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ANTHOCYANINS OF COLOR MUTANTS OF (*TRIFOLIUM INCARNATUM*)

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Key Word Index—*Trifolium incarnatum;* Leguminosae; flower color genetics; anthocyanins; peonidin and cyanidin 3-glucosides.

Abstract—Anthocyanins in color mutants of crimson clover, *Trifolium incarnatum*, were extracted and identified. All color mutants contained peonidin 3-glucoside and cyanidin 3-glucoside. The distinction between crimson and the varying pink forms was found to be due to differences in concentration. Maroon flowers contained two additional pigments, cyanidin 3-sambubioside and an unidentified cyanidin 3-glucoside.

INTRODUCTION

THIS investigation represents a preliminary study of flower color genetics in crimson clover. The only previous studies have been those of Sandal[1] and Picard[2] who established that white flower color is a monogenic recessive of crimson flower color. It was our objective to further elucidate genetic mechanisms in terms of the flavonoid pigments reagent in each color class. We now report the identification of these pigments.

RESULTS AND DISCUSSION

Maroon forms had the most pigments; these were designated 1–4, 1 having the highest R_f. 3 and 4 occurred only in the maroon flowers, but 1 and 2 were common to all types in varying concentrations. Two-dimensional PC substantiated TLC data and also showed that as the number of anthocyanins decreased, the number of other flavonoids increased (as determined by visual observation of spot intensity in UV light).

R_f values and spectral analysis indicated pigments 1–4 were peonidin 3-glucoside, cyanidin 3-glucoside, cyanidin 3-sambubioside, and an unidentified cyanidin glycoside. The lack of fluorescence on paper chromatograms and the presence of a shoulder in the spectrum at 460–440 nm indicate that pigment 4 is a 3-glycoside. There are two or more sugars present in the hydrolysate, but we were unable to fully identify them. The R_fs ($\times$ 100) for the pigments on TLC are: (1) 61, (2) 50, (3) 29, (4) 23.

All four pigments have a maximum absorption between 523 and 528 nm. Therefore, cyanidin 3-glucoside (525 nm) could be used as a standard for determining relative total anthocyanin concentrations of the various classes, particularly since the four compounds

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[1] P. C. SANDAL, *Agron. J.* **47**, 147 (1955).

[2] J. PICARD, *Ann. Inst. Nat. Rech. Agron. Paris* **B6**, 527 (1956).

also possesses similar extinction coefficients (personal communication, Dr. Sam Asen). The total average concentration of anthocyanins of each color type are presented in Table 1. These data agree with visual observation of pigment content on TLC plates.

TABLE 1. TOTAL ANTHOCYANIN CONCENTRATION OF FLOWER COLOR CLASSES OF CLOVER

	P Generation		F_2 Generation	
Color	Avg. Conc.*	Range	Avg. Conc.*	Range
Maroon	10·6†	6·9–19·1	16·8†	14·0–21·0
Maroon-crimson wavy			6·0	4·5–8·8
Crimson	1·28	0·71–1·99	3·07	1·57–4·21
Deep pink	0·41	0·28–0·77	1·35	0·98–2·16
Medium pink	0·22	0·07–0·31		
Light pink	0·12	0·05–0·24		
Lavender	0·15	0·11–0·18	1·06	0·94–1·24
pink F_2			2·65	2·14–3·83

* Values given in mg pigment/mg fresh petal weight $\times 10^{-5}$.
† Highly significant difference from those values not marked, as determined by DNMRT.

The difference in color of the various mutants is due not only to the number of pigments present, but also to the total concentration of the pigments. Genetic analysis shows that pigments 1 and 2 are under the control of one gene, **Cr**, so that crimson and pink flowers segregate in a monogenic ratio. Pigments 3 and 4 are under the control of a second gene, **M.** Maroon flowers, therefore, segregate in a modified dihybrid ratio in the F_2. Lavender coloration is also a dihybrid situation, the second gene governing concentration and flavonol co-pigmentation effects.[3]

EXPERIMENTAL

Materials. Six color mutants were chosen from inbred lines selected and bred for color over several generations. These were maroon, crimson, deep pink, medium pink, lavender and white. Heads were chosen after several days of bright, sunny weather. Florets of 2–3 heads were plucked, weighed and frozen. Sample weight ranged from 40–100 mg. Samples were also taken of each color segregating out in the F_2 generations.

Extraction. Frozen samples of florets of known fresh weight were extracted by maceration with 15 ml 0·01% HCl in MeOH + 1 ml of 1% HCl in MeOH. The residue was washed with 0·01% HCl in MeOH until the washings were clear. The combined washings were concentrated in N_2 at 50° to 5 cm^3. This extract was washed with light petroleum ($\times$ 3), and then taken to dryness. 5 ml of 0·01% HCl in MeOH was added, and this solution was used for chromatography, spectral analysis, and determination of concentration.

Chromatography. Two-dimensional PC was carried out on Whatman No. 3 paper in BAW (*n*-BuOH–HOAC–H_2O, 3:1:1) and 15% HOAc. Approximately 30 μl of the extract was spotted on a sheet. One-dimensional PC on Whatman No. 1 paper in BAW (4:1:5), H_2O–conc. HCl (97:3), HOAc–conc. HCl–H_2O (15:3:82); and Forestal, HOAc–conc. HCl–H_2O (30:3:10). TLC was on silica gel plates in EtOAc-butanone–HCO_2H–H_2O (10:6:3:3).

Concentration. A standard curve was set up by the method of Jorgensen and Geissman[4] using cyanidin 3-glucoside as a standard. The molar extinction coefficient was determined and used to convert absorbance readings of the extract to mg of pigment concentration. This was further converted to a mg of pigment/mg of fresh petal weight base for comparative purposes.

Pigment analysis was by standard procedures (see e.g.[5]). Sugars were identified by GLC (see e.g.[6]).

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