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DIFFERENCES IN PHENOLIC AND STEROIDAL CONSTITUENTS BETWEEN HEALTHY AND INFECTED FLORETS OF *MANGIFERA INDICA*

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Key Word Index—*Mangifera indica*; Anacardiaceae; malformation; fungal infection; *Fusarium moniliforme* var. *subglutinans*, lanosta-8-en-3β-ol-21-oic acid; *N*-2-(4′-hydroxyphenyl)-ethylcaffeamide, xanthones; phytoalexins.

Abstract—Differences in the low- and medium M_r phenolic and steroidal compounds in healthy and malformed florets of *Mangifera indica*, the latter infested with *Fusarium moniliforme* var. *subglutinans* (IMI 225231), and in those intentionally infected with this fungus are reported. The biochemical significance of the changes in these constituents, resulting from the hypersensitive responses in the host species, is appraised in respect to the etiology of the malformation disease of mango.

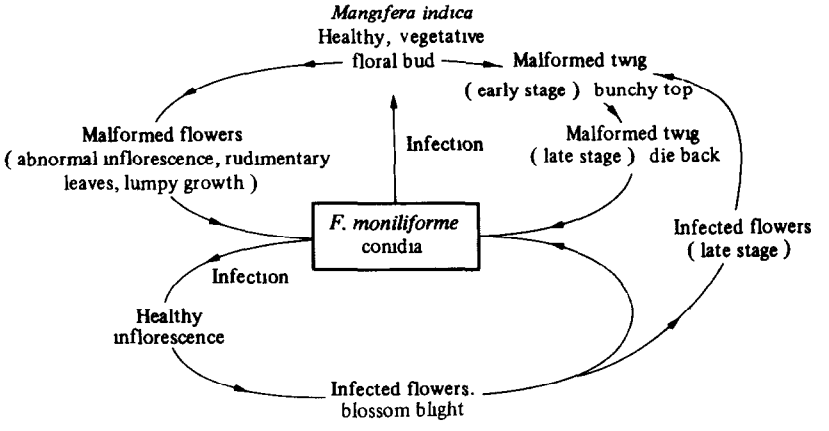
INTRODUCTION

The malformation disease of *Mangifera indica* makes superior quality mango trees totally abortive. Malformation is primarily the result of impaired floral sex expression due to interaction with *Fusarium moniliforme* var. *subglutinans* [1–3]. Studies conducted so far have not been directed at examining the nature of differences and biochemical significance of the individual phenolic and steroidal metabolites of healthy and malformed florets. The etiology of the malformation disease of mango is also unknown In this paper, we report the biochemical changes in the low and medium M_r phenolic and steroidal constituents of healthy and malformed florets and of those intentionally infected with *F moniliforme* var. *subglutinans* (IMI 225231).

RESULTS AND DISCUSSION

The malformation disease cycle of *Mangifera indica* (cv *Banarasi Langra*), postulated on the basis of the symptoms from the natural and artificial infections [1–3], is depicted in Scheme 1. The disease cycle suggests that any biochemical changes in the host species are a continuous development.

Significant changes were observed in the nature and contents of the phenolic and steroidal constituents of healthy and malformed florets and those artificially infected with the fungus. The healthy florets, at emergence, contained appreciable amounts of free sterols (FS), consisting of sitosterol, stigmasterol, campesterol, stigmasta-7-en-3β-ol and α-spinasterol, and their corresponding esters (steryl esters, SE) of palmitic and stearic acids The FS and SE were vicariously represented by the corresponding steryl glycosides (SG) and acyl steryl glycosides (ASG) in 10- to 12-week-old healthy florets. These constituents were characterized and quantified (Table 1) by HPLC, GC, UV and MS, according to a previously described procedure [4]. The malformed and the artificially infected florets, at no stage, produced any of the above sterols or their conjugates in noticeable



Scheme 1.

Table 1 Differences* in the phenolic and steroidal constituents of healthy and malformed florets of *M indica* and those intentionally infected with *F moniliforme*

Compound	Amount in mg/100 g florets (fr wt) one- to four-week-old/10- to 12-week-old		
	Healthy	Malformed	Intentionally infected
Compound **1**		8.0±0.2	1.5±0.2
		12.0±0.35	2.8±0.4
Compound **2**	14.0±2.6/18.0±2.5		
Gallotannins	1400±120/12000±438	164±32/	188±75
		178±78	162±22
Mangiferin	+	1450±12/	152±7
		3200±230	179±158
4-*O*-Me-gallic acid	-	+/165±14	+/22±2.5
Polymeric quinones	--	54±6/880±110	454±48
1,3,6,7-(OH)$_4$-Xanthone		+/12±1.5	2.5±0.7/24±3
Acyl steryl glycosides	12±1.5/40±2.5	-	
Free sterols	24±7.5/10±4.0	+	+
Steryl esters	10±2/3±0.5		+
Steryl glycosides	4±0.5/25±4		
Pregnenolone	-	1.5±0.3/1.8±0.7	1.8±0.1/1.1
Progesterone	-	2.5±0.6/4.7±0.4	5.1±0.9/4.8±0.7

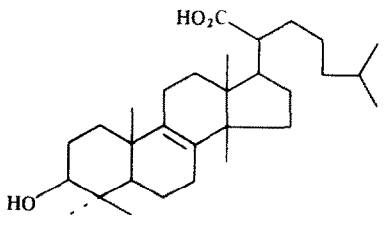
*Eight to ten replicates were maintained in each experiment, values (obtained from UV of prep TLC PPC, HPLC and GC isolations) represent mean + SE, ±, denotes traces, --, absent

amounts Instead, they were found to contain a mixture of zoosterols, viz pregnenolone (= 5-pregnen-3β-ol-20-one), progesterone, and a new lanostane derivative (compound 1) The quantities of all of these steroidal compounds progressively increased (Table 1) with the severity of the disease symptoms The presence of pregnenolone and progesterone, in the lipid-soluble fraction of the diseased florets, was established using tandem mass spectrometry [5] Collision-induced dissociation for both $[M]^+$ and $[M+H]^+$, at both high and low energy, was used to bring about the fragmentation (MS/ms) Subsequently, the two steroids were separated and quantitated by HPLC Compound **1** was isolated in quantities sufficient for its complete characterization None of these compounds was elaborated by the fungus in artificial cultures

In healthy florets, mangiferin, the major phenolic constituent of young leaves and bark of *M indica*, was either absent or present only in trace quantities Healthy florets, on the other hand, produced appreciable amounts of esters of glucose and gallic- and hexahydroxydiphenic acids (gallo- and ellagi-tannins) The amounts of these constituents rapidly increased with the growth of the florets (Table 1) Additionally, healthy florets were found to contain a new phenolic amide (compound **2**)

The gallic acid and hexahydroxydiphenic acid conjugates were present only as minor entities in both malformed and the intentionally infected florets, at any stage Compound **2** was not detected in these parts The major phenolic constituent of the diseased florets was mangiferin Its concentration was higher in malformed florets compared to that of intentionally infected florets Furthermore, the concentration of this compound in both parts increased rapidly with disease development Another phenolic metabolite, 1,3,6,7-tetrahydroxyxanthone, which was absent in healthy florets, appeared as a minor entity in both malformed and the intentionally infected florets Additionally, appreciable amounts of a brown polymeric quinone, and 4-*O*-methylgallic acid, instead of the gallo- and ellagi-tannins, were isolated from the diseased florets Structural elucidation of the two new compounds, **1** and **2**, are only described herein

Compound **1**, $C_{30}H_{50}O_3$ (from $[M]^+$ and elemental analyses), was optically inactive and did not exhibit any characteristic UV maximum above λ210 nm It showed significant IR bands due to OH, CO_2H and C=C groups but responded to the tetranitromethane test for unsaturation The EI mass spectral fragmentation pattern of the compound was reminiscent of a tetracyclic triterpene/-sterol [6] The fragmentation peak at *m/z* 314.2605,



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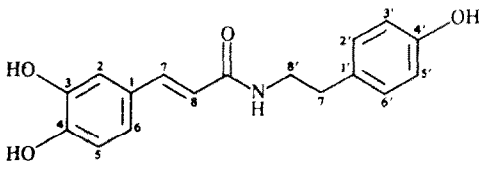

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ascribable to $C_{22}H_{34}O$, located the carboxyl function in the C-17 side chain. A further insight into its structure was obtained from the 100 MHz ^{1}H NMR spectrum and that of its methyl ester derivative. Both of these compounds showed seven C-Me signals. Two of these signals were associated with an isopropyl grouping, the remainder were singlets The two compounds further exhibited a methine proton quartet (H-3) but no olefinic proton signal. The methyl ester showed an additional signal due to the CO_2Me group The chemical shift of the Me-18 suggested the location of the double bond at C-8–C-9 position [7] The above spectral and chemical properties suggested lanosta-8-en-3β-ol-21-oic acid (**1**) to be structure of this compound It has not been encountered before in nature nor has it been prepared before synthetically The fungus, *F. moniliforme*, did not produce it in artificial cultures. Compound (**1**) was presumably produced in the host species from a cycloartenol intermediate by opening of the cyclopropane ring with the concomitant introduction of the 8,9-double bond Several C_{27}-oxygenated cycloartenol derivatives were reported earlier from other parts of *M indica* However, the occurrence of a C_{21}-carboxy-lanosteroid is rather unusual in higher plants and could be produced by the interaction of *M indica* (florets) with the pathogen, *F moniliforme*

Compound **2**, $C_{17}H_{17}NO_4$, exhibited UV maxima characteristic of amides of hydroxycinnamic acids. It produced a tri-*O*-acetyl derivative which showed acetoxyl bands in its IR spectrum between ν1765 and 1772 cm^{-1} (Ar-acetoxyl groups). These properties and the detailed ^{1}H NMR and mass spectra of the compound (see Experimental) suggested *N*-2-(4′-hydroxyphenyl)-ethylcaffeamide (**2**) to be its structure The fungus did not produce the compound in artificial cultures.

The elaboration and appreciable accumulation of mangiferin in the diseased florets would seem to be associated with the hypersensitive reaction in *M indica* due to interaction with the fungus. Hypersensitive responses are known in plants when they encounter potential pathogens The host species then produces antagonistic substances, eg. phytoalexins, in large quantities, to combat the pathogen. Other metabolic changes are also triggered which result in walling-off and death of the affected tissue [8] Thus, mangiferin and 1,3,6,7-tetrahydroxyxanthone, which are not normal constituents of healthy flowers of *M. indica*, were found in substantial amounts in the diseased flowers. It is also important to note that whenever the species was afflicted with any form of stress, e.g. cut injury or infection by pathogens, mangiferin and 1,3,6,7-tetrahydroxyxanthone were produced and accumulated in the injured organs [1, 9] Both mangiferin and 1,3,6,7-tetrahydroxyxanthone were found to be potent fungistatic agents [10, 11]. Thus, further ingress of the fungus, *Fusarium*, into the host organelles was prevented by mangiferin and 1,3,6,7-tetrahydroxyxanthone These entities and the gallic acid conjugates, present in the infected florets as minor components, in turn, were oxidized into polymeric quinones. The polymeric quinones caused collapse of the adjoining cells thereby removing the source of nutrition and/or multiplication of the fungus The following additional observations are consonant with the postulated sequence of the disease development

Mangiferin, which is a cytokinin augmentation factor [1, 10], was found to promote significantly vegetative growth in both producer and non-producer plants. Its accumulation in the diseased panicles, presumably, causes emergence of a large number of new shoots from the base of the panicles which, in the absence of normal biochemical function, assume typical characteristics of malformation. Likewise, the production and accumulation of the zoosteroids, pregnenolone and progesterone, instead of the normal phytosterols in the diseased flowers of mango, would seem to be associated with the impairment of the floral sex expression resulting in staminate, defective flowers with an emaciated ovary. The antifertility effects of progesterone in women [12, 13] and also in laboratory animals [14] are mediated by inhibition of the ovulatory surge of luteinizing hormone. It would be extremely interesting to study if a parallel situation exists in plants. The elaboration and accumulation of the phenolic amide (**2**) only in healthy florets is also physiologically significant. Amides of aromatic hydroxycinnamic acids have been frequently found to occur and accumulate in hermaphrodite flowers [15]. Such amides were found to be completely absent in staminate and defective flowers of the Araceae [16] Elaboration and accumulation of the amides of hydroxycinnamic acids would seem to be linked with the physiology of flowering They may also play important roles in the normal growth and protection of flowers as antiviral and antifungal agents as well as antifeedants [16].

EXPERIMENTAL

General procedures were the same as those reported previously [17].

Fungus. The fungus was isolated from the junction of ovary and thallamus of malformed flowers by blotting the surface-sterilized tissue on a water-agar medium. The only fungus that emerged was *Fusarium moniliforme* var *subglutinans* Wollnw & Reink.

Test plants Ten healthy and five malformed flower-bearing *M indica* L (cv *Banarasi Langra*, 6–8-year-old) trees, in the Banaras Hindu University Campus, were selected for the study, several weeks before the flowering season Healthy trees were divided into two groups of five. To one group, a spore suspension (1×10^4 spores/ml) of the fungus in sterile dist H_2O, was spread every week for four weeks during October At the emergence of flowering, panicles were washed with sterile dist H_2O and again once with a spore suspension of the fungus The control (healthy) group received only dist H_2O. The intentionally infected and the healthy group of trees were covered at the panicles with polythene bags loosely plugged with moist cotton balls to maintain the passage of air and moisture, and to provide protection against further ingress of any pathogen(s) About a week after inoculation, a pinkish growth of the fungus appeared on the infected florets Malformed florets also showed the presence of the fungus but less prominently Flower samples from the three groups (healthy, malformed, and intentionally infected) were collected, every week for 12 weeks, and chemically examined

Extraction of florets In a typical expt, intentionally infected florets (145 g, fr wt), collected after one week of inoculation of the fungus, were washed with dist H_2O and then macerated in a high-speed blender in MeOH (200 ml) The MeOH extract was kept overnight, filtered (Celite) and then evapd at 30° to give a brown residue (2 5 g) The marc (after extn with aq MeOH) was then continuously extracted (Soxhlet) with petrol and MeOH, in succession (30 hr, each) The residues from the hot petrol (0.71 g) and MeOH extracts (1 2 g) were combined with that of the cold MeOH extracts since their TLC patterns were similar The

combined residue was triturated with H_2O (100 ml) and the suspension extracted with Et_2O (fraction A), EtOAc (fraction B) and *n*-BuOH (fraction C) (100 × 3 ml portions each), in succession At the interface of the aq EtOAc extract, mangiferin and 1,3,6,7-tetrahydroxyxanthone were sepd and collected by filtration

Mangiferin The mixt was triturated with hot EtOH in which mangiferin was only sparingly soluble The identity of mangiferin, mp 268–271°, was established by direct comparison (mp, mmp, co-TLC, UV, MS) with an authentic sample [1, 2]

1,3,6,7-*Tetrahydroxyxanthone* The EtOH mother liquor after sepn of mangiferin was subjected to prep TLC, using *n*-BuOH–HOAc–H_2O (4 1 2) The R_F zone at 0 6 afforded the xanthone as dull yellow microcrystals, mp 370–372° (dec), UV λ_{max}(EtOH) nm (log ε) 240 (4 31), 258 (4 52), 315 (4 16), 365 (4 06), MS *m/z* (rel intensity %) 260 ([M]$^+$, 100), 232 (11), 231 (22). The identity of the compound was established by direct comparison (mp, mmp, co-TLC, UV, MS) with a synthetic sample of 1,3,6,7-tetrahydroxyxanthone [18]

Treatment of fraction A This fraction, consisting of a mixture of FS, SE and steryl carboxylic acid (compound 1), was sepd into neutral (fraction a_1), carboxylic (fraction a_2), and some intractable residue, in the usual way

Treatment of fraction a_1 Tandem MS of this fraction showed the presence of pregenenolone and progesterone besides a typical mixt of sitosterol, stigmasterol and campesterol (trace) Analytical HPLC [μ Bondapak-NH_2 (Waters), mobile phase, heptane–*iso* PrOH (4 1), detector, UV 217 nm, flow rate, 1 ml/min, 0 1 a u f s, temp ambient] of this fraction, using ref steroid samples (Sigma), showed the presence of pregnenolone (5-pregnen-3β-ol-20-one), R_t (min) 8 3, progesterone, R_t 7 8, sitosterol, R_t 5 8, campesterol, R_t 5 4, stigmasterol, R_t 5 35 HPLC plots were drawn using increasing amounts of pregnenolone and progesterone These were linear between 1 and 80 μg GC (3% OV-17 at 240) also established the identity of the above steroids (the plots of increasing amounts of pregnenolone and progesterone were linear between 60 ng and 7 μg)

Treatment of fraction a_2 The fraction was dissolved in MeOH and passed through a short column of silica gel (10 × 1 2 cm) Elution was done with C_6H_6–EtOAc (9 1) and fractions (15 ml) were collected and monitored by analytical TLC ($CHCl_3$–HOAC, 49 1)

Lanosta-8-en-3β-hydroxyl-21-oic acid (**1**) The middle C_6H_6–EtOAc eluates R_f 0 3, (Liebermann–Burchard reagent and I_2 vapour), were combined and concd to give the compound as colourless microcrystals, mp 210–212 °, $[\alpha]_D$ 0° ($CHCl_3$), IR ν_{max}(KBr) cm^{-1} 3550 (OH) 3450 (*br*, OH, carboxyl), 1718 (CO), 1665 (C=C), 950 (OH), MS *m/z* (rel int %) 458 ([M]$^+$, 68), 443 (72), 440 (18), 425 (100), 422 (14), 329 (10), 314 (18) [by accurate mass measurements *m/z* 314 2605 $C_{22}H_{34}O$ requires 314 2601], 311 (48), 299 (47), 273 (6), ^{1}H NMR ($CDCl_3$) δ 0 98 (6H, s, overlapping of C-19 and C-30 Me), 0 94 (6H, *d*, *J* = 6 5 Hz, C-26, 27 Me), 0 86 (3H, s, C-28 Me), 0 77 (3H, s, C-29 Me), 0 68 (3H, s, C-18 Me), 3 17 (1H, *q*, *J* = 10, 4 5 Hz, H-3), no olefinic H (Found C, 78 88, H, 10 45 $C_{30}H_{50}O_3$ requires C, 78 6, H, 10 9) Treatment of the compound with Et_2O–CH_2N_2 afforded the C-21 Me ester as an amorphous solid, ^{1}H NMR ($CDCl_3$) δ 3 70 (3H, s, CO_2Me), 0 65–0 96 (21 H, seven C-Me groups), MS *m/z* 472 ([M]$^+$, 75), 271 (100), 3-*O*-acetyl derivative, with Ac_2O–pyridine at 100 for 1 hr, crystallized from Me_2CO–hexane as needles, mp 118–121°, ^{1}H NMR ($CDCl_3$) δ 4 4 (1H, *q*, H-3), 2 12 (3H, s OAc), 0 65–0 98 (21 H, C-Me), MS *m/z* 500 ([M]$^+$, 27), 441 (9), 440 (11), 299 (100)

Treatment of fraction C The fraction contained a mixt of esters of glucose and gallic/hexahydroxydiphenic acids with M_rs ranging from 500 to 2500 The mixt was sepd by chromatography over Sephadex LH-20 followed by two dimensional prep PC [Whatman 3 MM, *n*-BuOH–AcOH–H_2O (14 1 5)/6% HOAc] The different R_f zones (0 2–0 5) were eluted to give the esters of glucose and phenols in different amounts (Table 1) Acid hydrolysis of the mixt afforded gallic acid (UV, HPLC, MS), hexahydroxydiphenic acid [MS, ^{1}H NMR (CD_3OD) δ 6 50 (1H, s), 6 42 (1H, s)], and ellagic acid (co-HPLC, UV MS) From the *n*-BuOH insol fraction, a dark brown material was obtained which was reduced with $NaHSO_3$ into a light brown powder which was re-oxidized in the presence of air

Polymeric quinones In another expt, dark brown florets, at an advanced stage of malformation, were extd in succession with Et_2O and Me_2CO, at room temp. The Me_2CO ext yielded a tan coloured spongy solid A part of this solid was triturated with H_2O and the resulting suspension extd with EtOAc The extract showed the presence of mangiferin, 1,3,6,7-tetrahedroxyxanthone and 4-*O*-methylgallic acid on TLC and HPLC A portion of the residue from the H_2O sol fraction, on KOH fusion, afforded protocatechuic acid (co-TLC, MS) and phloroglucinol (co-TLC, MS) Another portion of the dark brown solid was reduced with $NaHSO_3$ when a light brown spongy residue was obtained which, on autoxidation, reverted back to the polymeric quinone Oxidation of the residue from fraction C, containing mangiferin, 1,3,6,7-tetrahydroxyxanthone, and the oligoesters of glucose and the phenolic acids, with DDQ, in dioxane, afforded a similar polymeric quinone UV λ_{max} (MeOH) 227–232, 270–275 sh, 372 (sh) nm

The cold MeOH (fraction D), hot petrol (fraction E) and hot MeOH (fraction F) exts from the healthy florets were processed as before for the isolation of SG and ASG [17] CC (Florisil, $CHCl_3$–MeOH–H_2O, 190 9 0 10) of the phenolic components from fraction D, followed by prep PC [Whatman 3 MM, *n*-BuOH–HOAc -H_2O, 4 1 2] afforded compound 2 from the R_f zone 0 5

N-2-(4′-hydroxyphenyl)ethylcaffeic amide (**2**) The compound crystallized fromEt_2O–MeOH as straw coloured microcrystals, mp 140–143° (with prior shrinking at *ca* 95), UV λ_{max}nm (log ε) 220 (4 38), 232 sh (4 01), 293 (4 04), 320 (4 19), λ_{max} (MeOH–NaOMe) 242 (4 29), 306 (3 88), 362 (4 38), IR ν_{max}(KBr) cm^{-1} 1652, 1612, 1600, 1010, 930, MS *m/z* 299 ([M]$^+$, 42), 163 (22), 135 (17), 107 (100), ^{1}H NMR (CD_3OD) δ 7 54 (1H, *d*, *J* = 15 5 Hz, H-7), 7 17 1H, *d*, *J* = 15 5 Hz, H-8), 7 10 (2H, *dd*, *J* = 8 5, 2 5 Hz, H-2′, -6′), 7 01 (1H, *dd*, *J* = 8 5 2 5 Hz, H-6), 6 94 (1-H, *d*, *J* = 2 5 Hz, H-2), 6 88 (1H, *d*, *J* = 8 5 Hz, H-5), 6 84 (2H, *dd*, *J* = 8 5, 2 5 Hz, H-3′, -5′), 3 61 (2H, *m*, CH_2-8′), 2 80 (2H, *t*, *J* = 7 Hz, CH_2-7′) (Found C, 68 00, H, 5 44, N, 5 21 $C_{17}H_{17}NO_4$ requires C, 68 22, H, 5 68, N, 4 68) The tri-*O*-acetyl derivative prepd by treating (**2**) with Ac_2O–pyridine at 100 for 1 hr followed by removal of excess reagents with N_2, was obtained as an amorphous solid, *m/z* 425 ([M]$^+$, 35), 383 (7) 341 (9), 149 (14), 107 (100) Hydrolysis of (**2**) by refluxing with 1N NaOH for 1 hr, under N_2 produced caffeic acid (co-TLC, co-HPLC, UV, MS) and tyramine (co-TLC, co-HPLC, UV, MS)

In another expt, malformed florets (105 g) were macerated with MeOH and then kept at room temp overnight An aliquot of this ext was retained for analysis of free sterols and their conjugates by HPLC, GC and MS according to a previously described procedure [17] The remaining portion was saponified (10% MeOH–KOH, under reflux) and the non-saponifiable lipid fraction was partitioned into Et_2O–H_2O (2 1) The residue from the Et_2O layer was chromatographed on deactivated Al_2O_3 (grade IV) using hexane–Et_2O mixts followed by more polar eluting solvents Et_2O and MeOH The residue from the hexane–Et_2O (2 1) eluates when subjected to HPLC and GC showed the presence of pregnenolone and progesterone Healthy

florets also were processed in a similar way to determine whether there was any variation in the contents of their phenolic and steroidal constituents produced by the different ext methods, no significant variation was observed Results were analysed statistically according to an established procedure [19]

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