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LUTEOFOROL (3′,4,4′,5,7-PENTAHYDROXYFLAVAN) IN *SORGHUM VULGARE* L.

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Abstract—Luteoforol, prepared by reduction of eriodictyl with sodium borohydride, is characterized by the formation of luteolinidin when heated with aqueous HCl and by the formation of a blue-rose colour when treated with cold mineral acid. The latter property is used in a survey of the luteoforol content of the tissues of Kaffir Corn during a whole season's growth. The red colour of the seed-coat and the red or black colour of the glumes of the mature grain appear to be due to complexes ("phlobaphenes") embodying apigeninidin and/or luteolinidin, the latter arising from the luteoforol present in the green tissues.

INTRODUCTION

THE PRESENCE of constituents having the properties of flavan-4-ols has been reported in the leaves of grasses of the tribe Andropogoneae,[1] of which *Sorghum* is a member. One such compound, luteoforol, agreeing in properties with 3′,4′,5,7-tetrahydroxyflavan-4-ol, is richly present in the seed-coats of cultivated sorghum varieties.[2] Luteoforol is characterized by the production of a blue-rose colour in the cold when treated with concentrated mineral acid. This property was used[2] in a survey of the luteoforol content of some typical grain sorghums. An improved method of determination is now reported.

PREPARATION AND PROPERTIES OF LUTEOFOROL

A solution of luteoforol was prepared[2] by reduction of eriodictyol (3–12 mg) suspended in water (1–3 ml) at 0° with excess sodium borohydride over 30 min. After acidification to pH 6·5 with 6% acetic acid and addition of excess NaCl, the reduction product was extracted with *n*-propanol, the extract evaporated in a stream of air, and the solid residue extracted with methanol (MeOH). During these treatments an orange-yellow colour developed which intensified during subsequent attempts to purify the product. Its further purification was not therefore attempted.

The product, when dissolved in 2 N HCl, slowly became red at room temperature. On addition of amyl alcohol, the amyl alcoholic layer rapidly became crimson. When conc. HCl was added to the product dissolved in MeOH, the solution rapidly became deep purple. When cooled in ice and treated dropwise, with continuous shaking, with conc. H_2SO_4, the methanolic solution became blue-mauve in colour, deepening with continued addition of H_2SO_4 up to 50% (by vol.). This solution when diluted with methanol had λ_{max} 550 nm. A solution of the product in 2 N HCl became cherry red when heated, the λ_{max} being that of luteolinidin, 495 nm. On further heating, the colour rapidly faded to orange-yellow with the formation of a red, flocculent precipitate, in the same way as luteolinidin when similarly

[1] E. C. BATE-SMITH and T. SWAIN, *Nature* **213**, 1033 (1967).
[2] E. C. BATE-SMITH and V. RAŠPER, *J. Fd. Sci.* **34**, 203 (1969).

treated. The reduction product behaves, therefore, as a leuco-anthocyanin based on luteolinidin.

The reduction product undergoes, therefore, a number of independent reactions, depending on acidity and temperature:

(*a*) At pH 6·5 in aqueous solution at room temperature it slowly becomes orange-brown. In the cold (1–2°) it remains colourless, but after some days a white precipitate is formed.

(*b*) In alcoholic solution at room temperature, addition of mineral acid causes the formation of a blue-rose colour (λ_{max} 550 nm) together with some orange pigment (λ_{max} 465 nm). With time the orange pigment intensifies and the blue-rose fades. At 0° the formation of orange pigment is largely suppressed, but the blue-rose pigment fades, whereas at $-15°$ the formation of orange pigment is completely suppressed and the blue-rose pigment is stable.

(*c*) When an acid aqueous solution is heated, a cherry-red solution of luteolinidin is first formed (λ_{max} 495 nm), which on continued heating becomes orange, with the formation of an orange-brown precipitate.

(*d*) In aqueous or alcoholic solution the reduction product rapidly becomes yellow (λ_{max} 450 nm) on addition of alkali.

Of these reactions, the formation of the blue-rose pigment at $-15°$ offers the best possibility for the quantitative determination of luteoforol in plant material.

It had been found earlier[2] that the optimum concentration of H_2SO_4 in methanol for the development of the λ_{550} nm absorption at 0° was between 37·5 and 50 per cent (by vol.). For the development of colour at the lower temperature this concentration has been retained, by adding 0·1 ml of solution to 43% methanolic H_2SO_4 precooled to $-15°$. Using this method with varying amounts of eriodictyol reduction product and determining the absorbancy after 24 hr at $-15°$, gave a value for the $E_{550\ 1cm.}^{1\%}$ of the product from luteoforol of 1300 ± 50. This method was used for determination of luteoforol in the tissues. The absorption of the 550 nm pigment in 43% MeOH–H_2SO_4 decreased linearly at the rate of 25 per cent per 24 hr when kept at 0°, but at the rate of only 2 per cent per 24 hr at $-15°$ over 5 days. Solutions prepared at $-15°$ and kept at room temperature were completely decolorized after 24 hr. The absorbancy was also decreased by dilution with MeOH (25% reduction with an equal volume).

LUTEOFOROL CONTENT OF TISSUES OF *SORGHUM VULGARE* DURING A SEASON'S GROWTH

The sample of sorghum used for this investigation was the one previously described[2] as Kaffir Corn, a mixed sample of S. African origin. This had been shown to be divisible into two fractions, the one described as "bronze", amounting to about 20 per cent of the sample, and the other as "red", with some white and some red-white patterned grains. The bronze grains, but not the red, had a testa (nucellus) and contained not only luteoforol, but also leucocyanidin. Information received from Dr. P. van Twisk, National Nutrition Research Institute, Pretoria, suggests that the two fractions would correspond with the S. African Mealie Industry Control Board's classification of Kaffir Corn into the groups KF: varieties the kernels of which contain a red nucellar layer; KR or KM: varieties which have kernels red, or approaching red, but without a red nucellar layer. Fourteen grains of each type were sown in May in a heated greenhouse. Of these, five bronze (B) and nine red (R) germinated. All but one of these (B4) produced flowers and several of them produced mature seed during

the growing season. Many of them produced a second flowering culm before the end of the season, and also tillers from the root-stock. A second sowing of nine grains of each type was made in August.

Samples were taken of (*a*) seedling leaves, (*b*) mature leaves, (*c*) branchlets of the inflorescence without seeds, (*d*) branchlets with immature seeds and (*e*) branchlets with mature seeds. Since the luteoforol could not be extracted quantitatively from the tissues by any solvent treatment (cf. Ref. 2), the pigment was developed directly in the tissue and extracted into the methanolic H_2SO_4 by maceration. In the case of green tissues (leaves and immature inflorescences) the extract contained some chlorophyll, but since its major absorption peaks lay at λ_{665} and λ_{415} nm this interfered with the determination of luteoforol only when the concentration of the latter in the tissues was very low. The reaction is sufficiently sensitive that it is possible to determine luteoforol in a single grain (20 mg) or a single glume (1 mg). The results of such a determination of six grains of each of the bronze and red parents are given in Table 1.[1]

TABLE 1. LUTEOFOROL CONTENT OF SINGLE GRAINS

Bronze		Red	
Wt. of grain (mg)	Luteoforol (%)	Wt. of grain (mg)	Luteoforol (%)
29·5	0·071	46	0·030
22	0·123	36·5	0·029
24	0·128	35·5	0·0215
32	0·054	36·5	0·045
31	0·054	36	0·036
25	0·043	32	0·019

THE LEAVES

Very young leaves of a number of the seedlings of each type were examined at the second leaf stage both by heating in 2 N aq. HCl and extracting the pigments with *iso*amyl alcohol, and by determination of luteoforol as described above. None of the B seedlings contained measurable amounts of luteoforol, but one of them, B2, developed cyanidin when heated with HCl, presumably due to the presence of leucocyanidin in the leaf, since there was no preformed anthocyanin present. All the R seedlings examined, on the other hand, contained traces of luteoforol, the exact amount being difficult to determine because of the interference from chlorophyll. The amount in four of the seedlings tested was estimated to lie between 0·013 and 0·08 per cent.

At a later stage, when the leaves were fully developed and the inflorescence beginning to form, no luteoforol could be detected in them. The red coloration that frequently develops in the leaves as a result of injury or physiological stress, in these and other sorghum varieties, is due to a glycoside of cyanidin, probably chrysanthemin.

THE INFLORESCENCES

The inflorescence—spikelets—of sorghum, borne on a rachis, are of two kinds:[3] pedicelled, usually male or sterile; and sessile, bearing the grains. The former are seen as no more than

[3] J. D. SNOWDEN, *The Cultivated Races of Sorghum*, Adlard and Son, London (1936).

narrowly lanceolate, glume-like structures borne on the rachis below the ripe seed. The sessile spikelets, on the other hand, consist of a number of structures: two glumes (which may be deeply coloured, usually persist, and to a varying extent enclose the grain); two lemmas and a palea, usually membranous; two lodicules, often feathery in form; and the flower. As harvested, the ripe seed is either naked, or carries the glumes and perhaps one or two of the small pedicelled spikelets on a short branch of the rachis.

The first of the inflorescences of the B seedlings emerged about 12 weeks, the first of the R seedlings about 14 weeks, after sowing. The flowers were fully developed about a fortnight later, and seed, when present, was mature at 27 weeks. The inflorescences of the second culm of the R seedlings emerged at 27 weeks, at which time those of the B seedlings were in full flower.

TABLE 2. LUTEOFOROL CONTENT OF BRANCHLETS

		Luteoforol (% dry weight) Weeks after germination					
Seedling	Culm	0–1	1–3	3–6	6–12	12–18	>18
B0	1						0·30
	2			0·32			
B1	1	trace				trace	nil
B2	1						0·69
	2			1·83			
B3	1	0·08	0·10	0·70			
	2			0·78			
R1	2			0·75			
R3	1					0·79	
	2		2·20				
R4	2		1·12				
R5	1	0·07				1·86	
	2		2·51				
R6	1			2·0			
R8	1				0·60		
R9	1			2·10	0·75		

The stages in the development of the flower are reflected in the water content of the tissues, which decreases from about 70 per cent in the newly emerged inflorescence to 10 per cent or less in the ripe seed-head. During this time the luteoforol content rises dramatically, from virtually zero in the emergent inflorescence to values as high as 2·5 per cent (on a dry wt. basis and about 60 per cent water content) in the fully developed yet still green stage; but falling as the tissues lose their chlorophyll and become drier (Table 2). This fall in luteoforol is often accompanied by the formation of red pigment in the spikelets, the inflorescence as a whole assuming a reddish tinge. The values (on a dry wt. basis) for branchlets at 90 per cent dry weight, with only one exception, are lower than those for the fully developed branchlets of the same panicle at the green stage. The exception was B1, in which only a trace of luteoforol was present at any stage, including the seed.

The absolute values are unimportant not only because of the very different growing conditions of these plants compared with those where sorghum is grown as an economic crop, but because the material itself was inadequately characterized. It is clear, however,

that the synthesis of luteoforol provides an exceptional opportunity for the study of the ontogenesis of secondary constituents on account of the ease and unambiguity of its determination. An example of this is provided by the analysis of a number of branchlets dissected into their component parts.

DISSECTION OF THE INFLORESCENCE

A branchlet of seedling B3 was dissected, 1 week after emergence, into glumes, lemmas, paleas and residue; and at 3 weeks and 8 weeks into seeds, glumes and residue (the paleas and lemmas having then degenerated). A branchlet of seedling R5 was similarly dissected 1 week after emergence, and one of R8 after 5 weeks. The results, in Table 3, show that there is little differentiation in respect of luteoforol content until the seeds begin to enlarge, when the increase in endosperm results in only a small increase in their luteoforol content compared with that of the glumes and residues, which increases sharply.

TABLE 3. LUTEOFOROL CONTENT OF PARTS OF BRANCHLET

	Luteoforol (% fresh weight) Weeks after germination		
Seedling	1	3	5–8
B3	Glumes 0·0045 Lemmas 0·002 Paleas 0·0014 Residue 0·004	Seeds 0·030 Glumes 0·029 Residue 0·026	Seeds 0·058, 0·080 Glumes 0·304 Residue 0·265
R5	Glumes 0·052 Lemmas 0·020 Residue 0·004		
R8			Seeds 0·042, 0·054 Glumes 0·25

THE SEEDS

The seeds borne by the few seedlings of the B and R strains which produced mature seed were phenotypically identical with those of their respective parents. Typical grains of these had the luteoforol contents shown in Table 1.

The undeveloped seeds of seedling B3, when first examined about 2 weeks after emergence of the inflorescence, had the same luteoforol content as the glumes and residues, viz. 0·030 per cent on a fresh wt. basis. At 6 weeks, the seeds then having reached full size, but only 63 per cent dry wt. and still green at the apex, the luteoforol contents of two seeds were 0·089 and 0·123 per cent on a dry wt. basis, i.e. similar to that of the parent stock. However, seed harvested 6 weeks later and kept at room temperature for 2 months had lost most of this luteoforol; three of them contained only 0·030, 0·040 and 0·045 per cent.

The other fertile seedling of the B strain, B1, produced seeds which, though phenotypically similar, differed markedly in composition from any of the parent seed examined. The inflorescence had only traces of luteoforol and the seeds none at all, but like all seeds of

the B strain they contained leucocyanidin. The small amount of luteoforol present in the inflorescence was presumably in the upper glume, since this was deep black, the lower glume being colourless except for a small black area at the base.

The seeds of seedling R8 were examined at the green stage, 8 weeks after emergence of the inflorescence. The luteoforol contents, on a fresh wt. basis, were 0·042 and 0·054 per cent, equivalent to approximately 0·12 per cent on a dry wt. basis. At 18 weeks the seeds, then completely mature, were red in colour, dry wt. 96 per cent, the luteoforol content of two of them being 0·015 and 0·020 per cent. Seeds harvested at 14 weeks and kept at room temperature for 2 months had the same luteoforol content, viz. 0·015, 0·015 and 0·0165 per cent.

Two seeds of seedling R9 examined 14 weeks after emergence, shading from red at the base to green at the apex, dry wt. 64 per cent, had 0·110 and 0·120 per cent luteoforol on a dry wt. basis. At 20 weeks the luteoforol content had fallen to 0·028 and 0·042 per cent (dry wt. basis).

It can be concluded from these results that as the seeds mature the luteoforol present at the green stage is converted into the characteristic red pigment of the pericarp, and may at the same time polymerize so as no longer to give 550 nm absorption when treated with methanolic H_2SO_4. The product has all the properties regarded as characteristic of a phlobaphene, the only difference from the more familiar phlobaphenes of dicotyledonous plants and gymnosperms being the very unusual nature of the leucoanthocyanidin involved in its formation.

THE GLUMES

Next to the seeds, the glumes are the most prominent and distinctive features of the mature sorghum inflorescence. The varieties at present cultivated have, in fact, been selected for the retention of the seed on the panicle to enable them to be harvested, and this is effected by the induration of the tissues of the rachis and glume, and the development of the latter into a protective receptacle. In seems probable that the induration is due to the luteoforol present in the developing seed-heads, since this, as shown above, can amount to as much as 2·5 per cent of the dry wt. of these tissues.

The amount and subsequent fate of this luteoforol probably accounts also for much of the variation in colour found in grain sorghum varieties. The nature and extent of this variation in the glumes of Nigerian cultivated sorghums have been described and analysed by Stanton *et al.*[4] who detected three coloured components in ethanolic extracts by paper chromatography in butanol–acetic acid–water, but could not identify them as any of the commonly occurring anthocyanidins; black and dark mahogany-coloured glumes had a blue-purple component, (1) R_f 0·76–0·80, which were not present in those of red, paler mahogany, or sienna in colour. The latter had a red spot, (2) R_f 0·89–0·93, and a brown spot, (3) R_f 0·95–0·99, present also in most of the varieties with black glumes.

The glume colours of the seedlings in the present series ranged only narrowly from dark mahogany to black, but other samples with glume colours ranging from red to black have been available for comparison.

The glumes of seedling B1 (the inflorescences of which contained no more than a trace of luteoforol at any stage) were dark brown at maturity and contained no luteoforol. Those of seedling B3, 3 weeks after emergence, had 0·03 per cent of luteoforol on a fresh wt. basis. At 6 weeks, when the seeds were fully grown but still immature, the glumes, pale red in

[4] W. R. STANTON, T. B. MILLER and D. L. CURTIS, *Nature* **183**, 807 (1959).

colour, had 0·30 per cent (approximately 0·50 per cent on a dry wt. basis). After harvesting 6 weeks later and storing for 2 months, the glumes, then colourless except for some dark brown pigment at the base, had 0·03 per cent of luteoforol on a dry wt. basis.

At the 6-week stage the glumes of seedling R8 had 0·25 per cent of luteoforol and at full maturity were black in colour with 0·61 per cent (dry wt. basis). The glumes of seedling R9, on the other hand, were colourless except for a spot of deep red at the base, where the pedicel expanded into the blade of the glume. Their luteoforol content was about the same as that of the black glumes of R8, viz. 0·5 per cent.

Even when deeply coloured at maturity, therefore, the glumes still contain a large amount of luteoforol, but in deeply coloured glumes this is only about half the amount present at the green stage. This suggests that luteoforol may be the precursor of the pigment. The identification of the pigment extracted from black glumes by ethanol as luteolinidin strongly supports this suggestion.

Since the sample of Kaffir Corn used in the present experiments consisted of hulled grain, the glumes from a sample of Guinea Corn of similar origin to the sorghum varieties studied by Stanton[4] were used for the examination of the pigment, and also those from a genotype, NK 300A, bred in the U.S.A. and grown in Ghana. Extracts of the glumes, macerated with sand in ethanol, were chromatographed on paper in *n*-butanol–acetic acid–water, 4:1:5. Spots were obtained from black and red glumes agreeing in appearance with those described by Stanton,[4] but with lower R_f values. Thus the R_f of spot (1) was 0·62 (compared with 0·76–0·80); that of spot (2), 0·83 (0·89–0·93); and that of spot (3) 0·93 (0·95–0·99). However, the ΔRM value between each of these pairs was approximately the same (0·338, 0·301 and 0·368 for spots (1), (2) and (3), respectively) so that the R_f differences can be attributed to differences in experimental conditions.

Spot (1) was shown to be luteolinidin by co-chromatography and spectrophotometric comparison with an authentic specimen. By analogy and in view of Stafford's work,[5] spot (2) was expected to be apigeninidin, and this was confirmed by similar comparisons with an authentic specimen. Judging by its indeterminate colour, spot (3) seems likely to be a condensation product of one or both precursors.

The amount of pigment extracted from the deeply coloured glumes is actually very small, and it is possible, indeed probable, that their pigmentation is not due to the anthocyanidins as such, but to complexes from which small amounts of anthocyanidin can be detached. These complexes would be similar in nature to the phlobaphenes and phlobatannins formed in analogous circumstances by the flavan-3,4-diols, leucocyanidin and leucodelphinidin, and would be consistent with the tannin action previously reported. The remarkable feature about them is the intensely black colour of the tissues from which luteolinidin can be extracted.

While luteoforol is strongly indicated as the precursor of the luteolinidin extracted from the black glumes, there is so far no evidence that its lower homologue, 4′,5,7-trihydroxyflavan-4-ol, is present in juvenile tissues as the precursor of the apigeninidin present in red glumes. Aqueous solutions of this substance (apiforol) have been prepared by reduction of naringenin with sodium borohydride. These solutions produce apigeninidin when heated with 2 N aq. HCl, and a coloured ion with λ_{max} 537 nm when treated with 43% methanolic H_2SO_4 at $-15°$. Apiforol, if present, would therefore affect the quantitative determination of luteoforol, but the absorption curve should reveal the presence of apiforol by a shifting

[5] H. A. STAFFORD, *Plant Physiol.* **40**, 130 (1965).

of the 550 nm peak or by inflection at 537 nm. There is no sign of any such shift or inflection in pigments produced from the green tissues. It seems, therefore, that the apigeninidin must be formed during the later stages of enlargement and senescence of the glumes.

DISCUSSION

The conclusion to be drawn from this study is that the varying grain and glume colour of sorghum is a result of variations in the amount of colourless flavan-4-ol precursors of the pigments in the green tissues before the onset of the ripening process. This situation has many analogies in chemical genetics, especially the inheritance of leuco-anthocyanins in the seed-coats of leguminous grains. That here also the situation is under genetic control is supported by the genetical evidence.[6] What is at present lacking is any clear indication of the chemical and biochemical steps leading to the formation of the red, and eventually black, glume and seedcoat colours from the colourless precursors. The essentially orange colour of the pigment which can be extracted from these tissues with alcohol suggests that it is based on apigeninidin and luteolinidin in much the same way as the red phlobaphene colour of many gymnosperm and angiosperm heartwoods and barks is essentially based on cyanidin and delphinidin. An orange colour develops whenever luteoforol is kept at room temperature in aqueous or alcoholic solution or in the solid state and it seems likely therefore that it is a product of spontaneous oxidative condensation. The colour so formed has the same absorption maximum as luteolinidin, i.e. 495 nm, but it contains very little monomeric luteolinidin as judged by paper chromatography.

Conventional anthocyanic pigmentation based on cyanidin can occur in various parts of the sorghum plant, for instance at the base of the stem, at the nodes, and in damaged or senescent leaves, but this has not been encountered in any part of the inflorescence in the present series. The cyanidin produced when grains or young leaves of the "bronze" Kaffir Corn variety are heated with aqueous HCl originates from colourless leucoanthocyanin, and this has no biogenetic relationship to the anthocyanic pigmentation described above. This is only the second recorded instance of the occurrence of leucocyanidin in the Gramineae, the other being in barley, where it is present only in the endosperm.

In all these situations, the presence of leuco-anthocyanins, based on flavan-, whether 4-ols or -3,4-diols, is likely to be related to their tannin character. This was, in fact, the origin of the present investigation: to seek reasons for differences in palatability of tropical African grasses for the different species of the native animal population. One such reason may be that while luteoforol is present only in the young leaves of *Sorghum* spp., similar compounds are abundantly present in the mature leaves of *Hyparrhenia* spp., *Imperata cylindrica* (cotton grass), and other members of the tribe. The nature of the compounds in these species is at present being studied.

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[6] J. R. QUINBY and J. H. MARTIN, *Advan. Agron.* **6**, 305 (1954).