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THE EFFECT OF VARYING U.V. INTENSITIES ON THE CONCENTRATION OF SCOPOLIN AND CAFFEOYLQUINIC ACIDS IN TOBACCO AND SUNFLOWER

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Abstract—Using a simple but reliable method involving one-dimensional paper chromatography for the quantitative determination of chlorogenic acid, neochlorogenic acid, 4-*O*-caffeoylquinic acid ("band 510"), and scopolin, the effect of varying u.v. light intensities on the concentration of these compounds in tobacco and sunflowers has been determined. With increased u.v. intensity, an increase in scopolin concentration was found in tobacco leaves and stems and in sunflower leaves. Chlorogenic acid concentration was higher in roots, stems, and leaves of tobacco plants treated with low, stimulatory u.v. radiation than in comparable plants grown under control or under higher u.v. conditions. Similar experiments with sunflowers indicated that the chlorogenic acid concentration of sunflower leaves was less under the low u.v. radiation than in comparable sunflower plants of control or of higher u.v. treatments. In stems and roots of sunflower, no consistent pattern of change in the chlorogenic acid concentration was found under the u.v. conditions tested. The concentration of chlorogenic acid increased in sunflower older leaves, but no consistent pattern of chlorogenic acid change with age was observed in sunflower stems and roots. Neochlorogenic acid and the third isomer (band 510) were found in both tobacco and sunflower, but in much higher concentrations in tobacco. Both of these isomers were found in all parts of the two test plants, except tobacco stems or roots. Postulates are presented which point out the possible significance of chlorogenic acid and/or scopolin in lignification and in mediating environmental conditions through effects on internal regulatory mechanisms. Internal concentrations of these compounds possibly are correlated with their release from the plant, and subsequently with allelopathic relations among plants.

INTRODUCTION

SCOPOLIN (7-glucoside of 6-methoxy-7-hydroxycoumarin) and chlorogenic acid (3-*O*-caffeoylquinic acid) are widely distributed in nature and have been investigated by numerous research workers.[1,2] The amounts of both scopolin and its aglycone, scopoletin, have been determined in tobacco tissue in culture and in sunflower and tobacco plants treated with 2,4-dichlorophenoxyacetic acid,[3–5] in tobacco treated with maleic hydrazide,[1] and in boron-deficient sunflowers and tobacco.[6,7] Determinations were accomplished by the use of either paper or thin-layer chromatography[8] for the separation of individual compounds, and was

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[4] L. J. DIETERMAN, C-Y. LIN, L. M. ROHRBAUGH, V. THIESFELD and S. H. WENDER, *Anal. Biochem.* **9**, 139 (1964).
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followed by an individual, quantitative determination of fluorescence of the scopoletin and scopolin.

Reports of the isolation and quantitative determination of chlorogenic acid (CGA) are numerous[2] in contrast with only infrequent reports[9,10] in the literature on the isolation and individual quantitative determination of two of its isomers, neochlorogenic acid (5-*O*-caffeoylquinic acid) (neo CGA) and 4-*O*-caffeoylquinic acid ("band 510"; B510). Also, numerous reports have been published on physiological effects of scopoletin and of CGA in higher plants. Each of these two compounds has been postulated to have a synergistic effect on indoleacetic acid action, probably acting to inhibit the indoleacetic acid oxidase system.[11–13] Chlorogenic acid also has been suggested as being able to play a role in the synthesis of lignin.[14,15] Rice has shown that CGA and certain other phenolic compounds, have a significant phytotoxic effect.[16,17] These findings place additional importance on the acquisition of quantitative data involving conditions affecting the concentration of scopolin and CGA in plants.

Lott[18] reported that u.v. light exposure for 75 days in stimulatory amounts can cause a maximum increase above normal in CGA content of 79% in the case of open-air tobacco plants, and of 550% in that of greenhouse grown plants. Lott did not report any analyses on scopolin, B510, or neo CGA or results of experiments involving use of inhibitory and/or injurious amounts of u.v. radiation. The well-known fact that sufficiently elevated dosages of u.v. may cause injury in plants, and the discovery that certain stress conditions may cause accumulations of scopolin and scopoletin in tobacco, made it desirable to study the effect of inhibitory and injurious amounts of u.v. radiation—as well as stimulatory amounts—on the concentration of phenolic compounds in tobacco and sunflower. Experiments were designed, therefore, to determine the effects of varying intensities of u.v. radiation—as well as of age—not only on the concentration of CGA, but also on neo CGA, B510, and scopolin in tobacco and sunflower plants.

RESULTS

U.v. Effects on Tobacco

Concentrations of CGA, neo CGA, B510, and scopolin in younger leaves, older leaves, stems, and roots of control, low u.v., medium u.v., and high u.v. treated tobacco plants, as obtained by analysis of each, are recorded in Table 1. The recorded values are averages of two replicate sets of tobacco plants. Plants subjected to the low u.v. (stimulatory treatment) were consistently taller than control or higher u.v. plants, and their leaves were larger. In older leaves, total chlorogenic acid concentration correlated well with growth of the plants. Under high u.v., the plants became stunted and the leaves became bronze colored in 2–5 days. The leaves, however, remained turgid and did not dry out.

In both older and younger leaves, the concentration of CGA, B510, and neo CGA were

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greatest after the low, stimulatory u.v. treatment. In those plant parts exposed to u.v., scopolin generally increased in concentration with increased u.v. This was especially pronounced with inhibitory and injurious amounts of u.v. The roots contained a ratio of CGA: scopolin of approximately 5:1, with the greatest combination of both at the low u.v. level.

TABLE 1. CONCENTRATION OF CHLOROGENIC ACID, NEOCHLOROGENIC ACID, 4-*O*-CAFFEOYLQUINIC ACID, AND SCOPOLIN IN TOBACCO PLANTS TREATED WITH VARYING U.V. INTENSITIES

	μgs/g Fresh weight‡				
Treatment†	CGA	neo CGA	B510	Total caffeoyl-quinic acids	Scopolin
Older leaves					
Control	304	157	203	657	2·1
Low u.v.	448	199	263	910	3·1
Medium u.v.	364	130	180	675	11·3
High u.v.	275	73	116	464	59·3
Younger leaves					
Control	695	160	435	1290	4·5
Low u.v.	1240	166	670	2076	7·6
Medium u.v.	1030	149	436	1615	28·1
High u.v.	1060	107	630	1797	38·6
Stems					
Control	123	*	20	143	11·0
Low u.v.	253	*	37	290	15·9
Medium u.v.	196	*	33	229	38·7
High u.v.	271	*	52	323	34·1
Roots					
Control	222	*	*	222	39·4
Low u.v.	234	*	*	234	46·2
Medium u.v.	187	*	*	187	37·4
High u.v.	89	*	*	89	19·5

* Below amount determinable by procedure used.

† U.v. in mw/ft²: Low = 1–1·5; Medium = 4–5; High = 5–8.

‡ CGA = Chlorogenic acid; neo CGA = Neochlorogenic acid; B510 = (4-*O*-Caffeoylquinic acid) ("Band 510").

U.v. Effects on Sunflowers Correlated with Age

Sunflower plants were harvested 8, 15, 24, and 37 days after the start of the u.v. treatments. Effects of u.v. treatment were virtually the same morphologically in the sunflower plants as those indicated in the above paragraphs for tobacco. In the sunflower experiments, treatment was initiated on younger plants than with tobacco, and sunflower plants treated with high u.v. appeared even more stunted. The increase in height and in leaf size was also noted with the sunflowers receiving low, stimulatory u.v. as had been observed with the tobacco.

The concentration of scopolin in the sunflower parts exposed to u.v. was found to increase with increasing u.v. intensity (Table 2), as it did in tobacco. Again, this accumulation of

TABLE 2. CONCENTRATION OF PHENOLICS IN SUNFLOWER PLANTS TREATED WITH VARYING U.V. INTENSITIES

	μg/g Fresh weight‡																			
	CGA Harvest				B510 Harvest				neo CGA Harvest				Total caffeoyl-quinic acids Harvest				Scopolin Harvest			
Treatment†	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Control																				
Younger leaves	*	*	187	199	*	*	92	73	*	*	84	66	*	*	362	338	*	*	*	*
Older leaves	*	*	151	276	*	*	46	76	*	*	35	45	*	*	232	397	*	*	*	4·4
Stems	121	87	222	128	29	22	36	11	23	33	38	22	173	142	296	161	*	*	*	*
Roots	35	73	96	82	21	21	29	17	32	8	40	4	88	102	164	103	*	*	*	*
Cotyledons	548	519	*	*	65	93	*	*	54	48	*	*	667	660	*	*	*	*	*	*
Low u.v.																				
Younger leaves	*	*	126	130	*	*	70	68	*	*	68	59	*	*	264	256	*	*	*	*
Older leaves	*	*	56	165	*	*	28	58	*	*	39	43	*	*	123	266	*	*	*	9·3
Stems	130	121	116	91	34	21	26	22	33	31	37	25	197	173	179	138	*	*	*	*
Roots	32	78	56	68	22	26	44	51	15	24	26	*	69	128	126	119	*	*	*	*
Cotyledons	473	307	*	*	68	94	*	*	54	58	*	*	595	459	*	*	*	*	*	*
Medium u.v.																				
Younger leaves	*	*	156	314	*	*	63	119	*	46	61	90	*	46	280	523	10·4	9·8	6·0	11·8
Older leaves	*	59	196	368	*	*	34	109	*	*	27	88	*	59	258	565	*	33·9	23·2	41·1
Stems	115	106	88	150	35	24	31	44	5	26	10	48	155	150	129	242	*	*	*	*
Roots	37	29	64	90	22	17	23	59	17	14	7	*	76	60	93	149	*	*	*	*
Cotyledons	478	298	*	*	57	83	*	*	57	60	*	*	592	441	*	*	*	*	*	*
High u.v.																				
Younger leaves	*	37	230	170	*	21	120	74	57	27	126	64	57	85	474	308	45·3	43·1	39·6	20·1
Older leaves	*	102	266	475	*	64	39	90	*	64	*	78	*	230	305	643	*	59·3	59·5	35·3
Stems	65	32	56	78	43	25	18	18	46	7	19	8	154	64	93	104	*	*	*	*
Roots	33	60	67	63	20	22	57	30	23	18	56	17	76	100	180	110	*	*	*	*
Cotyledons	474	388	148	228	61	90	106	148	60	66	48	85	595	544	302	461	*	*	*	*

* Below amount determinable by procedure used.
† U.v. in mw/ft²: Low = 1–1·5; Medium = 4–5; High = 5–8.
‡ CGA = Chlorogenic acid; neo CGA = Neochlorogenic acid; B510 = (4-*O*-Caffeoylquinic acid).
§ Harvest made at 8, 15, 24 and 37 days after start of u.v. treatment.

scopolin was primarily found in the leaves subjected to inhibiting or injurious u.v. intensities, with older leaves having a larger buildup than younger leaves. Unlike tobacco, however, scopolin was found normally to be present in sunflowers only at a level that was minimal for quantitative studies. Scopolin did not occur in sunflower roots in amounts detectable by the procedure used.

In the case of more than 75% of the high u.v. treated sunflower plants, the cotyledons remained on the plant throughout the duration of the experiment, but in the controls and in the low and medium u.v. treated plants, they dried up and abscised within 25 days after planting. The cotyledons contained a high concentration of the caffeoylquinic acids studied, which decreased with increasing age of the plants. Prior to abscission, the cotyledon CGA concentration was least in the low and medium u.v. treated plants (Table 2). There was, however, no detectable scopolin in remaining cotyledons in any of the treatments, including the high u.v.

In contrast with tobacco leaves which had the highest CGA content in those leaves treated with low u.v. intensity, sunflower leaves had the lowest CGA content in this low u.v. treatment, and the content of CGA increased with increasing u.v. intensity. The leaves of control sunflower plants had a CGA concentration intermediate between those of the low u.v. and medium u.v. treated leaves. It appears likely that the concentration of caffeoylquinic acids in sunflower leaves is a function of both the age of the plant as well as of the intensity of u.v. irradiation. Up to 24 days after planting little, if any, CGA was present in the leaves of control and low u.v. treated plants, with only small amounts present in the medium and high u.v. treated plants.

Concentration of caffeoylquinic acids and scopolin in sunflower stems and roots, unlike those of tobacco, showed little correlation with age or u.v. conditions. Generally, the stems showed a decreased concentration of the caffeoylquinic acids after the first harvest, with a possible later increase with age.

DISCUSSION

Since the growth of tobacco plants correlated well with the CGA concentration, one possibility would be to postulate that this increased growth is a result of the effect of CGA in inhibiting indoleacetic acid oxidase, thus allowing more indoleacetic acid to be present, and thereby stimulating growth. One might also postulate from the experimental observations that the buildup of the caffeoylquinic acids under low u.v. conditions may have been due to greater lignification, thus requiring a larger CGA precursor pool.

Previously reported stress conditions in tobacco when produced after 2,4-D or maleic hydrazide treatment or in boron deficiency, resulted in large accumulations of scopolin in the treated or deficient plants. The accumulation of scopolin in those plants treated with high, injurious intensities of u.v. is consistent with the postulate that, in tobacco, increasing a stress condition to a point of injury commonly causes an increase in scopolin concentration. The results of further experiments recently completed in this laboratory indicate a similar type of increase in scopolin concentration in tobacco plants treated with near-lethal doses of ionizing radiation,[19] in plants receiving a cold treatment during the light period,[19] and in plants made deficient in potassium or nitrogen.[20]

In previous work from this laboratory involving accumulation of scopolin after 2,4-D

[19] D. E. KOEPPE, personal communication.
[20] G. M. ARMSTRONG, Ph.D. Dissertation, University of Oklahoma, Norman (1968).

treatment, a prime site of such accumulation occurred in the roots of the treated plant. Since no such accumulation occurred in the roots of the present stressed high u.v. plant, one might postulate that scopolin accumulated only *in situ* at the point of stress. The effects of 2,4-D application to the leaves and stem could occur in the roots, since this herbicide may be readily translocated throughout the plant.

Another interesting aspect of these quantitative studies is an apparent correlation found for the CGA to scopolin concentration (on the order of an approximately 5:1 ratio in all treatments) in tobacco roots. This fact; the lack of either neo CGA or B510 in the roots; and the knowledge that both CGA and scopolin can influence indoleacetic acid concentration *in vitro*, might lead one to speculate that such a concentration ratio of CGA to scopolin could be critical to the maintenance of an IAA concentration range conducive to growth.

The data obtained from the sunflower u.v. experiments show both direct, and inverse relationships to the tobacco data. The scopolin accumulation with higher u.v. treatments in sunflower leaves is consistent with the tobacco data. The fact that there is an early accumulation and then a falling off of scopolin concentration in younger sunflower leaves with age might appear, at first glance, to conflict with findings from stress experiments in tobacco where a continuing increase of scopolin generally has been found with increasing age of the plant after treatment. A difference, however, is not necessarily surprising since scopolin does not occur naturally in the control sunflower in the appreciable quantities that it does in tobacco. Also, the sunflowers received a dosage of u.v. for a longer period of time, and their period of treatment occurred at a much earlier age. There is also good evidence that scopolin is not translocated in sunflower, as both stems and roots contained no detectable scopolin even when the leaves contained as much as 60 μg/g fresh weight. It is somewhat surprising that in those cotyledons which remained on the plant, and in the stems of the sunflower plants exposed to high u.v. intensities, only traces of scopolin were found. Since these parts of the plant received high u.v. irradiation, it might be expected that they would produce significant quantities of scopolin.

The data for CGA in sunflower plants, when compared to those obtained from the tobacco experiments, indicate the possibility that CGA may play different roles in the two plants. In these growth chamber-grown sunflowers, there was apparently no large difference in CGA concentration between the younger and older leaves (except in the high u.v. treated plants), and these phenolic compounds were not present in detectable amounts during the first 3 weeks of the plant's very rapid growth. These results suggest the possibility that these phenolic compounds might be involved even more prominently in lignification, under certain circumstances, than in growth regulation. Working with chlorogenic acid labelled in the caffeoyl moiety from trans- (U^{14}-C) cinnamic acid, Taylor[15] has recently reported for *Xanthium* that as label is lost from CGA, radioactivity appears largely in 3,5-dicaffeoylquinic acid and in insoluble residues. Some apparently was used in the biosynthesis of lignin. The increased concentrations of the caffeoylquinic acids found in the studies being reported here for older leaves of sunflower in contrast to the lesser amounts found in the tobacco plants also suggest that the metabolism of these compounds—at least on a quantitative basis—is different in the two species.

The work reported here adds additional confirmation to previous reports that the changing of environmental conditions does affect the phenolic compound concentration of plants. Also, the increasing knowledge that some of these phenolic compounds have a phytotoxic effect on many higher plants and microorganisms, indicates the possibility that phenolic compounds may be of importance in mediating the effects of environmental conditions.

Martin[21] has pointed out that under favorable conditions the excretion of scopoletin is very low from intact roots, but that this excretion is increased under more unfavorable conditions. The increase found in scopolin concentration under the stress u.v. conditions reported in this paper is consistent with Martin's work and may correlate with the release of scopoletin from the plant. Only very small amounts of free scopoletin were found in the plant extracts. There is also a possibility that scopolin could first be hydrolyzed and then scopoletin released from the roots. Since scopoletin, above certain concentrations, has been found to be an effective inhibitor of germination and growth, this might place it and its glucoside, scopolin, in a key role in the allelopathic relations of plants. Wilson's[22] discovery that scopolin is leached from the leaves of native sunflower may indicate another mode of release from the plant.

Rice[17] has discussed the possible role of CGA in the inhibition of seed germination and growth of higher plants. From his work, it appears that the accumulation of chlorogenic acid with age might cause its allelopathic effects to be greater in the initial weed stage of succession in abandoned fields in Oklahoma.

The role of u.v. light in the growth of plants is well known to be significant, probably as a limiting factor in alpine, and northern taiga and tundra regions. The phenolic concentration changes observed with changes in u.v. intensity could affect the ability of plants to survive in these regions through either changes as metabolic regulators, or as phytotoxins.

EXPERIMENTAL

All plants were grown in Percival growth chambers under 16-hr light periods, at a light intensity of 8,000–10,000 lux. Plants were carefully selected for uniformity at the beginning of treatment, grown in pure quartz sand, and watered with a Fe-EDTA double-strength Hoagland's nutrient solution.[23] The double-strength nutrient solution was found necessary since the tobacco plants developed a severe chlorosis as they matured on the regular solution. The light period temperature was 32°, and the dark period 15·5°.

U.v. light was supplied from a GE germicidal lamp No. G30T8 suspended above the plants on one side of the growth chamber (control plants were isolated in a separate chamber under identical conditions, but without the u.v. source). When the lamp was covered by ten layers of window screen, the differential u.v. effect produced was as follows: (1) high u.v., 5–8 mW/ft^2, (2) medium u.v., 4–5 mW/ft^2, and (3) low u.v., 1–1·5 mW/ft^2. While most of the energy radiated by this lamp is in the range of 240–260 nm, it also irradiates to a limited extent in the middle u.v. range (280–320 nm), supplying some of the irradiation of this range that is lacking in the fluorescent tubes used in these chambers, but present under natural conditions. U.v. treatment of the tobacco plants (*Nicotiana tabacum* L. var. One Sucker) was initiated when the plants were approximately 80 days old and was maintained for a period of 21 days. Treatment lasted for 8 hr each day, being administered in the center of the 16-hr light period. Sunflower plants (*Helianthus annus* L. var. Russian Mammoth) received the same u.v. treatment, but it was initiated 7 days after planting.

After treatment, plants were harvested, weighed, and fixed in boiling isopropyl azeotrope (88% isopropyl alcohol to 12% water, w/w). The fixed plant matter was ground and extracted by the procedure used by Wilson *et al.*[24] The combined extracts were evaporated to dryness *in vacuo*, and then brought back to a known concentration with the solvent mixture IBMW (isopropyl alcohol:methyl alcohol:benzene:water, 2:1:1:1, v/v/v/v), the concentration of plant matter to solvent always exceeding a 3:1 ratio (grams fresh wt.:IBMW). All data reported were obtained during the period of maximum stability of the analyzed phenolic compounds in the IBMW solvent system.

Separation of scopolin, CGA, B510 and neo CGA, was accomplished through one-dimensional chromatography on Whatman No. 1 paper (9¼ × 22 in.), freshly washed with *ca.* 50 ml 5% MeOH and dried. The paper was developed for 20 hr using KFW (methyl-isobutylketone:formic acid:water, 14:3:2, v/v/v) as the solvent. Bands were located under u.v. light without exposure to NH_3, positive identification of the compounds was made by co-chromatography in different solvent systems and by u.v. spectra.

[21] P. MARTIN, *Z. Botany* **45**, 475 (1957).
[22] R. E. WILSON, Ph.D. Dissertation, University of Oklahoma, Norman (1968).
[23] D. R. HOAGLAND and D. I. ARNON, *Calif. Agr. Exp. Sta. Circ.* 347 (1950).
[24] J. L. WILSON, W. J. DUNLAP and S. H. WENDER, *J. Chromatog.* **35**, 329 (1968).

Compounds were eluted from the paper with 5–8 ml of 5% MeOH and determined spectrophotometrically (caffeic acid esters at 330 nm on a Hitachi-Perkin-Elmer model 139 spectrophotometer[10]) or fluorometrically (scopolin, with a model 110 Turner Fluorometer: primary filter No. 7–60, and secondary filter No. 2A plus No. 48, Kodak Wratten Filter). All data reported in this paper are the averages of at least two quantitative determinations.

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