Influence of Air Pollution on Quantities of Caffeic Acid Isolated from Leaves of Phaseolus vulgaris

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ABSTRACT

Phenolic composition was significantly altered in Tempo bean leaves cultured in polluted ambient air. Fluorescent compounds were concentrated in the reddish-brown leaves. Caffeic acid concentrations were present in injured leaves at twice the concentration detected in healthy leaves. An anthocyanin could not be detected in injured leaves. Air pollutants are now known to affect phenolic metabolism in plants, as do plant pathogens and other physiological stress factors. Phytopathology 60:1626-1629.

Localized lesions on foliage of many species of plants occur as a result of photochemical oxidant injury (12). Variously pigmented areas ranging in color from red to brown, depending on plant species, cultural conditions, phytotoxicants, and duration of time after exposure are associated with lesions. The anatomical and morphological aberrations of plant structures resulting from exposure to photochemical toxicants have been described (5, 6, 8), but little is known of the biochemistry of lesion induction and development. Recently, Koukol & Dugger (7) reported that anthocyanin was formed in leaves of *Rumex crispus* after exposure to smog and ozone. Higher levels of polyphenols were measured in weather-flecked than in healthy tobacco leaves (10). Injuries inflicted by plant pathogens, chemicals, physical wounds, and adverse temp, moisture, and nutrient levels, induce lesions in which there are increased levels of anthocyanins and phenolic constituents (3, 4). The contribution of *o*-diphenols to plant tissue discoloration has been reported (2, 11).

The relationship of oxidant symptoms and phenol metabolism with respect to plant tissue discoloration has not been investigated. The objectives of this investigation were (i) to determine if polluted ambient air containing primarily photochemical oxidants alter phenol metabolism in bean leaves; and (ii) to identify and quantitate *o*-diphenols in healthy leaves from plants cultured in activated carbon-filtered and in injured leaves from plants cultured in nonfiltered air.

MATERIALS AND METHODS.—*Plant culture.*—*Phaseolus vulgaris* L. 'Tempo' was seeded in 4-inch clay pots containing a soil-perlite (1:1) medium. Thirty to 40 plants were equally distributed between two greenhouses. Total oxidants and nearly all sulfur dioxide were removed from the air by activated carbon filters in one; in the other, the air pollutants were not removed. Moisture was supplied through an automatic watering system. Fourteen days after seeding, primary leaves were removed and lyophilized. Lamina were ground and passed through a 40-mesh screen.

Isolation.—One-g samples were homogenized with 50 ml 70% aq ethanol at 25 C. The supernatant was separated by filtration, and the residue was extracted 3 times with 70% ethanol. All filtrates were combined, and the ethanol was removed by flash evaporation. The aq fraction was placed on an Amberlite IR 120 ion exchange column having a resin bed 3×15 cm. Phenols were washed through the column with 200 ml deionized water. The water was removed by lyophilization, and the residue was dissolved in 10 ml deionized water. The pH was adjusted to 4.5 with N HCl. Twenty-five ml of redistilled diethyl ether was added, and the *o*-diphenols were partitioned into the organic phase. Four successive extractions were made; then all ether extracts were combined and evaporated under vacuum. The residue was dissolved in 2 ml of N NaOH and hydrolyzed for 4 hr at room temp. The hydrolyzate was adjusted to pH 4.5 with N HCl and extracted with four 25-ml volumes of ether. All ether extracts were combined, reduced to dryness, and brought to a volume of 5 ml in redistilled methanol.

Chromatography.—Whatman No. 3 paper was used in all chromatographic analyses. Three solvent systems (i) 2% aq acetic acid; (ii) ethyl acetate:formic acid:water 10:2:3; or (iii) butanol:acetic acid:water 4:1:2.2 were routinely used to develop chromatograms containing samples of bean extracts, caffeic, and chlorogenic acids. Relative locations were determined on papers by fluorescence and reactions with Arnow's reagent (1) or with ammonia fumes. For quantitative analyses, papers were developed in solvent (ii) and compounds were located by fluorescence, cut from papers, and eluted with absolute methanol. Absorbance values at 325 mμ were compared with those from standard concn of caffeic acid.

At least three samples of plant materials and six chromatographs from each sample were used in each determination. All quantitative results are expressed on a dry wt basis.

RESULTS.—During June, July, and August 1969, primary leaves of Tempo beans cultured in nonfiltered

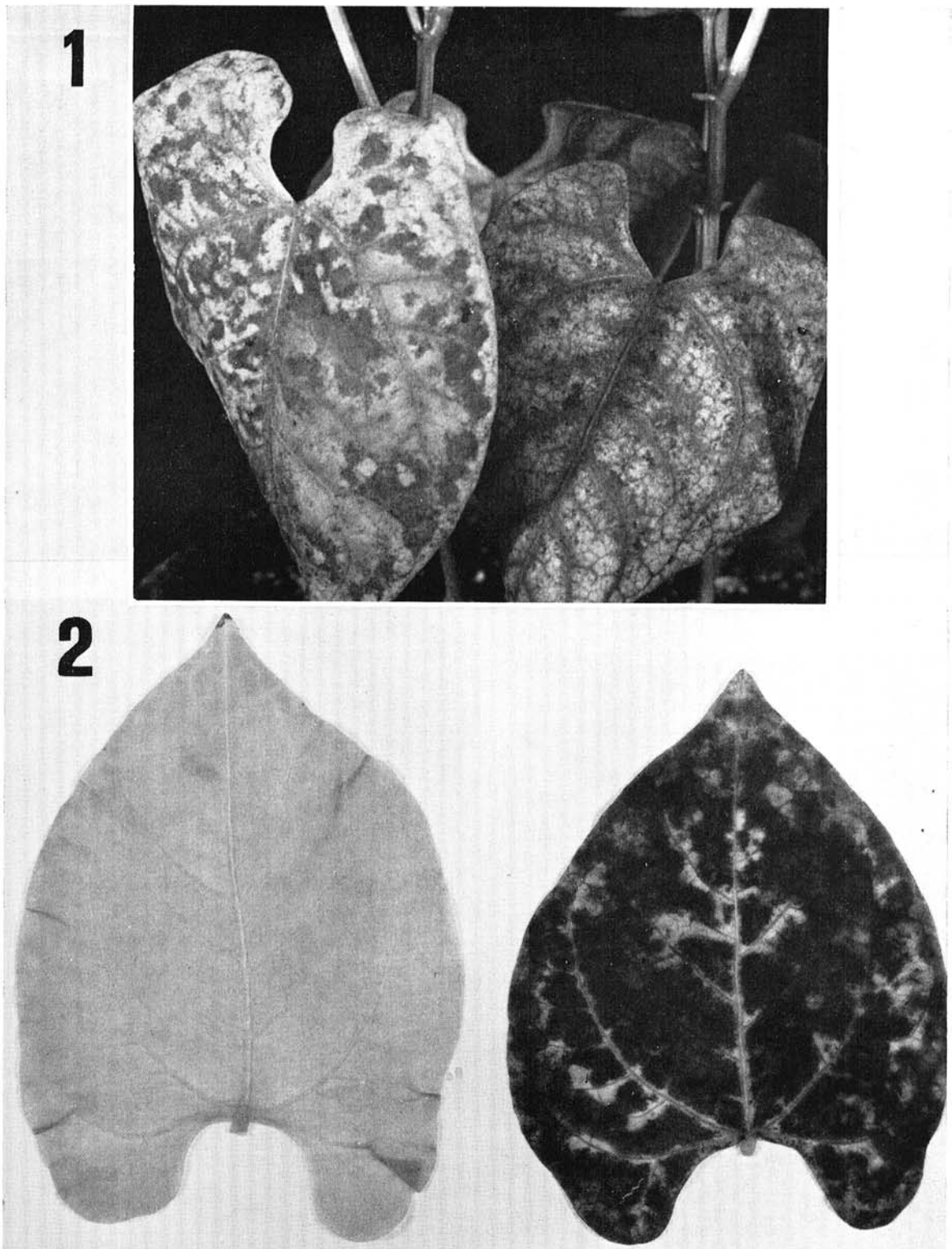

Fig. 1-2. **1)** Primary leaves of 14-day-old beans injured by air pollutants. **2)** Primary Tempo bean leaves after extraction in 70% ethanol for 24 hr. Left, noninjured; right, injured.

air were generally discolored, ranging in color from yellow to reddish-brown by 14 days after seeding (Fig. 1). Comparable plants cultured in filtered air were not discolored. During the sampling period, the highest levels of total oxidants were measured for the year by Mast 724-2 meters.

It was established that the coloring principle was not an anthocyanin, since the pigment could not be extracted from intact leaves by methanol, ethanol, or water (Fig. 2). Nonpolymerized phenols were removed by homogenizing the dried samples in 70% ethanol 4 times. Some pigment remained in samples from plants grown in nonfiltered air, and could not be completely extracted with further ethanol extractions. Chromatograms of plant extracts revealed that several fluorescent compounds are increased significantly by exposing bean plants to nonfiltered air.

Spectra (Fig. 3) show that the absorption of bean extracts A and C corresponds to caffeic acid B; viz, max absorption at 325 mμ. Relative migrations of components of bean hydrolyzates, chlorogenic, and caffeic acids appear in Table 1. In the three solvents used, R_F values of caffeic acid most nearly matched those of the unknowns. On a moisture-free basis, caffeic acid occurred in samples from plants cultured in nonfiltered air at approximately twice the concn (47 μg/mg) observed in comparable samples from those cultured in filtered air (23 μg/mg) on the basis of comparison with standards of known concn of caffeic acid.

R_F values of chlorogenic acid were dissimilar to those of the unknowns, indicating that chlorogenic acid was not a major *o*-diphenol in bean. No free quinic acid was present in hydrolyzates in either sample.

DISCUSSION.—Primary bean leaves were injured severely by components of nonfiltered air. Total oxidants are suspected of being responsible, as severity of injury paralleled the concn of total oxidant measured; i.e., the most severe injury was observed from June through September, when total oxidant levels were highest. During the 75-day sampling period from 1 June to 15 August 1969, there were 192 hr when total oxidant levels were above 5 pphm. Concn of 5 pphm for 7 or more hr occurred on 14 days. The highest oxidant level monitored was 11 pphm for a duration of 1 hr. In contrast, oxidant levels of 5 pphm were never detected during the months of October through March. Foliar discoloration was not observed between October and March. The possibility that sulfur dioxide was interacting with total oxidants is very remote, since on 1 day only was as much as 5 pphm detected. Other oxidants, such as PAN (Peroxyacetyl Nitrate) and NO_2 (Nitrogen Dioxide), were not monitored.

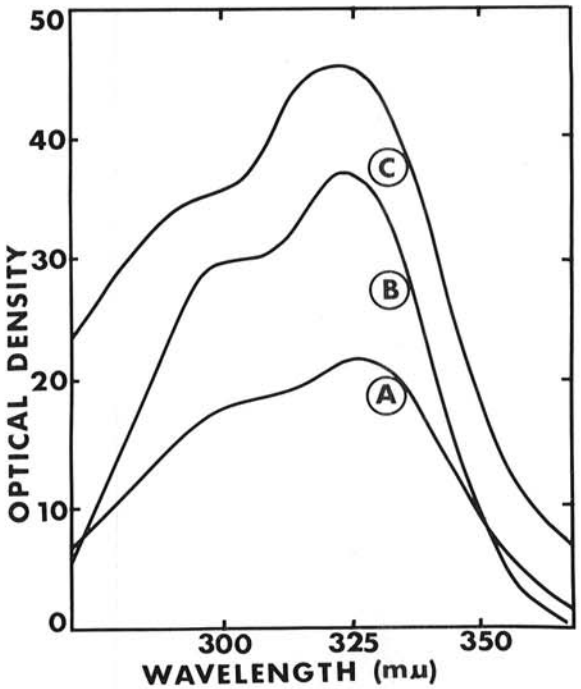

Fig. 3. Spectra of bean extracts and of caffeic acid. A = from filtered air; B = caffeic acid; C = from nonfiltered air.

Total ethanol extracts of bean leaves contained several fluorescing compounds, but they appeared in higher concn in leaf tissue exposed to ambient air than in similar tissue exposed to filtered air. Ferulic and *p*-coumaric acids are known to occur in bean leaves (15), but are not o-diphenols so were not considered in this study. Caffeic acid occurs in bean leaves as an ester of either meso-tartaric (15) or malic acid (13). The aliphatic acid in question has not been shown to contribute to leaf discoloration, so was not identified in Tempo beans.

An ether extract of the total phenol complex contained a single fluorescent compound on paper chromatograms which, after spraying with Arnow's (1)

TABLE 1. R_F values of chlorogenic and caffeic acids and phenolic extracts from Tempo bean leaves

	Compound			
			Bean hydrolyzates	
Solvent	Chlorogenic acid	Caffeic acid	Filtered air	Nonfiltered air
2% Acetic acid	0.67	0.54	0.51	0.53
Ethyl acetate:formic acid:water (10:2:3)	0.71	0.89	0.86	0.85
Butanol:acetic acid:water (4:1:2.2)	0.74	0.86	0.85	0.86

reagent, produced a yellow reaction product. After alkaline hydrolysis, followed by acidification, the ether fraction contained a compound that had spectral and chromatographic properties similar to those of caffeic acid. The polyphenol moiety, accepted as being caffeic acid, was present in both healthy and pollution injured leaves; however, it was nearly twice as concd in the injured leaves as in the noninjured ones.

The presence of elevated levels of caffeic acid could contribute significantly to the biochemistry of the browning reaction noted on oxidant-damaged leaves. According to Pierpoint (11), caffeic acid is oxidized to a quinone which then complexes with certain amino acids to form high mol-wt, brown polymers in tobacco leaves. These polymers would be insoluble in methanol, ethanol, and water, as are the brown pigments in the bean leaves. Leaves of Pinto beans, fumigated with ozone, yield increased levels of several amino acids (14), and leaves of Tempo beans yield higher levels of free amino acids after exposure to either nonfiltered air or to ozone (*unpublished data*).

It is significant that greater concn of phenolic compounds occur in both photochemically injured tissue and in that infected by plant pathogens. The reason for increased levels of caffeic acid in Tempo bean is not known. Presumably, as in other plants (9), the shikimic acid pathway is active, and intermediates, such as phenylalanine, which are known to be increased in ozone-injured tissue (14), could be deaminated and utilized in the synthesis of caffeic acid. The fate of caffeic acid in oxidant-injured tissue is not known. We are attempting to establish the role of caffeic acid in the development of the symptoms that result from air pollution injury to plants.

Tempo bean appears to be as susceptible as Pinto UI 111 bean to contaminants of polluted air or to ozone. It has the distinct advantage of being a dwarf type, which permits it to be interspersed among low-growing plants. The very pronounced symptoms observed on the leaves suggest that Tempo bean could be a useful biological monitor for air pollution investigations.

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