Registry No. Carbofuran, 1563-66-2; 2,3-dihydro-2,2-dimethyl-7-benzofuranol, 1563-38-8; 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol, 17781-16-7; 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol, 17781-15-6.

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Phenolic and Tannin Contents As Related to Anatomical Parameters of Soybean Resistance to Agromyzid Bean Flies

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Leaves and stems of four agromyzid bean fly resistant soybean (*Glycine soja*) varieties and two-susceptible ones (*Glycine max*) were assayed for phenolic, tannin, and condensed tannin contents. Phenolic content and the protein-binding or -precipitating capabilities of tannins both were higher in the leaf than in the stem of most studied soybean varieties, but differences did not correlate to bean fly resistance vs. susceptibility. No condensed tannins were detected in leaves or stems of any soybean variety. Through relating anatomical compartmentalizations of total phenols, tannins, and lignins in soybeans to the species-specific feeding behaviors of bean fly larvae in such plants, an understanding of the functions of such chemicals in the plant's defenses against insects seems to emerge. Thus, phenols and/or tannins are involved in the resistance of undifferentiated tissues, and phenols, tannins, and lignins are involved in the resistance of the differentiated soybean stem to bean fly larvae.

Tannins and other phenols have a broad spectrum of biological activity. They have been reported as being especially allomonic against viruses, bacteria, fungi, insects, and mammals (Feeny, 1970; Swain, 1979). Such phenolic compounds precipitate proteins (Swain, 1979) and have been extensively discussed as digestibility-reducing agents. As such, they may act as quantitative, i.e., dosage-dependent, defenses against herbivores (especially insects) in plants (Levin, 1971; Feeney, 1976; Rhoades and Cates, 1976).

Condensed tannins have been found in the tissues of several legumes including the leaves of some herbaceous species (Sarkar et al., 1976) and the grains of common bean (*Phaseolus vulgaris*) (Ma and Bliss, 1978), horsebean (*Vicia faba*) (Martin-Tanguy et al., 1977), cowpea (*Vigna unguiculata*), pigeon pea (*Cajanus cajan*), black gram (*Phaseolus mungo*), and azuki bean (*Vigna angularis*) (Price et al., 1980). They have been involved in southern pea, *Vigna unguiculata*, as a specific parameter of legume resistance to insects. An increased number of tannin sacs in the pods of such peas has been considered as a resistance factor against the cowpea curculio, *Chalcodermus aenueus* (Gundlach and Chambliss, 1977). In the present study efforts were made to identify any roles which phenolics and especially tannins play in the resistance of soybean plants to agromyzid bean flies.

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Larvae of agromyzid bean flies, *Melanagromyza sojae*, *Ophiomyia centrosematis*, and *Ophiomyia phaseoli*, kill soybean seedlings throughout the year in tropical areas of Asia by feeding inside the leaf and stem. A varietal screening program has identified four wild soybean (*Glycine soja*) varieties as being highly resistant and two commercialized (*Glycine max*) varieties as being susceptible to all three bean fly species (Chiang and Talekar, 1980). Some morphological, anatomical, and physiological parameters which correlate with this soybean resistance were previously established and discussed (Chiang and Norris, 1983a,b). Additional interrelated biochemical and anatomical plant parameters which also correlate positively with such soybean resistance are now presented in this paper. Dynamic interrelationships among the differentiation and development of the secondary plant body, the differentiation and lignification of sclerenchyma, and the total phenolic content in bean fly resistant soybeans are discussed.

EXPERIMENTAL SECTION

Materials. Five plants of each of four bean fly resistant soybean varieties, i.e., Asian Vegetable Research and Development Center (AVRDC) accession no. G3089, G3091, G3104 and G3122, and two bean fly susceptible varieties, i.e., AVRDC accession no. G1935 and G6, were grown from seeds in a University of Wisconsin—Madison greenhouse under the conditions previously detailed by Chiang and Norris (1983b). Leaves and stem portions for each of the experimental plants were collected separately at 8 weeks

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after germination, when they were in growth stages V14 to V16 (Fehr and Caviness, 1977) and designated according to the stem node of attachment or internode, respectively. None of the six studied varieties had developed into a reproductive stage. The sampled materials were immediately lyophilized and then stored in a desiccator in darkness at room temperature until the measurements of phenolic and tannin contents.

Estimation of Phenols. Five milligrams of lyophilized soybean tissue (i.e., leaves or stem internodes) was ground to 33 mesh or finer and shaken at a fixed oscillation for 1, 5, 20, or 48 h in 3 mL of methanol in a screw-capped 30-mL culture vial at 40 ± 0.5 °C. Each resultant extract was passed through a Whatman No. 1 filter paper in a Büchner funnel by using suction. Each vial was rinsed with an additional 3 mL of methanol. The total filtrate per sample extract was mixed with 50 mL of distilled water. A spectrophotometric analysis which detects phenols (including tannins) by the formation of the Prussian Blue complex (Price and Butler, 1977) was conducted within 1 h. A standard curve relating commercial (+)-catechin concentration to absorbance Δ720 nm under the experimental condition was prepared daily. Catechin equivalents (i.e., the absorbance that would be elicited by 1 mg of catechin/100 mg of ground, lyophilized soybean tissue) were used to express the phenolic content.

Hemanalysis of Tannins. Lyophilized soybean leaves taken from the sixth and eighth youngest nodes and stem–internodal sections taken between the fifth and ninth youngest nodes of each variety were ground to 33 mesh or finer. One-gram samples per tissue from a composite mixture from five plants per variety were extracted separately with 50 mL of methanol under continuous shaking for 20 h at room temperature. Methanol extracts were rotoevaporated until dry, and the residue was taken up with 5 mL of distilled water. The protein-binding or -precipitating capacity of tannins was determined by methods which were based on the concepts used by Bate-Smith (1973). A unit of whole blood (Wisconsin Red Cross, Madison, WI) was diluted 50 times with distilled water just before the assay. Out of the 5-mL aqueous solution of each sample extract, 2.5 mL was mixed with the same amount of diluted blood, and the mixture was shaken immediately. The mixture was centrifuged for 10 min at 5500*g*. The supernatant was then spectrophotometrically measured at 578 nm. A 2.5-mL quantity of the diluted blood solution and the remaining 2.5 mL of aqueous solution of sample extract were each mixed with 2.5 mL of distilled water and run through the same procedure to serve as a check and an individual-sample blank, respectively. The difference in the absorbance, ΔA_{578nm}, between the check and the supernatant from each blood–extract mixture was corrected by using each sample blank and then compared with values on a standard curve which was prepared by relating commercial tannic acid concentration to ΔA_{578nm} under the experimental conditions.

The precipitation of the blood proteins with tannic acid was linearly correlated with concentration between 0.1 and 0.3 mg/mL tannic acid. The derived equation, ΔA_{576nm} = 3.85(mg of tannic acid) − 0.36 (r = 0.99), allowed the calculation of tannic acid equivalents (TAE) for each sample, i.e., mg of tannic acid/100 mg of ground, lyophilized soybean tissue.

Vanillin Test for Condensed Tannins. Soybean tissues were extracted as described for hemanalysis of tannins. The vanillin test was then conducted according to the procedure described by Burns (1971), but a correction (Price and Butler, 1977) which subtracts the absorbance of each sample blank at 500 nm from that of the reacted sample was applied.

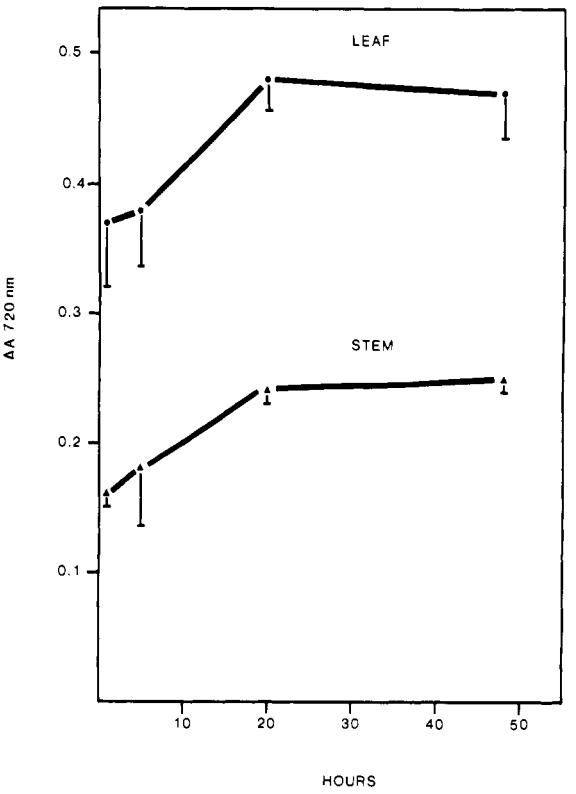

Figure 1. Quantity of soybean phenols extracted in methanol, as measured by ΔA_{720nm} according to the Prussian Blue test, as affected by the time interval of extraction.

The condensed tannin content was expressed as catechin equivalents (CE) through the use of the equation ΔA_{500nm} = 0.0057 (mg of catechin) + 0.005 (r = 0.99). This equation was derived from a standard curve linearly relating commerical (+)-catechin concentration (between 0.4 and 1.6 mg/mL) to ΔA_{500nm}.

Distribution of Tannins in Soybean Stems. The internode between the fifth and sixth youngest stem nodes of each of the six soybean varieties in growth stages V14 to V16, at 8 weeks after germination, was cross-sectioned. Internodal sections then were immersed in a special fixing solution (i.e., ferrous sulfate–formalin–distilled water, 2 g:10 mL:90 mL) for 1 week to precipitate tannins (Johansen, 1940). A counter treatment which immersed the soybean samples in FAA fixing solution (formalin–glacial acetic acid–50% alcohol, 5:5:90) was also applied as a check. The fixed tissue then was washed in distilled water, embedded, and cross-sectioned at 7 μm by using a rotary microtome. After removal of the paraffin, sections were mounted in Permount without staining. The distribution of the yellowish green tannin precipitates in the cells was detected by comparing and photographing internodal sections which were exposed to one of two fixation treatments using phase-contrast microscopy with an attached camera.

RESULTS

Estimation of Phenols. *Extraction from Soybean Leaves and Stems.* The influence of the duration of extraction on the amount of phenols removed from leaves and stems of a soybean variety (G6) is presented in Figure 1. Extracted phenols increased with the duration of the extraction period until 20 h. However, with a 5-h extraction, the percent of phenols which was extracted was comparable between the bean fly susceptible (G6) and resistant (G3089) varieties for leaves (i.e., 79 and 81%) and

Table I. Influence of Extraction Time (Duration) on the Removal of Phenols[a] from Soybean Leaves and Stems

plant sample	duration of extraction, h	soybean variety: G6 ΔA_{720nm}	G6 extraction, %	G3089 ΔA_{720nm}	G3089 extraction, %
leaf	1	0.37 ± 0.05	77	0.25 ± 0.02	58
	5	0.38 ± 0.05	79	0.35 ± 0.04	81
	20	0.48 ± 0.15	100	0.43 ± 0.07	100
stem	1	0.16 ± 0.01	66	0.10 ± 0.02	41
	5	0.18 ± 0.05	75	0.18 ± 0.02	75
	20	0.24 ± 0.01	100	0.24 ± 0.05	100

[a] Mean ± SD of five replicates; OD = ΔA_{720nm} = optical density as determined at 720 nm by using the Prussian Blue assay.

stems (i.e., 75 and 75%). This extracted amount also was a high percentage of that removed in 20 h. Such comparable values were not obtained with only a 1-h extraction (Table I).

In Soybean Leaves and Stems. Phenolic content after 5 h of extraction was generally higher in leaf than in stem extracts (Table II). In the stems, relatively high phenolic contents were found in the first, second, ninth, and tenth youngest internodes. The lower phenolic contents were usually detected in the two internodes between the fifth and seventh youngest nodes when plants were in the growth stages of V14 to V16 (Figure 2); however, variety G3091 was an exception in this regard.

Hemanalysis of Tannins. The protein-binding or -precipitating capabilities of tannins were higher in the leaf than in the stem extract of all varieties except G3091 (Table II). However, these capabilities in the six studied varieties did not correlate with bean fly resistance vs. susceptibility.

Vanillin Test for Condensed Tannins. Condensed tannins were not detected from the leaves or stems of the six soybean varieties.

Distribution of Tannins in Soybean Stems. Yellowish green tannin precipitates were detected extensively in cell vacuoles in the epidermis and cortex of the bean fly resistant varieties; however, they were scarce in pith cells. In bean fly susceptible varieties, such observed precipitates were mostly limited to the epidermis and the outer cellular layer of cortex (parts a, b, or c of Figure 3).

DISCUSSION

The reported biological actions of tannins and other polyphenols include especially repellency or deterrency which may affect palatability, growth inhibition through

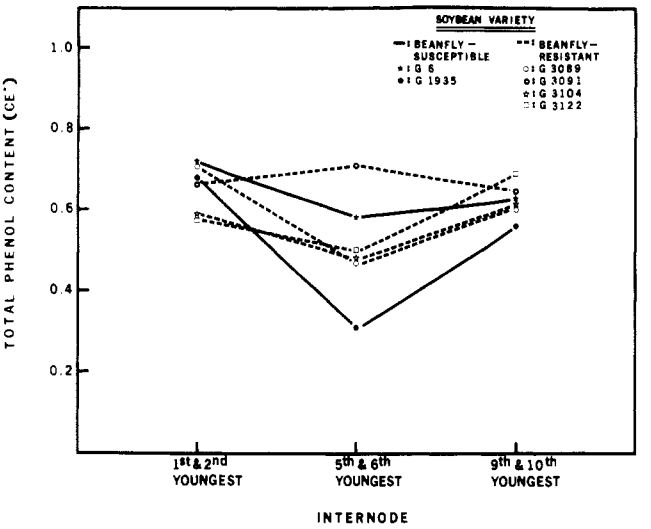

Figure 2. Comparison of total phenolic content in different stem portions of equal-aged (8 weeks after germination) soybeans of two bean fly susceptible vs. four-resistant varieties.

altered protein availability and enzyme inhibition, and direct toxicant. These jointly may contribute to the protection of plants against many herbivores and pathogens (Feeny, 1970; Levin, 1971; Bernays et al., 1981; Reese et al., 1982). However, some herbivores have counteradapted to tannins (Feeny, 1976; Fox and MacAuley, 1977; Bernays, 1978).

A high gut pH, commonly found in coleopterans and lepidopterans, has been suggested as a mechanism of counteradaptation to tannins. Such an alkaline condition strongly decreases the ability of tannins to form complexes with proteins (Goldstein and Swain, 1965). However, some other insects which feed on plant tissues containing high levels of tannins and which have a gut pH at or below neutral must have other mechanisms for preventing such polyphenols from being digestibility-reducing factors. It has been suggested that some caterpillars avoid tannins in oak leaves because they are potent antifeedants (Bernays et al., 1981). Feeny (1970) offered a more general "avoidance" hypothesis to explain how leaf-mining larvae of the winter moth might attack mature oak leaves in spite of their high tannin contents. It was proposed that such larvae succeed by feeding on the spongy mesophyll and ignoring the pallisade cells in which the tannins are believed to be concentrated.

Larvae of agromyzid bean flies are very tiny, and they only attack certain portions of the legume plant according

Table II. Estimation of Phenols,[a] Tannins,[b] and Condensed Tannins[a] in Soybean Leaves and Stems

soybean variety	phenolic and tannin contents, mean ± SD[c]: leaf Prussian Blue test, CE[a]	leaf hemanalysis, TAE[b]	leaf vanillin test, CE[a]	stem Prussian Blue test, CE[a]	stem hemanalysis, TAE[b]	stem vanillin, CE[a]
G6[d]	1.29 ± 0.06	0.23 ± 0	0	0.5 ± 0.5	0.15 ± 0.01	0
G1935[d]	0.89 ± 0.03	0.14 ± 0.01	0	0.4 ± 0.03	0.13 ± 0.01	0
G3089[e]	1.02 ± 0.07	0.18 ± 0.01	0	0.5 ± 0.05	0.15 ± 0.01	0
G3091[e]	1.06 ± 0.08	0.21 ± 0.06	0	0.73 ± 0.03	0.21 ± 0.01	0
G3104[e]	0.89 ± 0.09	0.18 ± 0.04	0	0.47 ± 0.02	0.17 ± 0	0
G3122[e]	0.99 ± 0.09	0.20 ± 0.05	0	0.43 ± 0.04	0.16 ± 0.01	0
correlation coeff[f]	$\gamma = 0.87$			$\gamma = 0.89$		

[a] Catechin equivalent (CE) = mg of catechin/100 mg of ground, lyophilized soybean tissue which would be required to give the observed absorbance. [b] Tannic acid equivalent (TAE) = mg of tannic acid/100 mg of ground, lyophilized soybean tissue which would be required to give the observed absorbance. [c] Data obtained from two replicate assays each for the vanillin test and hemanalysis and five replicate assays for the Prussian Blue test. [d] Bean fly susceptible cultivated soybean variety. [e] Bean fly resistant wild soybean variety. [f] Coefficient, r, for the correlation between the phenolic and tannin contents.

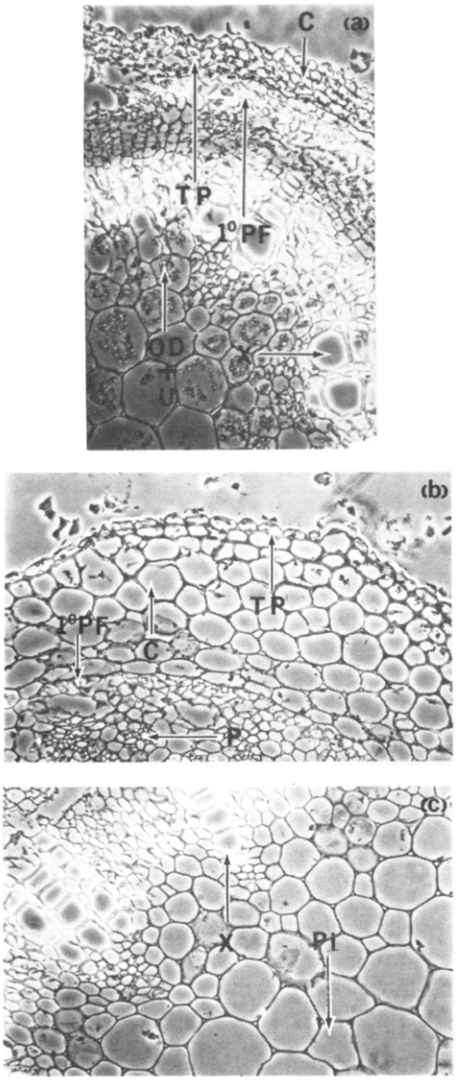

Figure 3. Transverse sections of the internode between the fifth and sixth youngest stem node of (a) the bean fly resistant variety, G3089, showing tannin precipitate in epidermal, cortical, and phloem and xylem parenchymal cells, (b) the bean fly susceptible variety, G6, showing a few tannin precipitates in the epidermis and the outermost two layers of cortical cells, and (c) variety G6, showing no tannins in the pith. (Magnification: 32×) (C, cortex; TP, tannin precipitates; 1°PF, primary phloem fibers; X, xylem; OD + U, oil drops and unknown particles which are also shown in counter-treated plant samples; P, phloem; Pi, pith).

to species-specific behaviors (Spencer, 1973). Therefore, compartmentalization, as well as presence vs. absence, of total phenols in such plants may play a particularly significant role in determining plant interactions with agromyzid larvae.

Our present findings regarding tannins and other phenols support a theory that cortex-feeding larvae of *O. centrosematis* and *O. phaseoli* should be largely able to avoid such chemicals which are localized specifically in the epidermis and outermost cortex in susceptible varieties and still obtain adequate food from the underlying cortex. Such avoidance does not seem possible in resistant varieties because all cortical cells contain tannins and other phenols. Such a phenolic-based defense mechanism also might contribute to soybean resistance to the bean fly, *Melanagromyza dolichostigma*. Its larvae feed in the stem tip of cultivated soybeans by making a coil-shaped mine within the cortex (Lee, 1976). However, it does not even attack wild soybean varieties which have phenols throughout their stem cortex (Lee, 1976; Spencer, 1973). Thus, the distribution of tannins and other phenols throughout the cortex in the stem is positively correlated with soybean resistance to bean flies which mine such tissues.

In the present study, a negative correlation between polyphenolic content and both differentiation of secondary growth, i.e., secondary phloem and secondary xylem, and differentiation and lignification of sclerenchyma in the stem of cultivated soybeans was evident. Such content was higher in portions of the stem where no secondary growth nor sclerenchyma cells were differentiated, lower where differentiation of secondary plant body and differentiation and lignification of sclerenchyma were actively proceeding, and higher in older stem where the differentiation of secondary plant body and differentiation and lignification of sclerenchyma had decreased and secondary xylem and lignified xylem fibers almost filled the pith cavity (Figure 2; Chiang and Norris, 1983b).

In intact growing plants, only water, inorganic ions, a few sugars, and vitamins appear to be moved appreciable distances (Milthorpe and Moorby, 1969). There is no evidence of translocation of polyphenols within the vascular systems of plants. They apparently accumulate within the tissues or cells in which they are formed (McClure, 1975). Therefore, the above-described negative correlation situation may implicate two principles in the biochemical aspects of soybean resistance to bean flies. First, a delicately balanced primary competition may occur between the biosyntheses of proteins (especially during secondary growth) vs. phenylpropanoids and, second, a possible secondary competition may develop between the biosyntheses of flavonoids vs. lignins. Being aware of these competitions is important because (a) the most likely limiting factor in controlling phenylpropanoid (derived from the shikimate pathway) accumulation is substrate (phenylalanine and/or tyrosine) supply rather than enzymic (PAL) activity (Margna, 1977) and (b) phenylpropanoids are precursors for both lignins and flavonoids which are synthesized simultaneously by soybean cells (Hahlbrock and Grisebach, 1979).

Thus, one apparently can significantly explain soybean resistance to bean flies in terms of undifferentiated (i.e., a lack of secondary growth and sclerenchyma) vs. differentiated (i.e., presence of secondary growth and sclerenchyma) plant tissues. Therefore, phenols and/or tannins are involved in the resistance of the undifferentiated stem and phenols, tannins, and lignins are involved in the resistance of the differentiated stem.

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Some Phosphorothionate Imported Fire Ant Toxicants with Delayed Kill

Thomas H. Fisher,* William E. McHenry, Earl G. Alley, Clifford S. Lofgren, and David F. Williams

Twenty-one diethyl aryl phosphorothionates (**2**), $(EtO)_2P(=S)OC_6H_3XY$, were prepared and tested as imported fire ant (IFA) toxicants. The substituents placed on the aryl ring include bromo esters, ketones, imines, an aldehyde, an oxime, a semicarbazone, a phenylhydrazone, a trifluoromethyl, an acylhydrazone, and a benzylidene malononitrile substituent. The phosphorothionates with both a bromo and a CO_2R substituent on the phenyl ring were poorer toxicants than the corresponding phosphorothionates with a single Br or CO_2R substituent on it. Phosphorothionates **2** [X = 4-C(=O)CH_2Ph, Y = H; X = 4-C(=O)$NHNH_2$, Y = H] were both found to give delayed kill at 0.1% toxicant in soybean oil, while **2** (X = CF_3, Y = H) was found to be nearly as good an IFA toxicant. A QSAR study found the toxicity of **2** to be correlated with the steric parameter MR and to a lesser extent with Π and σ.

Much effort has recently been directed to finding a nonpersistent replacement for the now-banned insecticide mirex, for use in bait formulations to control the imported fire ants (IFA), *Solenopsis richteri* Forel and *Solenopsis invicta* Buren. Both tranditional insecticides and other types of toxicants such as insect growth regulators (Vinson and Robeau, 1974; Banks et al., 1978), the dye Phloxin B (David and Heitz, 1978), fluoroacetyl derivatives of sterols and fatty acids (Kochansky et al., 1979), and some amidinohydrazones (Williams et al., 1980) have been examined.

Of the many organophosphorus insecticides studied, most do not have the delayed action (less than 15% kill after 1 day) necessary for a bait toxicant. Several dialkyl halophenyl phosphorothionates showed good delayed kill in laboratory tests but were not effective in the field (Banks et al., 1977); some analogous alkoxycarbonylphenyl compounds, especially those of low hydrophobicity, showed promise, but delay was inadequate (Fisher et al., 1980).

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In the present study, a further range of substituents on the phenyl ring is examined, as well as disubstituted compounds, with sufficient variation in electronic, lipophilic, and steric properties to allow a quantitative structure–activity analysis.

EXPERIMENTAL SECTION

Materials and Instrumentation. Diethyl phosphorochloridothionate (Aldrich) and refined soybean oil were used as obtained commercially. Acetone was distilled over phosphorus pentoxide prior to use. IR and NMR spectra were run on a Perkin-Elmer Model 283B and a Varian A-60 spectrophotometer, respectively.

Phenols (1). The following phenols were commercially available and were used without further purification: *p*-hydroxyacetophenone; *p*-hydroxypropiophenone; *p*-hydroxybenzophenone; 4-hydroxybenzaldehyde; benzyl 4-hydroxyphenyl ketone; α,α,α-trifluoro-*p*-cresol; *p*-hydroxybenzoic acid hydrazine; *p*-hydroxybenzylidene malononitrile.

Salicyclic acid and *p*-hydroxybenzoic acid were brominated by the procedure of Hewitt et al. (1904) and *m*-hydroxybenzoic acid was brominated by the method of Buchler et al. (1946) to give 5-bromosalicyclic acid, 3-bromo-4-hydroxybenzoic acid, and 4-bromo-3-hydroxybenzoic acid, respectively. These three bromohydroxybenzoic acids were esterified with methanol or isopropyl alcohol in ethylene dichloride solvent and a sulfuric acid

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