Biosynthesis of Lipids Containing Isoricinoleic (9-Hydroxy-*cis*-12-octadecenoic) Acid in Seeds of *Wrightia* Species

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Seeds of two *Wrightia* species, *i.e. W. tinctoria* and *W. coccinea,* synthesize triacylglycerols containing isoricinoleoyl (9-hydroxy-*cis*-12-octadecenoyl, 9-OH-Δ^{12}18:1) moieties as the major lipid constituents. Seed maturation is accompanied by lipid accumulation and steep increase in the level of 9-OH-Δ^{12}18:1 in the total lipids, while relative proportions of palmitic (16:0), oleic (18:1), and linoleic (18:2) acids decrease. 9-Hydroxy stearic acid (9-OH-18:0) is also detected in these seed lipids, and both 9-OH-Δ^{12}18:1 and 9-OH-18:0 occur exclusively in the neutral lipids of mature and developing seeds. Developing seeds of both *Wrightia* species synthesize upon incubation with sodium [1-^{14}C]acetate, besides common long-chain fatty acids, considerable proportions of 9-OH-Δ^{12}18:1 and 9-OH-18:0, which are predominantly esterified in triacylglycerols. Substantial proportions of radiolabeled 9-OH-Δ^{12}18:1 and 9-OH-18:0 are also formed upon anaerobic incubation of the developing seeds with the ammonium salts of [1-^{14}C]18:2 and [1-^{14}C]18:1, respectively. The data presented suggest that hydration of the Δ^9 olefinic bond of 18:2 is a possible pathway of synthesis of 9-OH-Δ^{12}18:1, however, hydration of the Δ^9 olefinic bond of 18:1 followed by Δ^{12} desaturation of 9-OH-18:0 cannot be ruled out.

Introduction

Hydroxy fatty acids are known to be major constituents of seed lipids in several plant species [1], yet little is known so far on their biosynthesis in higher plants [2–6]. In general, two separate pathways seem to exist for the biosynthesis of hydroxy acids in various organisms [7]. In the aerobic pathway they are formed by mixed function oxygenases which require molecular oxygen and NADH and/or NADPH. Ricinoleic (12-hydroxy-*cis*-9-octadecenoic) acid, for example, is formed in castor *(Ricinus communis)* bean by the aerobic pathway [2, 3, 5]. In the pathway that does not require molecular oxygen the hydroxyl group is formed by hydration of a preexisting olefinic bond of a fatty acid. Ricinoleic acid is formed in the fungus *Claviceps purpurea,* for example, by the anaerobic pathway through hydration of the Δ^{12} olefinic bond of 18:2 [8]. The anaerobic pathway of formation of hydroxy acids has been demonstrated in several microorganisms [7, 9–13]. To our knowledge, it is not known so far whether the hydration of an olefinic bond is also involved in the synthesis of hydroxy acids in plants.

We have shown recently that lipids in mature seeds of *Wrightia* species contain isoricinoleic acid (9-OH-Δ^{12}18:1), closely related in structure with ricinoleic acid, as the major constituent fatty acid [14]. The synthesis of 9-OH-Δ^{12}18:1 was envisaged to occur by hydration of the Δ^{12} olefinic bond of 18:2 or by Δ^{12} desaturation of 9-OH-18:0, which was also detected in the mature *Wrightia* seeds [14]. In the present communication we report the compositional changes in the seed lipids of two *Wrightia* species during seed maturation and radiolabeling experiments with the developing seeds. These studies are aimed to elucidate the pathway of synthesis of 9-OH-Δ^{12}18:1.

Materials and Methods

Plant material

Seed pods containing mature seeds and those containing developing seeds at two different stages of maturation (I, 3–4 weeks after flowering, II 6–7 weeks after flowering) of *W. tinctoria* and *W. coccinea* were collected from the plants grown at National Botanical Research Institute, Lucknow, India. The developing seeds were kept frozen at −70 °C.

Abbreviations: Common long-chain fatty acids or acyl moieties having X carbon atoms and Y *cis*-double bonds are designated X:Y; 9-OH-Δ^{12}18:1, isoricinoleic acid (isoricinoleoyl moieties); 9-OH-18:0, 9-hydroxy stearic acid (9-hydroxy stearoyl moieties).

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Incubations and lipid extraction

Developing seeds were removed from the pods, incised into halves with a scalpel, and 10 seeds each, weighing about 200 mg at the developmental stage I and about 300 mg at the developmental stage II, were incubated with the radioactive substrates in 300 µl sodium phosphate buffer (0.1 M, pH 6.0). The incubations were carried out in screw-cap tubes by reciprocal shaking (130 strokes/min) at a temperature of 26 °C in a water bath for various periods. The mode of incubation was either aerobic, with the tubes being loosely stoppered, or anaerobic, in which the tubes were flushed with nitrogen and sealed before starting the incubations.

The substrates (Amersham-Buchler, D-3300 Braunschweig) were 185 kBq sodium [1-^{14}C]acetate (2.227 kBq.nmol^{-1}), 92.5 kBq [1-^{14}C]oleic acid (2.227 kBq.nmol^{-1}) or 74 kBq [1-^{14}C]linoleic acid (2.146 kBq.nmol^{-1}). The fatty acids were added as ammonium salts in a mixture consisting of 20 µl aqueous ethanol (20%, v/v) and 2 µl aqueous ammonium hydroxide (33%, w/v).

Incubations were terminated by heating the reaction mixture with isopropanol and the lipids were extracted by homogenizing with chloroform: methanol (2:1, v/v) followed by partitioning with aqueous sodium chloride [15].

Fractionation and analysis of lipids

The methods described previously [14] were used for thin-layer chromatography (TLC) of total lipids into neutral lipids and polar lipids, conversion of lipids to methyl esters, and their gas chromatography. Methyl esters were fractionated by TLC on Silica Gel G containing 20% (w/w) silver nitrate by developing twice with hexane : diethyl ether (70:30, v/v) into methyl esters of saturated, monounsaturated, diunsaturated, triunsaturated, and hydroxy fatty acids (9-OH-Δ^{12}18:1 plus 9-OH-18:0). Alternatively, the chromatoplates carrying the layer of Silica Gel G containing 20% (w/w) silver nitrate were developed twice up to 2 cm with diethyl ether and then up to 19 cm with hexane : diethyl ether : acetic acid (70:30:1, v/v). This resulted in the simultaneous separation of the methyl esters of both common long-chain fatty acids and the hydroxy acids according to the number of olefinic bonds, *i.e.* 9-OH-Δ^{12}18:1 was separated from 9-OH-18:0.

Radioactivity was measured in a Packard Tri-Carb C2425 liquid scintillation spectrometer (Packard Instruments Co. Inc., Downers Grove, Ill., U.S.A.) using Aquasol-2 (NEN-Chemicals, D-6072 Dreieich) for aqueous samples and Toluene Scintillator (Packard) for lipid samples. Silica Gel scrapings from TLC plates were counted in a mixture (1:1, v/v) of Aquasol-2 and Toluene Scintillator containing 1% (v/v) water. Silica Gel scrapings containing silver nitrate were counted in Toluene Scintillator.

Aliquots of the seed lipids that were extracted into the chloroform phase and those partitioning into the aqueous phase after treatment of the crude lipid extract with aqueous sodium chloride [15] were hydrolyzed [16] and the radioactivity in the fatty acids and water-soluble hydrolysis products was measured.

Results and Discussion

Compositional changes in lipids of the seeds of two *Wrightia* species, *i.e. W. tinctoria* and *W. coccinea*, during seed maturation are shown in Table I. It is evident that seed maturation is accompanied by accumulation of total lipids and increase in the proportion of neutral lipids, which are mainly composed of triacylglycerols, such as triisoricinoleoylglycerol and diisoricinoleoylacylglycerols, as shown earlier for the mature seeds [14]. Concomitantly, the level of polar lipids, which are composed of phospholipids and glycolipids, decreases (Table I).

Changes in the composition of acyl moieties of seed lipids (Table I) show that maturation of the *Wrightia* seeds is accompanied by decrease in the relative proportions of 16:0, 18:1, and 18:2 in the total lipids and a steep increase in that of 9-OH-Δ^{12}18:1 in both total lipids and neutral lipids. The pattern of accumulation of the constituent acids of the total lipids during seed development shows, in terms of mg/g seed tissue, a net increase in the levels of 18:1 and 18:2 and a massive rise in that of 9-OH-Δ^{12}18:1 (Table I). These findings suggest that 9-OH-Δ^{12}18:1 is possibly synthesized by hydration of the Δ^9 olefinic bond of 18:2, which, in turn, might be derived from 18:1 by Δ^{12} desaturation as observed in many plant tissues [17]. In this context it is of interest to note that ricinoleic acid is known to be synthesized in the fungus *Claviceps purpurea* by hydration of the Δ^{12} olefinic bond of 18:2 [8], rather than by hydroxylation of 18:1 at C-12 as reported for castor bean [2, 3, 5].

Table I. Composition of lipids in developing and mature seeds[a] of *Wrightia* species.

Seed: Lipids	Distribution [%]	Composition of acyl moieties (w % of acyl moieties; figures in parentheses are mg per g seed) 16:0	18:0	18:1	18:2	18:3	9-OH-Δ^{12} 18:1	others[b]
W. tinctoria								
Developing seed I								
Total lipids		25(5)	6(1)	24(5)	21(4)	17(3)	4(<1)	3(<1)
Polar lipids	24	29	8	30	20	12	tr	1
Neutral lipids	76	28	6	22	21	19	3	1
Developing seed II								
Total lipids		11(9)	4(3)	10(8)	13(10)	7(6)	53(42)	2(2)
Polar lipids	18	25	9	20	30	14	tr	2
Neutral lipids	82	15	5	10	18	7	42	3
Mature seed								
Total lipids		6(18)	3(9)	8(24)	11(33)	1(3)	69(207)	2(6)
Polar lipids	2	23	12	22	29	3	2	9
Neutral lipids	98	7	4	7	12	2	67	1
W. coccinea								
Developing seed I								
Total lipids		21(8)	2(<1)	19(8)	30(12)	4(2)	22(9)	2(<1)
Polar lipids	10	35	8	23	23	8	tr	3
Neutral lipids	90	19	3	19	34	4	20	1
Developing seed II								
Total lipids		9(6)	3(2)	15(11)	15(11)	5(4)	52(36)	2(1)
Polar lipids	8	22	6	22	38	11	tr	1
Neutral lipids	92	10	4	11	12	13	49	1
Mature seed								
Total lipids		6(14)	3(7)	6(14)	9(21)	<1(<1)	74(170)	1(2)
Polar lipids	1	31	13	24	23	1	7	1
Neutral lipids	99	5	3	7	10	<1	73	2

[a] Lipid content (wt %) of the seeds: *W. tinctoria*, developing seed I = 2%, developing seed II = 8%, mature seed = 30%; *W. coccinea*, developing seed I = 4%, developing seed II = 7%, mature seed = 23%.
[b] Including 20:0 and 9-OH-18:0.

The occurrence of minor proportions of 9-OH-18:0 in the lipids of *Wrightia* seeds at all the three stages of development (Table I and reference [14]) does not, however, rule out the role of 9-OH-18:0, derived by hydration of 18:1, as a possible precursor for the synthesis of 9-OH-Δ^{12}18:1 by Δ^{12} desaturation. The operation of this pathway is supported by the pattern of accumulation of 18:1 and 9-OH-Δ^{12}18:1, as shown in Table I. The occurrence of only minor proportions of 9-OH-18:0 may imply that 9-OH-18:0 formed from 18:1 is rapidly desaturated to 9-OH-Δ^{12}18:1.

It is interesting to note that although 9-OH-Δ^{12}18:1 is the most predominant constituent of the total lipids and neutral lipids of all the samples of *Wrightia* seeds, not more than traces of 9-OH-Δ^{12}18:1 are detectable in the phospholipids and glycolipids of the developing *Wrightia* seeds (Table I). Obviously, this unusual hydroxy acid synthesized in the *Wrightia* seeds is channelled almost exclusively to the triacylglycerols without the intermediate accumulation of phospholipids containing 9-OH-Δ^{12}18:1. Similar observations have been reported for other seeds that synthesize fatty acids having unusual structure rather than common long-chain fatty acids [18].

In order to obtain further insight into the pathway of synthesis of 9-OH-Δ^{12}18:1, the developing seeds of *W. tinctoria* and *W. coccinea* were incubated with sodium [1-^{14}C]acetate and the formation of lipids containing radiolabeled fatty acids was examined. Table II shows the pattern of incorporation radioactivity from acetate into the lipids of developing *Wrightia* seeds at two different stages of devel-

opment. For both *Wrightia* species, extensive incorporation of radioactivity into the seed lipids occurs at the earlier stage (I) as compared to the later stage (II). For the developing seeds at stage I of both species, the extent of incorporation of radioactivity increases with time, reaching a plateau after about 72 h (Table II). Most (> 90%) of radioactivity incorporated into the lipids is located in the acyl moieties and unesterified fatty acids, but very little radioactivity is found in the water-soluble hydrolysis products of the lipids (Table II). This shows that most of the acetate is utilized for fatty acid synthesis. The data given in Table II show for most of the seed samples incubated with acetate that over 90% of radioactivity incorporated into the lipids are located in phospholipids and glycolipids; extensive radiolabeling of neutral lipids occurs in *W. coccinea* seeds at stage II of development.

Table III records the distribution of radioactivity in acyl moieties (including unesterified fatty acids) of total lipids and lipid classes of developing *Wrightia* seeds upon incubation with sodium [1-^{14}C]acetate. The data show that most of the radioactivity is located in 16:0, 18:0, and 18:1, which are very likely formed by *de novo* synthesis from acetate. Although the proportion of labeled hydroxy acids (9-OH-Δ^{12}18:1 plus 9-OH-18:0) are relatively small compared to the labeled common long-chain fatty acids, the labeled hydroxy acids are located predominantly in the neutral lipids, mostly triacylglycerols (Table III). These data are in good agreement with the occurrence of the hydroxy acids almost exclusively in the neutral lipids of the developing *Wrightia* seeds (Table I).

The data given in Table III also show for the developing seeds of both *Wrightia* species at stage I that some increase during the course of incubation with acetate occurs in the levels of labeled 18:2 and 9-OH-Δ^{12}18:1; concomitantly, the level of labeled 18:1 is decreased. These findings seem to support the operation of the pathway:

$$18{:}1 \rightarrow 18{:}2 \rightarrow \text{9-OH-}\Delta^{12}18{:}1$$

However, formation of labeled 9-OH-18:0 is also observed during the entire period of incubation with acetate (Table III), which does not rule out the formation of 9-OH-Δ^{12}18:1 as follows:

$$18{:}1 \rightarrow \text{9-OH-}18{:}0 \rightarrow \text{9-OH-}\Delta^{12}18{:}1$$

The developing *Wrightia* seeds were also incubated under both anaerobic and aerobic conditions with the ammonium salts of the radioactive fatty acids, *i.e.* [1-^{14}C]18:2 and [1-^{14}C]18:1, and the seed lipids were examined for the formation of radiolabeled hydroxy acids. With both substrates, about 90% or more of the added radioactivity have been recovered in the chloroform-soluble lipids in most cases (Table IV).

The results given in Table IV also show that only small proportions of exogenous [1-^{14}C]18:2 or [1-^{14}C]18:1 are incorporated into the polar lipids in most incubations. The relative proportions of labeled

Table II. Incorporation of radioactivity into lipids of developing *Wrightia* seeds upon incubation with sodium [1-^{14}C]acetate.

Seed	Incubation time [h]	Radioactivity in chloroform-soluble lipids		Distribution of radioactivity in chloroform-soluble lipids (% of dpm in total lipids)	
		Total (% of dpm in substrate)	Fatty acids plus acyl moieties (% of dpm in total lipids)	Polar lipids	Neutral lipids
W. tinctoria					
Developing seed I	24	45	99	96	4
Developing seed I	72	65	98	98	2
Developing seed II	24	2	95	94	6
W. coccinea					
Developing seed I	24	69	99	94	6
Developing seed I	48	71	91	97	3
Developing seed I	72	80	89	92	8
Developing seed II	24	4	92	79	21

Table III. Distribution of radioactivity in fatty acids and acyl moieties of lipids of *Wrightia* seeds upon incubation with sodium [1-^{14}C]acetate.

Seed	Incubation time	Distribution of radioactivity in fatty acids and acyl moieties [%]														
		Total lipids					Polar lipids					Neutral lipids				
		16:0 + 18:0	18:1	18:2	18:3	Hydroxy acids	16:0 + 18:0	18:1	18:2	18:3	Hydroxy acids	16:0 + 18:0	18:1	18:2	18:3	Hydroxy acids
W. tinctoria																
Developing seed I	24	33	64	<1	<1	1	34	57	4	2	2	38	35	7	8	12
Developing seed I	72	43	44	6	5	3	43	44	9	2	2	23	30	13	11	22
Developing seed II	24	27	66	1	3	3										
W. coccinea																
Developing seed I	24	37	62	<1	<1	1	41	53	3	1	2	36	46	6	5	8
Developing seed I	48	32	57	3	3	5[a]	27	56	5	6	4	29	45	8	7	11
Developing seed I	72	29	54	9	3	4[b]	36	43	11	4	6	45	23	16	6	9
Developing seed II	24	25	69	1	2	2										

[a] Composed of 3% 9-OH-Δ^{12}18:1 and 97% 9-OH-18:0.
[b] Composed of 19% 9-OH-Δ^{12}18:1 and 81% 9-OH-18:0.

Table IV. Incorporation of radioactivity into lipids and their constituent hydroxy acids of developing *Wrightia* seeds upon incubation with ammonium [1-^{14}C]linoleate or ammonium [1-^{14}C]oleate under aerobic (A) or anaerobic (AN) conditions.

Seed	Substrate	Incubation Mode/Time [h]	Distribution of radioactivity in chloroform-soluble lipids			Labeled hydroxy acids synthesized (nmol)[b]	
			Total (% of dpm in substrate)	Polar lipids	Neutral lipids[a]	9-OH-Δ^{12}18:1	9-OH-18:0
				(% of dpm in total lipids)			
W. coccinea							
Developing seed I	[1-^{14}C]18:2	A / 24	93	1	99	0.6 (2)	0.6 (2)
	[1-^{14}C]18:2	A / 48	71	10	90 (34)	0.2 (1)	0.1 (0.5)
	[1-^{14}C]18:2	AN / 24	88	1	99	1.5 (5)	1.2 (4)
	[1-^{14}C]18:2	AN / 48	95	2	98 (89)	3.8 (12)	2.5 (8)
	[1-^{14}C]18:1	A / 24	89	7	93	0.7 (2)	2.9 (8)
	[1-^{14}C]18:1	A / 48	84	12	88 (66)	0.7 (2)	1.4 (4)
	[1-^{14}C]18:1	AN / 24	92	6	94	1.1 (3)	3.0 (8)
	[1-^{14}C]18:1	AN / 48	93	4	96 (60)	0.8 (2)	2.3 (6)
Developing seed II	[1-^{14}C]18:2	AN / 48	95	5	95	1.3 (4)	0.3 (1)
W. tinctoria							
Developing seed II	[1-^{14}C]18:2	AN / 48	90	22	78	0.9 (3)	0.3 (1)

[a] Figures in parentheses represent radioactivity in unesterified fatty acids, expressed as % of radioactivity in total lipids.
[b] Figures in parantheses represent radioactivity in hydroxy acids, expressed as % of radioactivity in total lipids.

unesterified fatty acids in the radioactive neutral lipid fraction of the developing *W. coccinea* seeds (Table IV) indicate that substantial proportions of [1-^{14}C]18:2 and [1-^{14}C]18:1 are esterified to the acyl lipids, mostly triacylglycerols.

It is also evident from the data given in Table IV that varying proportions of 9-OH-Δ^{12}18:1 and 9-OH-18:0 are formed upon incubation of developing *Wrightia* seeds with [1-^{14}C]18:2 or [1-^{14}C]18:1. Distinctly higher proportions of labeled 9-OH-Δ^{12}18:1 are formed upon incubation of the developing *W. coccinea* seeds at stage I of development with [1-^{14}C]18:2 under anaerobic than aerobic conditions (Table IV). Under identical conditions of anaerobic

incubation with [1-^{14}C]18:2, the *Wrightia* seeds at stage II of development synthesize distinctly lower proportions of labeled 9-OH-Δ^{12}18:1 than at stage I (Table IV). The formation of higher proportions of labeled 9-OH-Δ^{12}18:1 from [1-^{14}C]18:2 under anaerobic as compared to aerobic conditions (Table IV) further support the anaerobic pathway of synthesis of 9-OH-Δ^{12}18:1 by hydration of the Δ^9 olefinic bond of 18:2 in a similar manner as observed in many organisms [7–13].

Substantial proportions of labeled 9-OH-18:0 are also formed upon incubation of the developing *W. coccinea* seeds at the stage I of maturation with [1-^{14}C]18:1 under both anaerobic and aerobic conditions, and concomitantly, the formation of labeled 9-OH-Δ^{12}18:1 is also observed (Table IV). Moreover, the total amount of labeled hydroxy acids (9-OH-Δ^{12}18:1 plus 9-OH-18:0) formed from [1-^{14}C]18:1 is higher in anaerobic than in aerobic incubations (Table IV). These findings do not rule out the operation of the pathway by which 9-OH-18:0, formed by hydration of 18:1, is desaturated to 9-OH-Δ^{12}18:1. Further studies are required to establish unequivocally the pathway of synthesis of hydroxy acids in *Wrightia* seeds.

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