2. The pH optimum was found to be near 7.0. The activity was completely inhibited by 0.1 mm-*p*-chloromercuribenzoate and the inhibition was partially reversed by L-cysteine. The activity of an acid-treated extract was stimulated by flavin-adenine dinucleotide, but not by riboflavin 5'-phosphate. Phosphate and, to a lesser extent, arsenate stimulated the enzymic action.

3. A possible role of the enzyme in the oxidation of elemental sulphur by the organism is discussed.

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A Comparative Study of the Seed Polyphenols of the Genus *Theobroma*

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The importance of the polyphenolic constituents of the cacao bean in determining the flavour character of processed cacao has long been recognized. Earlier studies have, however, been largely limited to the clone, Imperial College Selection 1 (Forsyth, 1952, 1955; Griffiths, 1958) and West African Amelonado cacao (Rohan, 1958). During the past 30 years a collection of varieties of *T. cacao* and related species has been established at the Imperial College of Tropical Agriculture and more recently plants of the related genus *Herrania* have also been established for studies on their suitability for hybridization. The study of the distribution of the polyphenols within this group of plants appeared desirable for several reasons.

First, it is important to establish whether the flavour of a clone or variety of cacao is related to the type and quantity of the polyphenols present. Since it has been suggested (Forsyth, 1951) that not only the astringency but also the chocolate-flavour principle derives from the phenolic constituents of the cacao bean, it was of interest to determine whether the characteristic polyphenols of cacao are restricted to the seeds of the species used in cocoa manufacture or whether they are also found in the seeds of related species which do not yield chocolate-flavoured products.

Secondly, the suitability of material for inter-specific crossing will largely depend upon the cotyledon containing similar phenolics to those of *T. cacao* and lacking phenolic constituents which would give rise to a decrease in palatability or to undesirable pharmacological properties.

Thirdly, it was of interest to observe the distribution pattern of polyphenols in relation to the taxonomy of the group. Although the phenolic constituents of leaves (Bate-Smith, 1957; Roberts, Wight & Wood, 1958) and woody tissues (Erdtman, 1958) have been extensively studied from the taxonomic viewpoint, the literature relating to the comparative study of the phenolics of seeds is sparse.

Mature seeds of the genus *Theobroma* are relatively large (1–3 cm. in length) and soft in texture. The cotyledons, which are white or purple, are closely invested by a fibrous testa to which mucilaginous material, the 'pulp' of the cacao bean, adheres. The ripe seeds of *Theobroma* spp. are particularly suitable for comparative studies since the relative proportions of the seed phenolics remains constant at maturity. Comparative studies on leaf material are complicated by the complex changes undergone by the phenolics during development and senescence (Griffiths, 1958).

EXPERIMENTAL

Source of plant material. All seeds employed in these studies were obtained from ripe pods harvested from trees growing at the Imperial College of Tropical Agriculture, Trinidad, B.W.I.

Extraction and separation. The polyphenols were extracted by homogenizing 5 g. of peeled cotyledons in 25 ml. of cold, absolute methanol or methanolic 1% HCl (where indicated), in a M.S.E. micro-blender for 5 min. A volume (0.05 ml.) of the filtered extract was then applied to the chromatogram (Whatman Chromatography Paper no. 1).

Separation was effected by two-dimensional chromatography; water was employed as the primary solvent and butan-1-ol-acetic acid-water (40:10:50) as the secondary solvent.

Spray reagents. The *p*-nitroaniline spray reagent (Swain, 1953), which gives rise to yellow-brown spots with catechins and leucoanthocyanins (Griffiths, 1958), was used as a routine. It was possible to gauge the amount of each of the polyphenols present by the size and intensity of the resulting spot.

The $\text{FeCl}_3\text{-K}_3\text{Fe(CN)}_6$ reagent of Barton, Evans & Gardner (1952) was also used as a confirmatory spray reagent.

The presence of leucoanthocyanins was confirmed by the use of the reagent of Roux (1957), which gave rise to pink spots with leucoanthocyanins.

The ferricyanide reagent of James was employed for the detection of catecholamines, the presence of a catecholamine being indicated by the development of a red spot on the chromatogram due to indole ring closure (James, 1948).

RESULTS

The general distribution of polyphenols in the genus *Theobroma* is shown in Table 1. It will be seen that (-)-epicatechin and the leucoanthocyanins are of widespread occurrence within the group, (-)-epicatechin being present in all the species of *Theobroma* and *Herrania* that were examined. The leucoanthocyanins L1 and L2 were present in all species of *Theobroma* except

Table 1. *Seed phenolics of the genera Theobroma and Herrania*

1, (-)-epiCatechin; 2, leucoanthocyanin, L1; 3, leucoanthocyanin, L2; 4, leucoanthocyanin, L3; 5, leucoanthocyanin, L4; 6, (+)-catechin; 7, (-)-epigallocatechin; 8, (+)-gallocatechin; 9, 3-β-D-galactosidylcyanidin chloride; 10, 3-α-L-arabinosidylcyanidin chloride; 11, fluorescent polyphenol giving purple spot with *p*-nitroaniline (major); 12, fluorescent polyphenol giving purple spot with *p*-nitroaniline (minor); 13, *p*-nitroaniline: red spot; 14, *p*-nitroaniline: yellow-brown (non-fluorescent); 15, *p*-nitroaniline: yellow; 16, green fluorescence in NH_3 (*p*-nitroaniline: colourless); 17, *p*-nitroaniline: brown; 18, *p*-nitroaniline: yellow-green; 19-21, flavonols; brown fluorescence in u.v. radiation and yellow in visible light in the presence of NH_3 ; 19, *p*-nitroaniline: grey-brown; 20, *p*-nitroaniline: yellow; 21, *p*-nitroaniline: brown.

Spot no. ...	1	2	3	4	5	6	7	8	9	10	11
<i>T. cacao</i>											
SCA 6 (Forastero)	++++	+++	++	+	+	+	+	+	++	++	.
ICS 45 (Criollo)	+++	++	+	+	+	+	+	+	.	.	.
<i>T. bicolor</i>	++	.	.	.	.	.	.	.	.	.	.
<i>T. microcarpa</i>	++++	+++	+	.	.	.	.	.	.	.	+++
<i>T. subincana</i>	+++	++	+	.	.	.	.	.	.	.	+++
<i>T. angustifolia</i>	+++	+++	+	.	.	+	.	+	.	.	+++
<i>T. grandiflora</i>	+++	++	+	+	+	.	.	.	.	.	+++
<i>T. mammosa</i>	+++	+++	+	+	.	.	.	.	.	.	+++
<i>H. albiflora</i>	++	+	+	.	.	++	.	.	.	.	+++
<i>H. purpurea</i>	++	+	+	.	.	++	.	.	.	.	+++
<i>H. balaensis</i>	++	+	+	.	.	++	.	.	.	.	+++
<i>H. cuatrecasana</i>	++	+	+	.	.	++	.	.	.	.	+++
<i>H. nycterodendron</i>	++	+	+	.	.	++	.	.	.	.	+++
<i>H. nitida</i>	++	+	+	.	.	++	.	.	.	.	+++
<i>H. umbricata</i>	++	+	+	.	.	++	.	.	.	.	+++
Spot no. ...	12	13	14	15	16	17	18	19	20	21	
<i>T. cacao</i>											
SCA 6 (Forastero)	.	.	.	.	.	.	.	.	.	.	
ICS 45 (Criollo)	.	.	.	.	.	.	.	.	.	.	
<i>T. bicolor</i>	.	++	.	.	.	.	.	.	.	.	
<i>T. microcarpa</i>	.	.	.	.	.	.	.	.	.	.	
<i>T. subincana</i>	.	.	.	.	.	.	++	.	.	.	
<i>T. angustifolia</i>	.	.	++	+	.	+	+	.	.	.	
<i>T. grandiflora</i>	.	.	.	.	.	.	++	.	.	.	
<i>T. mammosa</i>	.	.	+	.	.	+	.	.	.	.	
<i>H. albiflora</i>	+	+	.	.	+	.	+	+	+	+	
<i>H. purpurea</i>	+	+	.	.	+	.	+	+	+	+	
<i>H. balaensis</i>	+	.	.	.	+	.	+	+	+	+	
<i>H. cuatrecasana</i>	+	+	.	.	+	.	+	+	+	+	
<i>H. nycterodendron</i>	+	.	.	.	+	.	+	+	+	+	
<i>H. nitida</i>	+	+	.	.	+	.	+	+	+	+	
<i>H. umbricata</i>	+	+	.	.	+	.	+	+	+	+	

T. bicolor and in all species of *Herrania* examined. In certain *Theobroma* species, e.g. *T. cacao*, *T. grandiflora* and *T. mammosa*, additional leucoanthocyanins were detected. In addition to those leucoanthocyanins earlier described by Forsyth (1955) in *T. cacao*, a further leucoanthocyanin, L4, has been detected. This was made possible by the use of methanolic 1% HCl as the extractant and by improvement in the equilibration of the solvent system. Under the original conditions (Forsyth, 1955) a diffuse zone of *p*-nitroaniline-positive material was found near to the point of origin in the upper left-hand corner of the chromatogram (Fig. 1) but, with the modifications described, an improvement in the definition of the leucoanthocyanin L4 makes its detection possible.

Of the species examined, only *T. bicolor* showed a complete absence of leucoanthocyanins and in this species only two polyphenols were detected. The ability to synthesize leucoanthocyanins is, however, shown by leaf material of *T. bicolor*, since chromatographic examination of the leaf polyphenols by the technique of Griffiths (1958) revealed their presence.

Anthocyanins were absent from all *Theobroma* spp. other than *T. cacao*. An association between the presence of these compounds and the chocolate-flavour principle is contraindicated by the occurrence of a variety of *T. cacao* (Criollo), which, although unpigmented, is widely used in chocolate manufacture.

Fig. 1. Composite diagram showing the position of seed phenolics of *Theobroma* and *Herrania*. Numbering is as given in Table 1. Spots shown diagrammatically with a complete outline were detected by the use of the diazotized *p*-nitroaniline reagent; spots shown with a dotted outline were detected by u.v. fluorescence or by diazotized *p*-nitroaniline reagent.

A major phenolic of the *Theobroma* and *Herrania* genera (spot 11) possesses a strong blue fluorescence in u.v. radiation and gives rise to a strong purple with diazotized *p*-nitroaniline. This compound is absent only from *T. cacao* and *T. bicolor*. However, although the compound is not present in the cotyledons of *T. cacao* it has been detected in the pod wall of this species (Griffiths, 1958). The compound has not yet been identified, although it has been shown to give a pink spot with the ferricyanide reagent of James (1948), which has been held by Waalkes, Sjoerdsma, Creveling, Weissbach & Udenfriend (1958) to be characteristic of a catecholamine. The *R_f* does not, however, correspond to that of any of the commoner catecholamines. In view of the known pharmacological activity of certain of the catecholamines it appears desirable that the nature and non-toxicity of this compound should be established before material containing it is used for hybridization.

Flavonols were found to be wholly absent from the cotyledons of all *Theobroma* species examined, although their presence in leaf material of *T. cacao* has been reported (Griffiths, 1958). In the *Herrania* spp. examined several flavonols were detected.

To determine whether any variation in polyphenolic distribution occurred within a species, a large number of clonal varieties of *T. cacao*, including SCA 6, SCA 12, ICS 1, ICS 95, ICS 60 and ICS 45, were examined. No marked variation in phenolics other than anthocyanins was noted; the chromatograms showed the same polyphenols in the same relative amounts. The anthocyanin pigments, α -L-arabinosidyl cyanidin and β -D-galactosidyl cyanidin, did, however, vary appreciably in amount: strong purple colorations were present on chromatograms of the SCA varieties and of the clone ICS 95, but smaller amounts were present in the clones ICS 1 and ICS 60, indicated by relatively weak spots on the corresponding chromatograms, and in the Criollo clone, ICS 45, the anthocyanins were absent.

DISCUSSION

The association of a group of leucoanthocyanins with a large (–)-epicatechin spot is the most constant feature of chromatograms of *Theobroma* spp. *T. bicolor* is an exception to this rule. The *Herrania* spp. are characterized by a large (+)-catechin spot, which equals that of (–)-epicatechin in intensity; the cacao leucoanthocyanins L 1 and L 2 are present and spot 11, which is given by all *Theobroma* spp. except *T. cacao* and *T. bicolor*, is always present. It is reasonable to suppose therefore that the genus *Herrania* is closely related to *Theobroma*. In view of the sparse information at present available on the distribution pattern of polyphenols in other genera and species of the Sterculiaceae, a detailed

taxonomic analysis of affinities, based on polyphenol distribution is not at present possible.

The results given in Table 1 indicate that the seeds of *Theobroma* spp. may be distinguished from each other by chromatographic analysis of their phenolic constituents. In most cases the differences between species are marked and qualitative in nature but in a few instances (e.g. *T. grandiflora* and *T. mammosa*) the differences are slight and of a quantitative character but are sufficiently reproducible to permit identification.

The situation for *Herrania* spp., however, is very different. Little variation in the phenolic constituents of the seed was found although in a few cases qualitative and quantitative differences could be detected among the minor constituents.

The polyphenols detected in the cotyledons of *Theobroma* are not peculiar to the seeds as the major seed phenolics of *Theobroma* have been detected in the vegetative parts of *T. cacao* (Griffiths, 1958). The role of these substances in the normal biochemical and physiological processes of the plant remains obscure. That the anthocyanins of the seed of *T. cacao* are not essential to either germination or growth of the seedling is indicated by the normal development of seedlings of the Criollo type, in the cotyledons of which anthocyanins are completely absent.

Since the leucoanthocyanins have been held to be of particular significance in relation to the basic flavour properties of cacao beans (Forsyth, 1951), their widespread occurrence in seeds which are not used in cocoa manufacture is of interest. The cacao beans of commerce are seeds of *T. cacao*, of which several distinct varieties are recognized, e.g. Amelonado, Forastero and Criollo, etc. As a result of the researches of Pound (1935) and others a large collection of clonal types covering a wide range of hybrid varieties of *T. cacao* was established in Trinidad. Earlier research at the Imperial College of Tropical Agriculture (Cope & Jolly, 1955) resulted in the recognition of considerable variation in the flavour of cacao of different clones. The chemical basis of the differences in flavour has, however, received little attention.

Chromatographic examination of various clonal varieties has not led to the recognition of any variation in the phenolic constituents of the different clones. Although the morphology and pigmentation of the pod varies appreciably from clone to clone, the phenolic constituents of the cotyledons were indistinguishable. The only clear-cut difference noted was the reduction or absence

of anthocyanin pigmentation in beans of the Criollo type (e.g. ICS 45). It appears probable therefore that flavour differences among the non-Criollo types of cacao are not to be attributed to variation in polyphenol content of the fresh beans.

SUMMARY

1. The distribution of methanol-soluble polyphenols in the genera *Theobroma* and *Herrania* is described.

2. (-)-*epi*Catechin, certain leucoanthocyanins and an unidentified compound showing blue fluorescence in u.v. radiation were found to be present in most of the species examined.

3. The major bean polyphenols of *T. cacao* were found to be present in a wide range of related species which are not used for cocoa manufacture.

4. The polyphenols of the *Theobroma* species are sufficiently characteristic to permit the identification of seeds of unknown species by paper chromatography of their phenolic constituents.

5. The seeds of *Herrania* spp. may be distinguished chromatographically from those of *Theobroma* spp. by the large (+)-catechin spots of the former genus and by the presence of flavonols.

6. The presence of a hitherto unreported cacao leucoanthocyanin L 4 is noted.

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