ANTHOCYANINS IN BRACTS OF EUPHORBIA PULCHERRIMA AS REVEALED BY PAPER CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS[1]

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Lawrence et al (4) identified the red pigment in bracts of poinsettia as an anthocyanin. The methods employed served to identify the principal anthocyanidin (cyanidin), but not the sugar moiety. Blank (2), Robinson and Robinson (5, 6, 7), and Scott-Moncrieff (8) reported that variation in the sugar moiety of the anthocyanin pigment affected color. They found that monoglycosides were redder than the corresponding pentose glycosides, while 3-biosides and 3,5-diglycosides were bluer. The purpose of the present investigation was to isolate and identify by paper chromatographic and spectrophotometric methods the anthocyanidin as well as the sugar moiety of the anthocyanin in bracts of *Euphorbia pulcherrima*.

METHODS AND MATERIALS

Bracts from plants of diploid Ruth Ecke (standard red) poinsettia, a seedling of tetraploid Ruth Ecke (dark red), and a cross between Ruth Ecke and Ecke's White (scarlet red) were used. The bracts were dried at 70° C in a forced-draft oven and then ground separately in a Wiley mill to pass a 20-mesh screen. A 4-g sample of the ground tissue was extracted with three successive 100-ml portions of cold 1 % hydrochloric acid by macerating in a Waring blendor for five minutes. The combined extracts were filtered, and the anthocyanin was precipitated with neutral lead acetate. The supernatant was separated by filtration and the lead salt was washed with distilled water and then dried in vacuo in the dark at room temperature. Ten ml of a methanolic solvent containing 2 % hydrochloric acid was added to a 300-mg sample of the lead salt and held in the dark at 5° C for 24 hours. The lead chloride precipitate was removed by centrifugation and the clear supernatant solution containing the anthocyanin was subjected to paper chromatographic separation. Chromatograms were prepared by streaking the red solution in a band approximately 1 cm wide across the narrow width of Whatman No. 3 MM paper (18 by 22 inches). The papers were irrigated in a chromatographic cabinet by the descending method with a solvent of 1-butanol : acetic acid : water (4 : 1 : 5 v/v). The separated anthocyanin bands were cut out and eluted with 50 % methanol and the eluant was concentrated under reduced pressure. The anthocyanin was further puri-

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TABLE I

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR THE ANTHOCYANINS IN BRACTS OF POINSETTIA

COMPOUNDS	R_f VALUE IN				λ MAX. IN ETHANOL CONTAINING .01 *N* HYDROCHLORIC ACID (mμ)
	ACETIC ACID (5 %)	1-BUTANOL: ACETIC ACID: WATER (4:1:5 v/v)	PHENOL: WATER (73:27 w/w)	M-CRESOL: ACETIC ACID: WATER (50:2:48 v/v)	
Poinsettia anthocyanins:					
Band 1 from —					
Ruth Ecke(diploid)	.26	.32	.51	.43	538
Ruth Ecke (tetraploid)	.25	.33	.51	.42	538
Ruth Ecke × Ecke's White	.26	.32	.51	.44	538
Band 2 from —					
Ruth Ecke (diploid)	.39	.28	.49	.31	539
Ruth Ecke (tetraploid)	.38	.29	.47	.32	539
Ruth Ecke × Ecke's White	.39	.28	.49	.32	539
Band 3 from —					
Ruth Ecke (diploid)	.36	.52	.78	.62	518
Ruth Ecke (tetraploid)	.36	.51	.78	.64	518
Ruth Ecke × Ecke's White	.36	.52	.78	.63	518
Band 4 from —					
Ruth Ecke (diploid)	.50	.44	.75	.50	518
Ruth Ecke (tetraploid)	.50	.44	.75	.50	518
Ruth Ecke × Ecke's White	.50	.44	.75	.50	518
Authentic anthocyanins:					
Idaein (cyanidin-3-galactoside)	.25	.32	.65	.50	538
Chrysanthemin (cyanidin-3-glucoside)	.27	.32	.53	.44	538
Antirrhinin (cyanidin-3-rhamnoglucoside)	.37	.28	.49	.32	540
Callistephin (pelargonidin-3-glucoside)	.34	.55	.82	.68	517
Pelargonin (pelargonidin-3,5-diglucoside)	—	.34	.68	.42	518

TABLE II

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR ANTHOCYANIDINS FROM ACID HYDROLYSIS OF THE ANTHOCYANINS IN BRACTS OF POINSETTIA

COMPOUNDS	R_f VALUE IN			λ MAX. IN ETHANOL CONTAINING .01 *N* HYDROCHLORIC ACID (mμ)
	ACETIC ACID : HYDROCHLORIC ACID : WATER (30:3:10 v/v)	M-CRESOL : 5.5 *N* HYDROCHLORIC ACID : ACETIC ACID (1:1:1 v/v)	1-BUTANOL : 2 *N* HYDROCHLORIC ACID (1:1 v/v)	
Poinsettia anthocyanidins:				
Band 1 from —				
Ruth Ecke (diploid)	.59	.75	.69	545
Ruth Ecke (tetraploid)	.60	.74	.67	545
Ruth Ecke × Ecke's White	.60	.74	.68	545
Band 2 from —				
Ruth Ecke (diploid)	.60	.74	.70	544
Ruth Ecke (tetraploid)	.60	.74	.68	544
Ruth Ecke × Ecke's White	.60	.75	.69	544
Band 3 from —				
Ruth Ecke (diploid)	.76	.80	.80	532
Ruth Ecke (tetraploid)	.76	.80	.80	532
Ruth Ecke × Ecke's White	.76	.80	.80	532
Band 4 from —				
Ruth Ecke (diploid)	.75	.81	.80	532
Ruth Ecke (tetraploid)	.75	.81	.80	532
Ruth Ecke × Ecke's White	.75	.81	.80	532
Authentic anthocyanidins:				
Delphinidin	.38	.57	.35	555
Cyanidin	.60	.74	.69	545
Pelargonidin	.74	.82	.80	530

TABLE III

R_g VALUES AND COLOR WITH ANILINE HYDROGEN PHTHLATE OF SUGARS FROM ACID HYDROLYSIS OF ANTHOCYANINS IN BRACTS OF POINSETTIA

COMPOUNDS	R_g VALUE IN			COLOR WITH ANILINE HYDROGEN PHTHALATE
	ETHYL ACETATE : PYRIDINE : WATER (8:2:1 v/v)	1-BUTANOL : ETHANOL : WATER (40:11:19 v/v)	PHENOL : WATER (73:27 w/w)	
Sugars from acid hydrolysis of anthocyanins:				
Band 1 from —				
Ruth Ecke (diploid)	.97	.98	1.00	Brown
Ruth Ecke (tetraploid)	.97	.98	.97	Brown
Ruth Ecke × Ecke's White	1.00	1.00	1.00	Brown
Band 2 from —				
Ruth Ecke (diploid)	.97; 1.95	.99; 1.76	1.00; 1.62	Brown
Ruth Ecke (tetraploid)	.97; 1.96	1.00; 1.79	1.00; 1.61	Brown
Ruth Ecke × Ecke's White	.97; 1.96	1.00; 1.78	.98; 1.60	Brown
Band 3 from —				
Ruth Ecke (diploid)	1.00	1.00	.97	Brown
Ruth Ecke (tetraploid)	.99	.98	.99	Brown
Ruth Ecke × Ecke's White	1.00	1.00	1.00	Brown
Band 4 from —				
Ruth Ecke (diploid)	1.00; 1.97	.99; 1.76	.99; 1.60	Brown
Ruth Ecke (tetraploid)	1.00; 1.97	1.00; 1.79	1.00; 1.61	Brown
Ruth Ecke × Ecke's White	1.00; 1.97	1.00; 1.78	1.00; 1.62	Brown
Authentic sugars:				
Galactose	.89	.92	1.07	Brown
Glucose	1.00	1.00	1.00	Brown
Arabinose	1.41	1.21	1.38	Red
Fucose	1.63	1.51	1.64	Brown
Lyxose	1.81	1.43	1.34	Red
Xylose	1.70	1.31	1.24	Red
Rhamnose	1.97	1.78	1.62	Brown

fied by rechromatographing as previously described but using 5 % acetic acid as the solvent.

For the determination of R_f values a suitable portion of the purified anthocyanin extract was applied with a capillary tube to Whatman No. 1 paper (7 by 22 inches) at intervals of 2.5 cm along the starting line and irrigated by the descending method with the solvents listed in table I.

A 1-ml portion of the concentrated anthocyanin eluate was hydrolyzed with 1 ml of 2 *N* hydrochloric acid by heating at 100° C under reflux for 30 minutes. The anthocyanidin fraction was extracted three times with a minimum amount of *n*-amyl alcohol and the aqueous phase was examined for sugars.

R_f values for the anthocyanidin were determined by ascending chromatography. A suitable portion of the extract was spotted on 15- by 16-inch sheets of Whatman No. 1 paper rolled into cylinders as described by Irreverre and Martin (3), and irrigated with the solvents listed in table II. Identification of the anthocyanidins was made by co-chromatography with authentic compounds.

The aqueous phase containing the sugars was deacidified by passage through a weak anion-exchange resin (Deacidite E) and then concentrated to about 0.1 ml under reduced pressure at 40° C. Aliquots of 5 to 20 μl of the concentrated sugar fraction and 5 μl of 1 % solutions of authentic sugars, respectively, were applied with capillary tubes to Whatman No. 1 paper (7 by 22 inches) at intervals of 2.5 cm along the starting line. The front edge of the paper was trimmed with pinking shears to promote faster and more uniform movement of the solvent front. The papers were irrigated by the descending method with the solvents listed in table III. The sugars were located on the air-dried chromatograms by momentarily dipping them into aniline hydrogen phthalate (1), removing the excess by blotting, and then heating them at 100° C for three minutes. Identification was made by co-chromatography with authentic compounds.

The absorption spectra of anthocyanins and anthocyanidins were determined with a Beckman DU spectrophotometer using an ethanolic solvent containing .01 *N* hydrochloric acid.

Results and Discussion

The anthocyanins extracted from the bracts from each of the poinsettia plants, when originally separated by paper chromatography resolved into two distinct red bands and one obscure orange-scarlet band. When the orange-scarlet band was examined in several solvent systems (table I) it was found to contain two components. The R_f values and wave lengths of maximum absorption for the anthocyanins are shown in table I. These data indicate that the anthocyanins in the bracts of all the poinsettia plants examined were probably the same. Those in bands one and two were cyanidin glycosides and those in bands three and four were pelargonidin glycosides.

To identify further the anthocyanins, the four pigments from the bracts from each of the poinsettia plants were subjected to acid hydrolysis, and the products were identified by paper chromatographic and spectrophotometric methods. The anthocyanidin produced from bands one and two was cyanidin and that from bands three and four pelargonidin (table II). Hydrolysis of bands one and three yielded one sugar identified as glucose and of bands two and four, two sugars identified as glucose and rhamnose (table III).

Although the positions of the individual sugars were not determined it is apparent from the data in tables I, II and III that the anthocyanins in the bracts of all the poinsettia plants examined were identical. The anthocyanins were cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin) and pelargonidin rhamnoglucoside. The R_f values of the pelargonidin rhamnoglucoside indicate that the sugars are probably attached at the 3-position as rutinose. If the sugars were attached at the 3- and 5-positions, the R_f values for this anthocyanin probably would have been similar to those shown in table I for pelargonidin-3,5-diglucoside (pelargonin).

On the basis of the quantity of pigment in the bracts of the poinsettia plants examined, the cyanidin glycosides constituted the major components and the pelargonidin glycosides the minor ones.

Summary

The anthocyanins in the bracts from poinsettia plants of diploid Ruth Ecke (standard red), a seedling of tetraploid Ruth Ecke (dark red), and a cross between Ruth Ecke and Ecke's White (scarlet red) were isolated and identified by paper chromatographic and spectrophotometric methods. Acid hydrolysis of the anthocyanins and identification of the anthocyanidin and sugar moieties indicated that the anthocyanins in the bracts from all the poinsettia plants examined were identical. They were cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin), and pelargonidin-3-rhamnoglucoside.

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CURVATURES AND MALFORMATIONS IN BEAN PLANTS CAUSED BY CULTURE FILTRATE OF ASPERGILLUS NIGER [1,2]

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The fungus, *Aspergillus niger,* has been demonstrated to produce a growth substance active in the Avena test (4, 16). Boysen-Jensen (5) showed that the active material was soluble in ether under slightly acid conditions and that the fungus could convert tryptophan, lysine, leucine, tyrosine, histidine and phenylalanine into growth substance. The work of Kögl and Kostermans (12) established further that the molecular weight of the growth regulator was 169, a value which closely approximates that expected for indoleacetic acid (IAA). These lines of evidence suggest that IAA is produced by *A. niger.* IAA has been clearly shown to be the growth substance in the culture filtrate of *Rhizopus suinus* (18).

As a part of a routine screening program, seeking to detect fungus products that influence the growth of higher plants, it was recently discovered that culture filtrates of a fungus isolated from soil induced in plants effects which somewhat resemble those caused by known growth substances. However, the effects are distinctly different from those caused by IAA and evidence will be presented which eliminate IAA as the causative agent. Other preliminary studies concerning the active compound will also be reported in the present paper. The fungus responsible for these effects is designated as 56-39 and has been identified as *A. niger.*

Materials and Methods

Culture Conditions: The methods used to obtain filtrates from fungi routinely isolated from soil have been described (6). The fungi were grown in 500-ml Erlenmeyer flasks containing 100 ml of corn steep-Cerelose [3] medium (Staley's corn steep liquor, 40.0 g; Cerelose, 40.0 g; $CaCO_3$, 3.5 g; $NaNO_3$, 3.0 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.25 g; deionized water, 1000 ml). The inoculated flasks were incubated on a reciprocating shaker at 28° C for 7 days. The growth in each flask was removed by filtration (Whatman No. 1) and discarded. One drop of Tween 80 was added to approximately 100 ml of culture filtrate, adjusted to pH 5.0. All filtrates were initially tested without dilution.

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[2] Journal Paper No. 1124 of the Purdue Agricultural Experiment Station.

[3] Trade name for technical grade glucose produced by Corn Products Refining Company.

Treatment of Plants with Culture Filtrates: For purposes of routine screening, seedlings of corn (the single cross WF9 × 38-11) and bean (var. Black Valentine) have been used.

Corn seedlings were treated when 6 to 8 cm in height by filling the whorls with the culture filtrate on each of two alternate days. Plant heights were measured after 10 to 12 days and other effects of the treatment were noted. However, the work reported here concerns only the activity of the culture filtrate on bean plants, since little or no effect has been observed on corn.

Bean seedlings were ordinarily treated when the primary leaves had expanded to about one third of their full size and before elongation of the epicotyl had begun. This was done by placing 40 to 50 droplets of culture filtrate on one of the primary leaves on each of two alternate days. For reasons which will be made clear later, this technique was modified so that plants were treated only on the young growing point before the epicotyl began to elongate.

Results

Description of Activity of Culture Filtrate *A. niger* 56-39: The fungus in question was originally designated as 56-39, one of some 1500 culture filtrates tested for activity on higher plants. In the initial experiment four bean plants were treated on one of the simple leaves as described. Normally, the subsequent elongation of the stem follows a straight vertical line with the compound leaves produced more or less perpendicular to the elongating stem. However, at the end of 7 to 10 days two of the plants treated with the culture filtrate of 56-39 showed marked downward curvatures of the elongating stem and of the first compound leaf, as well as malformations on the stem near the junction of the first compound leaf and on the petiole of this leaf. The experiment was repeated a number of times on a large scale using a new supply of culture filtrate. In each experiment it was only possible to produce these striking effects on approximately one half of the treated plants. A variety of experiments were conducted in an attempt to induce the effects of the treatment on all of the treated plants. These included growing the plants under a variety of conditions, treating them at different ages, treating both of the simple leaves, incubating the cultures of 56-39