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Correlation between Phenylphenalenone Phytoalexins and Phytopathological Properties in *Musa* and the Role of a Dihydrophenylphenalene Triol

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Abstract: A correlation has been established between production of specific phenylphenalenones and resistance of various banana and plantain varieties towards certain pathogens. In addition a dihydrotrihydroxyphenylphenalene was isolated from the resistant 'Pelipita' plantain variety in relatively high concentrations and its structure and relative configuration were assigned on the basis of 1D and 2D NMR and NOE information. This compound is considered a key intermediate in the biosynthesis of phenylphenalenone phytoalexins.

Keywords: *Musa acuminata*; biosynthesis; phytoalexins; pathogens; resistance; susceptibility

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Introduction

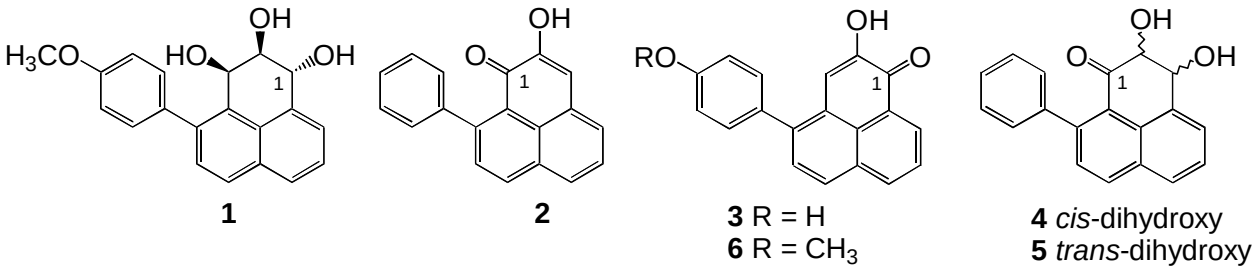
Phytoalexins are antibiotic compounds produced by plants under the influence of chemical, physical or microbial stress factors [1]. Production of these secondary metabolites is part of the complex defense mechanisms of plants against pathogenic microorganisms. Thus, a comprehensive analysis of phytoalexin production and biogenesis regulation could contribute to the design of plants possessing enhanced resistance against pests. We are interested in carrying out screening experiments to determine the occurrence of secondary metabolites, especially phytoalexins, in *Musa* varieties, and to establish correlations between phytoalexin production and susceptibility to pathogens, specifically bacteria and fungi. Phenylphenalenones represent a class of compounds isolated from the Musaceae (genus *Musa* and *Ensete*), Haemodoracea and Strelitziaceae families [2-5]. Modified phenylphenalenones were found in a Pontederiaceae species [6]. We describe herein the correlation between the concentration of phenylphenalenones and the resistance against *Mycosphaerella fidjiensis* and *Fusarium oxysporum* fungi, both pathogens towards *Musa* plants. In addition, we report the structure and relative configuration of ($1R^*$,$2S^*$,$3R^*$)-2,3-dihydro-1,2,3-trihydroxy-9-(4´-methoxyphenyl)-*1H*-phenalene (**1**), which is a possible biosynthetic intermediate in phenylphenalenone biosynthesis in bananas.

Results and Discussion

Phytoalexin production and pathogen resistance

Ethyl acetate extracts from leaves and roots of two banana varieties (‘Gross Michel’ and ‘Cavendish’) and two varieties of plantain (‘Pelipita’ and ‘Dominico’) were analyzed by TLC and HPLC for the occurrence of phenylphenalenone type compounds. The known 9-phenylphenalenones anigorufone (**2**) [7], *cis*- and *trans*-2,3-dihydro-2,3-dihydroxy-9-phenylphenalenones (**4** and **5**) [2], and also the 4-phenylphenalenones irenolone (**3**) [8], and 4'-methoxyirenolone (**6**) [9] were identified in all samples (Figure 1).

Figure 1. Phenylphenalenone structures.



1 **2** **3** R = H, **6** R = CH_3 **4** *cis*-dihydroxy, **5** *trans*-dihydroxy

In general, phenylphenalenone concentrations were higher in the rhizomes than in the leaves of the same species (Table 1). Banana and plantain (*Musa acuminata)* varieties 'Gross Michel' (colla AAA), 'Cavendish' (colla AAA), and 'Dominico' (colla AAB), containing low or moderate concentrations of phenylphenalenones in leaves, showed a lower degree of resistance against *Mycosphaerella fidjiensis* compared with the 'Pelipita' variety (*M. acuminata* colla ABB).

Table 1. Relative concentrations of phenylphenalenones in some *Musa* varieties and resistance against *Mycosphaerella fidjiensis* and *Fusarium oxysporum* var. *cubensis* race 4.

Compounds						
6	R	x	x	xxxx	x
	L	----	----	xxxx	----
5	R	xxx	xxx	----	xxx
	L	----	----	----	----
4	R	xxx	xxx	----	xxx
	L	----	----	----	----
3	R	xx	xxx	xxxx	xx
	L	x	xx	xxxx	xx
2	R	xx	xxx	xxxx	xxx
	L	x	xx	xxxx	x
1	R[b]	x[c]	x	xxxx	x
	L[a]	----	----	----	----
Resistance *F. oxysporum*		+	++++	++++	++
Resistance *M. fijiensis*		+[b]	+	++++	++
Cultivar		Gross Michel	Cavendish	Pelipita	Dominico
		Banana		*Plantain*	

[a] L: leaf, R: rhizome
[b]: Increasing resistance to fungi, +: susceptible, ++++: resistant.
[c]: Relative production levels of compounds, x: < 0.1 mg kg^{-1}, xx: 0.1-0.2 mg kg^{-1}, xxx: 0.2-0.5 mg kg^{-}, xxxx: 0.5-1 mg kg^{-1}.

Interestingly, the 'Pelipita' variety, having non-commercial and non-edible fruits, and being reported as highly resistant against *M. fidjiensis* and tolerant against the rhizome pathogen *Fusarium oxysporum* [10], showed a somewhat different phytoalexin pattern. Compounds **2**, **3** and **6** occurred in relatively high concentrations both in roots and leaves while dihydrodiols **4** and **5** were absent. Instead of the latter products, the root extracts exhibited an enhanced peak of compound **1**, which was only detectable in minor amounts in other varieties. Clearly, a correlation between phytoalexin production

pattern and resistance can be seen. However, it has not yet been demonstrated to which extent the individual compounds of the phenylphenalenone type contribute to this tolerance.

The high concentration of 2,3-dihydro-1,2,3-trihydroxy-9-(4´-methoxyphenyl)-phenalene (**1**) in the highly resistant 'Pelipita' plantain variety emphasizes its key role in the generation of antibiotic phenylphenalenone phytoalexins. Moreover, compounds carrying three oxygen functions at C-1, C-2 and C-3 might be of special importance as biosynthetic intermediates. Another 2,3-dihydro-1,2,3-trihydroxy-9-(4´-methoxyphenyl)-phenalene has been already reported from *Musa acuminata* var. 'Gran Dwarf' [11]. However, the ^{13}C-NMR chemical shifts of **1** (Table 2) did not completely resemble those of the compound isolated previously and the $[\alpha]_D$ values were also different. Thus, it is not completely clear whether both trihydroxy compounds are identical or if they represent stereoisomers. In any case, intermediates of that type (as well as 4'-hydroxy and hypothetical 4'-nonsubstituted analogues) are suggested to be involved in the biosynthesis of 4-phenylphenalenones from regioisomeric 9-phenylphenalenones [11]. Therefore, compound **1** was isolated in appropriate amounts, its structure was elucidated, and its relative configuration was determined.

*Structure elucidation and stereochemistry of compound **1***

Compound **1** is an amorphous white solid ($[\alpha]_D^{24}$ = -8.9°, c 0.1, $CHCl_3$). A molecular formula of $C_{20}H_{18}O_4$ (*m/z* obs. 322.12038, calcd. 322.12051) was assigned using HR-EIMS. The structure of compound **1** and the chemical shifts of all protons and carbon atoms were assigned using ^{1}H-, ^{13}C-, and 2D-NMR (COSY, HMQC, HMBC) techniques. The ^{1}H-NMR data (Table 2) and a ^{1}H-^{1}H COSY experiment indicated the presence of nine aromatic protons, distributed in three sets of spin systems, AA'BB' (δ 7.53/7.00) of the phenyl ring, AB of H-5/H-6 (δ 7.48/7.90) and AMX due to H-7/H-8/H-9 (δ 7.83/7.57/7.83). The chemical shift values of another spin system of three methine protons H-1 (δ 5.28), H-2 (δ 3.83), and H-3 (δ 5.02) indicated attachment of these protons to oxygenated carbon atoms. Final structure elucidation and unambiguous assignment of chemical shifts was done on the basis of HMQC and HMBC experiments. For example, H-3 showed long-range correlation to C-1 (δ 70.6), C-4 (δ 140.9), and C-9b (δ 128.0), while H-2 exhibited connectivities with C-1 and C-3 (δ 68.7), and H-1 displayed couplings to C-3, C-9 (δ 123.3) and C-9b. Finally, a cross peak of the OCH_3 singlet (δ 3.88) with C-4' (δ 159.6) indicated the position of the methoxyl group at the phenyl ring. The relative configuration of the molecule was determined from the values of vicinal coupling constants (Table 2). Thus, the large coupling constant $J_{H\text{-}1\text{–}H\text{-}2}$ = 9.6 Hz indicated a diaxial relationship for H-1 and H-2, while the small coupling $J_{H\text{-}2\text{–}H\text{-}3}$ = 2.9 Hz indicated that H-3 must be equatorial.

The acetonide **7** of compound **1** was prepared in order to confirm the stereochemistry. Downfield shifts in the ^{13}C-NMR spectra of C-2 and C-3 of **1** from δ 75.3 and δ 68.7, respectively, to δ 80.1 and δ 73.5 of **7**, and HMBC correlations of H-2 (δ 4.17) and H-3 (δ 4.99) with the quarternary carbon (δ 109.5) of the acetonide moiety proved the 2,3-acetonide structure. Moreover, these ^{13}C- and HMBC data and the vicinal coupling constant $J_{H\text{-}2\text{–}H\text{-}3}$ = 4.9 Hz confirmed the *cis* relationship between H-2 and H-3 (Table 2).

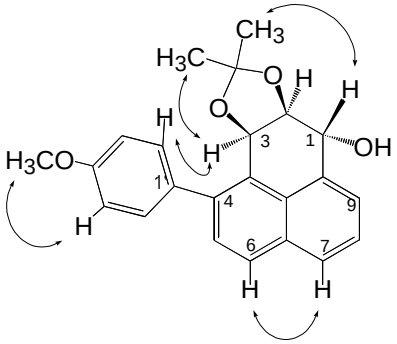
The circular dichroism (CD) spectrum of **1** showed three optically active absorption bands at 220, 250, and 305 nm with positive, negative and negative Cotton effects respectively, confirming the chirality of this compound. Based on these data, compound **1** was assigned as (1*R**,2*S**,3*R**)-2,3-dihydro-1,2,3-trihydroxy-4-(4'-methoxyphenyl)-*1H*-phenalene.

Table 2. ^{1}H and ^{13}C NMR data of compound **1** and acetonide derivative **7**.

	Compound 1		**Compound 7**	
	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
1	5.28 (*d*, *J*=9.6)	70.6	5.19 (*d*, *J*=8.3)	71.6
2	3.83 (*dd*, *J*=9.6, 2.9)	75.3	4.17 (*dd*, *J*=8.3, 4.9)	80.1
3	5.02 (*d*, *J*=2.9)	68.7	4.99 (*d*, *J*=4.9)	73.5
3a	-	130.0	-	129.3
4	-	140.9	-	143.0
5	7.48 (*d*, *J*=8.5)	129.3	7.56 (*d*, *J*=8.5)	129.8
6	7.90 (*d*, *J*=8.5)	129.2	7.96 (*d*, *J*=8.5)	123.2
6a	-	133.0	-	132.7
7	7.83 (*d*, *J*=7.7)	127.8	7.84 (*d*, *J*=7.6)	129.3
8	7.57 (*t*, *J* =7.7)	126.4	7.57 (*t*, *J*=7.6)	126.4
9	7.83 (*d*, *J*=7.7)	123.3	7.86 (*d*, *J*=7.6)	125.2
9a	-	136.0	-	134.9
9b	-	128.0	-	125.3
1´	-	133.2	-	133.1
2´- 6´	7.53 (*d*, *J*=8.6)	131.3	7.66 (*d*, *J*=8.6)	131.9
3´- 5´	7.00 (*d*, *J*=8.6)	114.1	7.00 (*d*, *J*=8.6)	113.6
4´	-	159.6	-	159.4
-OCH_3	3.88 (*s*)	55.8	3.90 (*s*)	55.8
C*Me*$_2$	-	-	1.65 (*s*), 1.41 (*s*)	29.5, 26.7
CMe_2	-	-	-	109.5

In the 2D NOESY spectrum of the acetonide **7** intensive cross signals were observed between CH_{3eq}–H-2 and CH_{3ax}–H-3, respectively (Figure 2). This finding was confirmed by ROESY and NOE difference spectra.

Figure 2. NOE's in 2,3-acetonide derivative of (1*R**,2*S**,3*R**)-2,3-dihydro-1,2,3-trihydroxy-4-(4'-methoxy-phenyl)-*1H*-phenalene (**7**).

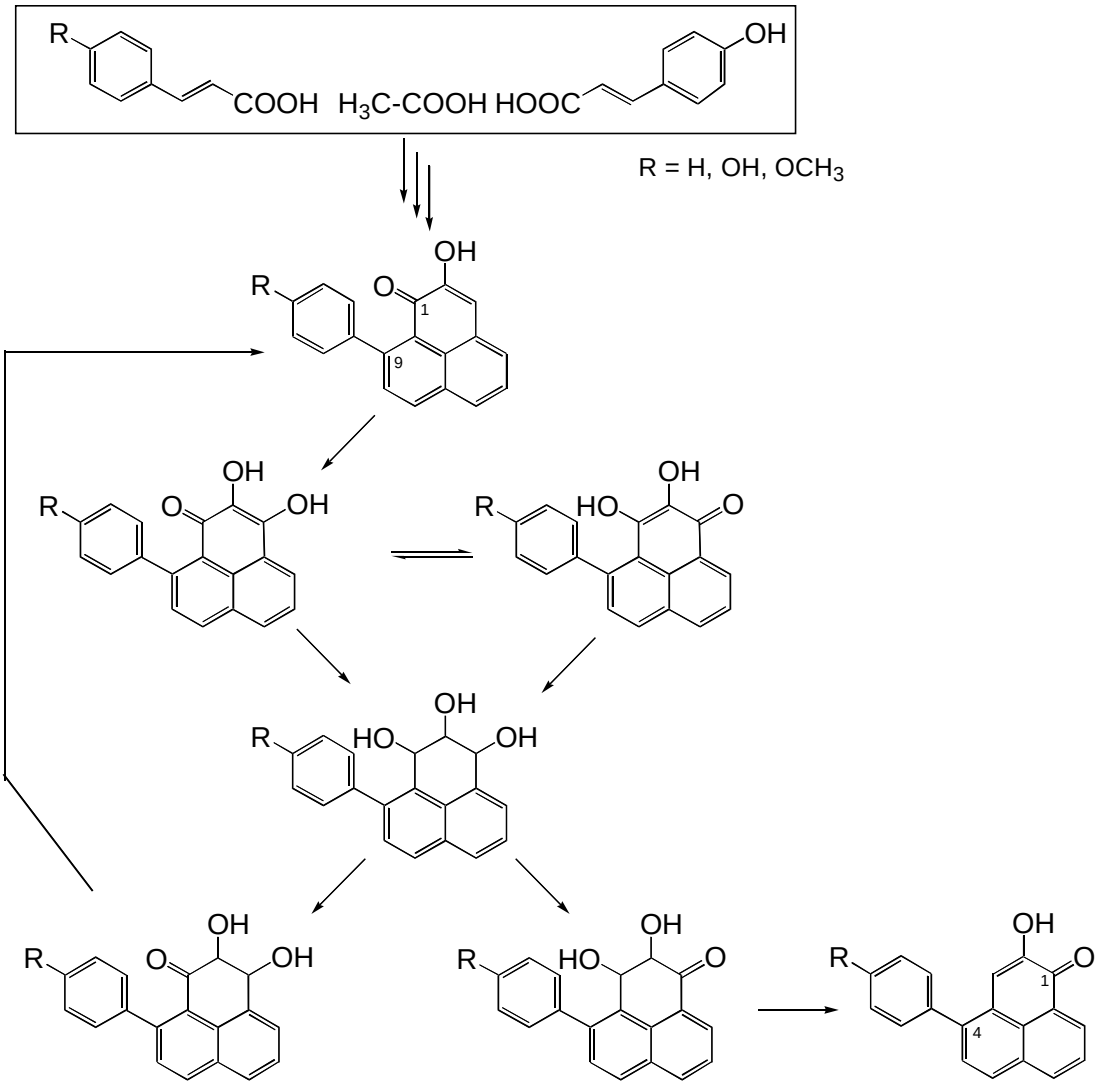


Biosynthetic pathway model

The general pathway of 9-phenylphenalenone biosynthesis from two phenylpropanoid units has been elaborated in *Anigozanthos* [12,13] and was confirmed recently in *Musa* [14]. However, while 9-phenylphenalenones have been found exclusively in *Anigozanthos* (some minor 4-phenyl-phenalenones [15] in *Anigozanthos* might be formed through keto-enol tautomerism), both regioisomeric 9- and 4-phenylphenalenones were isolated from *Musa*. The occurrence of phenalene-1,2,3-trioles, which is known from *Musa* only, could be a clue to the biosynthesis of 4-phenyl-phenalenones. Only few of these phenalene-1,2,3-triols have been isolated so far [11,14,15]; recently the absolute configuration of some di- and trihydroxy compounds were assigned by degradation techniques. A hypothetical biosynthetic sequence is depicted in Figure 3. Involvement of 1,2,3-triol intermediates in the biosynthesis of phenylphenalenones has been suggested also by Luis et al. [11] and Kamo et al. [16].

Anigorufone (**2**) (and its 4‘-hydroxyl or 4‘-methoxyl analogues as well) very likely represents a relatively early product in phenylphenalenone biosynthesis. The next step seems to be hydroxylation at C-3 resulting in 2,3-dihydroxy-9-phenylphenalenones, which subsequently may undergo tautomerism to form 2,3-dihydroxy-4-phenylphenalenones. Either tautomer could be converted by two reductive steps to an array of stereoisomeric compounds of the 1,2,3-triol type (e.g. compound **1**). Oxidation and, in the final step, dehydration, could result in the formation of 4-phenylphenalenones. This hypothesis is in accord with published suggestions involving oxidation and dehydration of precursors in the course of formation of 4-phenylphenalenones [15]. Oxidation and dehydration of phenalene-1,2,3-triols in part may also lead to recycling of 9-phenylphenalenones. The pathway proposed here involves a relatively large number of individual steps, which, however, are almost simple oxidations, reductions, and dehydrations. Probably a metabolic network may exist in *Musa* capable of generating an array of phenylphenalenone type compounds which may posses specific activities against different pathogens. In addition, the previously reported occurrence of musanolones C and D [2], steroisomeric 2,3-dihydro-2,3-dihydroxy-9-phenylphenalenones in rhizomes [17] and in unripe fruits of *M. acuminata* [14] could be explained smoothly from the pathway suggested in Figure 3.

Figure 3. Hypothetical biogenetic pathways to phenylphenalenones in *Musa*.



Variable substitution pattern at the phenyl ring indicates involvement of different phenylpropanoids as a precursor of the exocyclic phenyl ring. Variation in concentrations of several phenylphenalenones and production of phenalene-1,2,3-trioles seems to indicate a role in response to pathogen attack. Additionally, a correlation among resistance, PAL activation and phytoalexin production in the 'Yangambi' and 'Dominico' cultivars was observed [18]. The high concentration of compound **1** in the resistant/tolerant 'Pelipita' plantain variety emphasizes its key role in the generation of antibiotic phenylphenalenone phytoalexins in this plant.

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Experimental

General

Spectroscopic methods. ^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMBC and HMQC spectra were recorded on a Bruker AMX 300 spectrometer (300 MHz for ^{1}H; 75 MHz for ^{13}C; $CDCl_3$). NOESY, NOE difference and ROESY spectra were acquired on a Bruker Avance DRX 500 spectrometer. HR-EIMS resolution mass spectra were run on a VG-Micromass ZAB-2F mass spectrometer at 70 eV. CD spectra were recorded on a Jasco J-720 CD spectrometer.

Plant material and infection. Healthy and infected rhizomes and leaves of *Musa acuminata* (colla AAA) cv. 'Gross Michel' and cv. 'Cavendish', *M. acuminata* (colla ABB) cv. 'Pelipita', *M. acuminata* (colla AAB) cv. 'Dominico' were supplied by ICA (Instituto Colombiano Agropecuario). *Mycosphaerella fidjiensis* and *Fusarium oxysporum* were used for infection.

Extraction and HPLC analysis. Freshly collected rhizomes (20 kg) and leaves (500 g) of each variety (healthy and infected) were separately processed. They were washed, chopped, pressed to eliminate water, and immediately extracted with ethanol (2 L) in a percolator system. The crude extracts were evaporated to 25% of its initial volume and the residue was extracted with EtOAc [11,17]. For HPLC analysis, EtOAc extracts were evaporated to a semisolid material and chromatographed over a Sephadex LH-20 column using 2: 1: 1 *n*-hexane-CH_2Cl_2-MeOH as eluent; six fractions (100 mL) were collected, evaporated to dryness, and taken up by 10 mL of MeOH. Aliquots of 20 µL of fractions obtained in this manner were analyzed by reversed-phase HPLC (Waters Spherisorb ODS 2, 5 µm, 4.6 x 150 mm), using a linear gradient MeCN containing 0.1% trifluoroacetic acid (TFA) – H_2O (0.1% TFA) from 30 to 70% MeCN in 35 min at a flow rate of 1 mL min^{-1}. Authentic phenylphenalenones were used as internal standards for identification purposes and to compare relative concentrations between species tested. Compound **1** was isolated by preparative TLC from the last colourless Sephadex fraction using Et_2O / *n*-hexane (7:1 v/v) as eluent (R_f 0.17).

*Preparation of Acetonide **7**.* To **1** (5 mg) dissolved in C_6H_6 (1 mL) were added 2,2-dimethoxypropane (10 µL) and traces of p-toluenesulfonic acid. The mixture was stirred under reflux for 1 h., K_2CO_3 (0.2 mg) was added and the mixture stirred for an additional 4 h at room temperature and then extracted with CH_2Cl_2 to give **7** (4.7 mg, 87%); HRMS *m/z* obs. 362.1519 (calcd. for $C_{23}H_{22}O_4$, 362.1518)

Spectral data. Spectroscopic data of new compound **1** and its acetonide derivative **7** are listed in Table 2 or mentioned in the text. Data of known compounds matched those of authentic reference samples.

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Sample Availability: Samples are available from the authors.