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FLAVONOIDS IN GENOTYPES OF *TRIFOLIUM SUBTERRANEUM*—III.

VARIETAL DIFFERENCES

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Abstract—Flavonoid patterns in leaves of the Yarloop, Clare and Mt. Barker varieties of subterranean clover have been determined and compared quantitatively with that of the Geraldton variety. A "Red Leaf" mutant has also been studied and compared with its Mt. Barker parent. This mutation results in increased cyanidin and quercetin production.

INTRODUCTION

THE DIFFERENT varieties of subterranean clover are known to vary widely in their isoflavone concentrations.[1] Little is known, however, of other flavonoid constituents in these plants. In the preceding papers of this series the total flavonoid pattern of the Geraldton variety,[2] and the variations from this pattern in chemically induced mutants of Geraldton[3] were reported. In this work the pattern of flavonoids in leaves of three of the natural varieties of subterranean clover, Yarloop, Clare and Mt. Barker have been determined and compared quantitatively with that of Geraldton.

The three clovers compared with Geraldton represented the three subspecies of *Trifolium subterraneum* L. viz. ssp. *yanninicum* (Yarloop), ssp. *brachycalycinum* (Clare), ssp. *subterraneum* (Mt. Barker), and as such are representative of groups of genetic diversity within the species.[4] The "Red Leaf" variety of subterranean clover was established as a mutant of Mt. Barker and brought into direct comparison with it in this study.

RESULTS

Hydrolysed extracts of the various clovers were examined by means of two-dimensional paper chromatography and the individual flavonoids were determined spectrophotometrically as described in the previous paper,[3] with the results given in Table 1. From these results and from visual comparisons of the other constituents, the patterns of flavonoids in

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[1] C. M. FRANCIS and A. J. MILLINGTON, *Australian J. Agri. Res.* **16**, (1965).
[2] E. WONG and C. M. FRANCIS, *Phytochem.* **7**, 2123 (1968).
[3] E. WONG and C. M. FRANCIS, *Phytochem.* **7**, 2131 (1968).
[4] J. KATZNELSON and F. H. W. MORLEY, *Israel J. Botany* **14**, 112 (1966).

TABLE 1. CONCENTRATION OF FLAVONOID COMPOUNDS IN LEAVES OF DIFFERENT VARIETIES OF SUBTERRANEAN CLOVER (mg % fresh wt.)

	Geraldton	Yarloop	Clare	Mt. Barker	Red Leaf
Isoflavones					
Genistein	14·6	109	242	45·0	24·6
Biochanin A	23·4	13·3	4·1	174	94·6
Pratensein	1·6	+		7·4	3·8
Daidzein	<0·2	+			
Formononetin	42·7	37·8	4·0	1·2	1·3
Flavonols					
Kaempferol	38·8	18·3		11·6	4·6
Quercetin	7·6	9·8		10·8	76·2
Isorhamnetin	0·9	2·0		1·5	2·8
4′,7-Dihydroxyflavonol	0·8	+			
Fisetin	0·4	+			
Geraldol	<0·2	+			
Flavones					
Luteolin	0·7	+	7·3		
4′,7-Dihydroxyflavone	0·4	+			
3′,4′,7-Trihydroxyflavone	<0·2	+			
Geraldone	<0·2	+			
Chalcones					
Isoliquiritigenin	<0·1	+			
Homobutein	<0·1	+			

+ = Present in low amounts, comparable to Geraldton. Blank space indicates compound absent from chromatograms.

the other varieties relative to Geraldton can be summarized as follows:

Yarloop. This variety is very similar to Geraldton in flavonoid pattern. Isoflavones again predominate, but with genistein here being the most abundant component.

Clare. The pattern of this variety is vastly different from Geraldton. Genistein is present in very high concentration, with a much smaller amount of biochanin A. Formononetin is also greatly reduced and is the only one of the 5-deoxy compounds detectable. Another striking effect is the complete absence of flavonols in this variety. In contrast, the flavone luteolin is greatly increased.

Mt. Barker. This variety is again characterized by very low formononetin content and the absence of other 5-deoxy compounds. The normal isoflavones genistein and biochanin A are much higher than in Geraldton, with the latter compound predominating. The other methylated isoflavone, pratensein, is also increased in this variety.

Red Leaf. This is a mutant of Mt. Barker and is characterized by a pronounced red colour in the leaf. This colour has now been shown to be due predominantly to a cyanidin derivative, probably its 3-xylosylglucoside (see Experimental). Although no quantitative measurement was made of anthocyanins in the sample of Red Leaf studied here, measurements on Red Leaf genotype obtained from unpublished cross-breeding experiments show that the anthocyanin concentration is of the order of 30 mg% fresh wt., the anthocyanin content in the normal clover being by contrast less than one-twentieth of this value. In association with this large increase in cayanidin, there is also a large increase in the corresponding flavonol, quercetin (Table 1). In contrast, other 3′,4′-dihydroxy compounds such as luteolin and pratensein appear to be little affected.

DISCUSSION

In comparison with Geraldton, Yarloop shows little change in the flavonoid pattern as a whole, but Clare and Mt. Barker show many interesting variations. With reference to the biosynthetic scheme for the clover flavonoids presented earlier,[2] these biochemical differences can again be discussed in terms of genetic modifications at various stages of the pathway. In contrast to the isogenic mutants, however, biochemical differences in each variety relative to Geraldton require modification at more than one point of the pathway.

Clare. The differences in the operation of the biosynthetic pathways relative to the Geraldton variety are as follows: (1) Inhibition of the 5-deoxy pathway at the beginning, (2) blockage of the route to flavonols, and (3) decrease in the capacity for 4′-*O*-methylation of isoflavones. Inhibitions (1) and (2) together would have the indirect effect of allowing for greater synthesis of isoflavones and flavones of the normal series.

The distinctive flavonoid pattern of Clare is of interest for the variety is representative of a subspecies (*brachycalycinum*) whose reproductive barriers against other taxa of *Trifolium subterraneum* seem to have existed long enough to permit morphological divergence and perhaps recognition as a distinct species.[4] Morley and Francis[5] have reported the leaves of most varieties of ssp. *brachycalycinum*, like Clare, to contain minimal quantities of the isoflavones formononetin and biochanin A. An extension of the latter study to determine

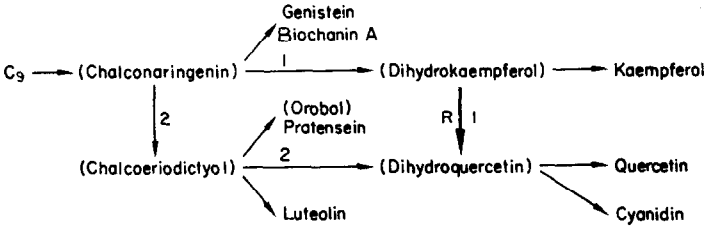
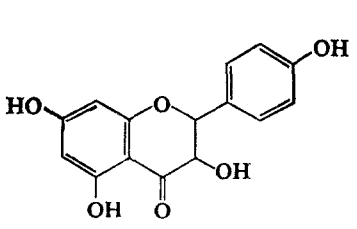


FIG. 1. POSSIBLE BIOSYNTHETIC ROUTES TO QUERCETIN AND CYANIDIN AND THE PROBABLE SITE OF ACTION OF GENE R IN THE RED LEAF MUTANT.
Compounds in brackets not detectable in this work.

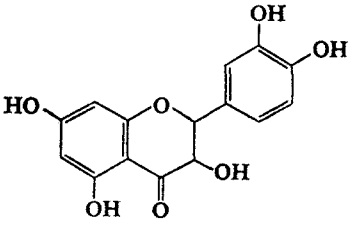
the extent of Clare's distinctive chemical features, such as absence of flavonols or production of luteolin, is likely to be of considerable chemotaxonomic value for it may enable some new strains to be placed within ssp. *brachycalycinum* without recourse to the laborious and time-consuming hybridization techniques hitherto employed.

Red Leaf. When this mutant is compared with its parent, Mt. Barker, the most striking effect is that cyanidin and quercetin are increased together. If this is the effect of a single dominant gene (R), as we believe, then the most obvious interpretation is that R enhances the formation of a common intermediate to the 3′,4′-hydroxy flavonol and anthocyanin. Traces studies have shown that 5,7,4′-trihydroxyflavanonol (I) (dihydrokaempferol) is a precursor for cyanidin and quercetin whereas kaempferol is not.[6] The formation of 5,7,3′,4′-tetrahydroxyflavanonol (II) (dihydroquercetin) from (I) is thus the likely key common step to cyanidin and quercetin.[7] This scheme is shown in route 1 in Fig. 1.

[5] F. H. W. MORLEY and C. M. FRANCIS, *Australian J. Agri. Res.* **19**, 15 (1968).
[6] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966).
[7] L. PATSCHKE and H. GRISEBACH, *Phytochem.* 7, 235 (1968).



(I)



(II)

Another possible route to the 3′,4′-hydroxy flavanonol (II) (and thus to cyanidin and quercetin) is via the corresponding 3′,4′-dihydroxychalcone (chalcoeriodictyol), as discussed previously.[3] This diversion, being earlier in the pathway, would be expected to affect the corresponding flavone and isoflavone as well (route 2, Fig. 1). Since, however, pratensein and luteolin are not found to be increased in this mutant, it can be concluded that route 1 (Fig. 1) is the pathway operating in clover and that the effect of R is the enhancement of the formation of the common 3′,4′-hydroxy flavanonol intermediate from the 4′-hydroxy flavanonol (Fig. 1).

It is interesting to contrast the effects of this R gene with that of gene M in *Antirrhinum majus*,[8] which enhances cyanidin and quercetin formation, but affects luteolin at the same time. Genes affecting hydroxylation of anthocyanin and flavonol together have been reported in other plants.[9]

EXPERIMENTAL

Plant Materials

Leaf samples were collected from plants grown together in replicated field plots and harvested when 10 weeks old. The genetic background of the Red Leaf variety was established by backcrosses in the suspected parent variety, viz. Mt. Barker.

Extraction, Chromatography and Spectrophotometric Analysis

These were carried out as described in the preceding paper.[3]

Identification of Anthocyanin Pigment in the Red Leaf Mutant and in Geraldton

An aqueous ethanolic extract of this mutant after washing with petrol. ether was concentrated *in vacuo* and the aqueous residue then washed several times with ether. The residue was concentrated to a thick red syrup, taken up in 1 per cent HCl–methanol and chromatographed as bands on 3MM paper in BAW, giving one main magenta band at R_f 0·33. This band was eluted and further chromatographed in the following solvent systems[10] (R_f in brackets): 1 per cent HCl (0·26); HAc–HCl (0·58); Bu–HCl (0·23); HCOOH–HCl (0·83). This compound had λ_{max} at 523 nm in 1 per cent HCl–methanol. On the basis of these chromatographic and spectral properties the compound was provisionally identified as cyanidin 3-xylosylglucoside by comparison with values given by Harborne.[10,11] Further evidence for the identity of this anthocyanin was provided by its hydrolysis in N HCl at 100° for 45 min. Paper chromatography in the systems referred to above revealed the presence of two magenta components, one identical in R_f with cyanidin 3-glucoside,[10] the other with cyanidin.[10] The isolated cyanidin had λ_{max} at 536 nm in methanolic HCl. The same pigment was found to be the main anthocyanin in Geraldton clover. Anthocyanin concentrations in leaves of Geraldton and the other clovers studied in this work are, however, generally very low.

Acknowledgement—Financial assistance from the Australian Wool Board is gratefully acknowledged.

[8] T. A. GEISSMAN, E. C. JORGENSEN and B. L. JOHNSON, *Arch. Biochem. Biophys.* **49**, 368 (1954).
[9] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoid Compounds*, p. 250, Academic Press, London (1967).
[10] J. B. HARBORNE, *J. Chromatogr.* **1**, 473 (1958).
[11] Reference 9, p. 1.