graph fits the equation, $y = (0.80)x + 5.8$. Although all of the leaves in this study showed some visible injury, the y intercept of 5.8 suggests that there can be a reduction in Chl without visible necrosis or chlorosis.

An analysis was made of the variability in the results obtained by Chl determination and by visual evaluation. The data for leaves subjected to the 10 ozone fumigations were evaluated using an analysis of variance in which six plants of each fumigation were samples and the two leaves of a plant were subsamples. The standard errors were 7.1 for per cent Chl reduction and 10 for per cent necrosis and chlorosis. The variability in Chl reduction between leaves on a plant was found to be one-seventh the magnitude of the variability between plants in a treatment. Thus, it would conserve time and provide almost as much precision to make a single, combined Chl extraction of both leaves on a plant rather than analyzing each leaf separately. For a sample size of six plants per treatment, and separate or combined extraction of two leaves on a

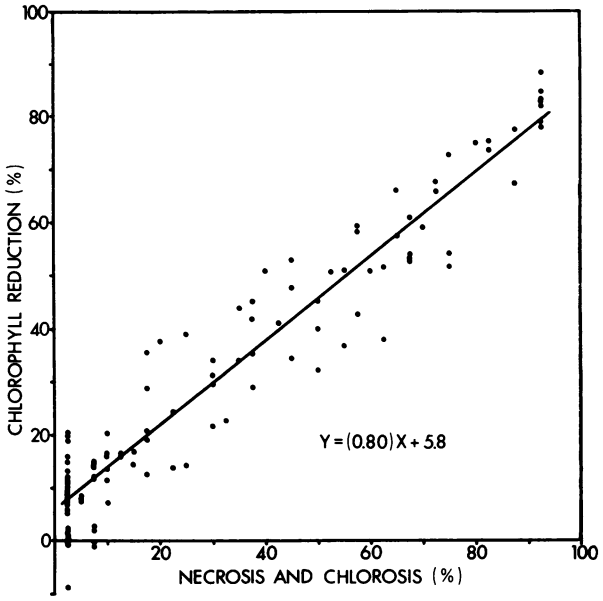

FIG. 1. Correlation of Chl reduction with visible necrosis and chlorosis of Pinto bean leaves fumigated with different ozone concentrations; $r = 0.92$, $n = 116$, and $s_{y \cdot x} = 7.20$.

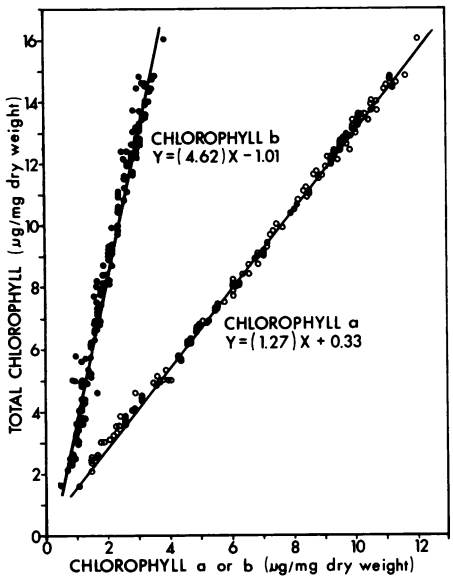

FIG. 2. Concentrations of Chl *a* and *b* plotted individually against total Chl (Chl *a* + Chl *b*) for Pinto bean leaves fumigated with different ozone concentrations. For Chl *a*, $r = 1.00$; for Chl b, $r = 0.99$; $n = 142$.

plant, a difference between two treatment means of approximately 15% in Chl reduction and 20% in visible necrosis and chlorosis would be significant at the 5% level.

Chl *a* and *b* concentrations for the leaves treated at different concentrations of ozone are plotted individually against total Chl concentration in Figure 2. The data were analyzed by the least squares method. The plotting for Chl *a* fits the equation $y = (1.27)x + 0.33, r = 1.00$; that for Chl *b* fits the equation $y = (4.62)x - 1.01, r = 0.99$. The Chl *a*/*b* ratios decreased as the total concentration of Chl decreased. For example, at 14 µg total Chl/mg dry wt the Chl *a*/*b* ratio is 3.31, at 8 µg total Chl/mg dry wt the ratio is 3.10, and at 2 µg total Chl/mg dry wt the ratio is 2.02. The average Chl *a* and *b* concentrations for the control plants fell exactly on the plotted lines with 14.23 µg total Chl/mg dry wt, and a Chl *a*/*b* ratio of 3.34.

There are two possible explanations for the decrease in Chl *a*/*b* ratios. First, Chl a may be more readily degraded by ozone than Chl *b*. Second, ozone may affect the synthesis of new Chl so that the synthesis of Chl *a* is reduced or the synthesis of Chl *b* is increased relative to uninjured leaves. Presently, it is not known whether ozone affects Chl directly, by degradation, or indirectly, by impairing the synthesis of new Chl. Further research on this is necessary in order to detemine the reason for the decrease in Chl *a*/*b* ratios with ozone injury.

CONCLUSIONS

The Chl determination procedure described herein is a practical, precise method of evaluating ozone injury to bean leaves. The method appears to estimate the same aspect of injury as visual observation with more precision than the latter and without human bias. The procedure does not require extensive training or experience for the person making the measurements. It requires more time than visual observation, but the stability of ethanol-Chl solutions for several weeks in the dark permits flexibility in the time of spectrophotometric analysis.

We feel that this Chl extraction procedure should prove particularly useful in the evaluation of cultivar responses, where differences in type of symptom expression make it difficult to estimate visible necrosis and chlorosis consistently. Preliminary investigations with other species, including corn, sorghum, peas, tobacco, barley, and wheat indicate that this procedure has wide applicability. It should also be useful in the evaluation of injury induced by other pollutants.

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