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CHEMICAL COMPONENTS OF CALLUS TISSUES OF RICE*

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Abstract—Chemical components of callus and other tissues (leaf, root, and seed) of rice were investigated to find the variation in callus metabolism. The isolated compounds were squalene, three sterols, three triterpenes and a fatty alcohol; the last being present only in the callus. Cylindrin, isoarborinol and chlorophyll were not found in the callus. Fatty acids were detected in all tissues in varying amounts, the callus containing lauric acid in greater quantity than the leaves, roots, and seeds.

INTRODUCTION

SEVERAL workers have shown that under certain abnormal conditions plants synthesize metabolites which are not normally produced.[1-6] Such metabolites can be induced by hormones, nutritional conditions, injury,[7-10] pathogens,[11-16] temperature, light, humidity and so on. It is also well known that the tissues of higher plants are altered by hormones such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) and indoleacetic acid (IAA) to form undifferentiated tissues.

From both chemical and biochemical points of view, we have been much interested in the 'abnormal metabolism' of callus tissues induced by the action of a hormone, 2,4-D, since this might lead to callus specific metabolites. It is also intriguing to examine such chemical components to try to elucidate the mechanism of the dedifferentiation process.

* Part II in the series "Chemical Components of Callus Tissues". For Part I see Ref. 17.

[1] I. URITANI and T. AKAZAWA, *Plant Pathology*, Vol I, p. 349, Academic Press, New York (1959).
[2] K. O. MÜLLER, *Plant Pathology*, Vol. I, p. 469, Academic Press, New York (1959).
[3] I. A. M. CRUICKSHANK, *Austral. J. Biol. Sci.* **15**, 147 (1962).
[4] I. A. M. CRUICKSHANK, *Ann. Rev. Phytopathol.* **1**, 351 (1963).
[5] K. TAMARI and J. KATO, *Nippon Nogeikagakukaishi* **31**, 538 (1957).
[6] H. SHICHI and I. URITANI, *J. Biochem., Tokyo* **55**, 11 (1964); I. URITANI and K. OHSHIMA, *Agric. Biol. Chem.* **29**, 641 (1965).
[7] R. GMELIN, *Acta. Chem. Scand.* **16**, 1378 (1968).
[8] Y. NAGASHIMA, *Nippon Nogeikagakukaishi* **33**, 1144 (1959).
[9] K. MORITA and S. KOBAYASHI, *Tetrahedron Letters* 573 (1966).
[10] I. URITANI and K. MURAMATSU, *Nippon Nogeikagakukaishi* **27**, 29 (1953); I. URITANI and M. MIYANO, *ibid* **29**, 151, 156 (1955).
[11] T. KUBOTA and T. MATSUURA, *Nippon Kagakukaishi* **74**, 44, 101, 105, 197, 208; *ibid.* **75**, 447 (1952).
[12] I. A. M. CRUICKSHANK and D. R. PERRIN, *Austral. J. Biol. Sci.* **14**, 336 (1961).
[13] D. R. PERRIN, *Tetrahedron Letters* 29 (1964).
[14] T. MINAMIKAWA, T. AKAZAWA and I. URITANI, *Nature, Lond.* **195**, 4842 (1962).
[15] L. D. SCHEEL, V. B. PERNON, R. L. LARKIN and R. E. KUPEL, *Biochem.* **2**, 1127 (1963).
[16] D. TOMIYAMA, T. SAKUMA, N. ISHIZAKA, N. SATO, N. KATSUI, M. TAKASUGI and T. MASAMUNE, *Phytopathol.* **58**, 115 (1968).
[17] H. YANAGAWA, T. KATO, Y. KITAHARA, T. KAMEYA and N. TAKAHASHI, *Phytochem.* **10**, 2775 (1971).

Previously, we have compared the chemical components of normal and callus tissues of pumpkin and reported that the callus produced several specific metabolites while at the same time not producing certain compounds usually found in the normal tissues.[17] In the present paper we reported the results using rice, *Oryza sativa* L.

Rice has been reported to contain sterols,[18–22] wax,[24, 25] pigments,[26, 27] carotenoids,[20] phenolic compounds,[28] several organic acids including fatty acids,[20,29] phytic acid,[30] amino acids, and other chemical components. Several investigations have been carried out on the induction of the callus tissues of rice using auxins.[31,32] No studies on the chemical components of the callus tissues have been reported excepting for their overall composition, including crude proteins and crude fats.[33]

Chemical Components from Tissue Cultures

Callus was grown using Murashige and Skoog's medium. The induction of the callus was best done by using 3 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D); yeast extract (0·2%, w/v) and coconut milk (10%, v/v) were used to induce callus growth. The callus was subcultured under continuous light, which increased the growth, as compared to dark.

TABLE 1. RELATIVE AMOUNTS OF FATTY ACIDS IN DIFFERENT TISSUES OF RICE

Tissue	Fatty acid						
	Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Callus	32	13	38	6	8	0	3
Root	12	8	65	5	10	0	0
Leaf	trace	9	28	9	3	26	25
Embryo	8	7	49	3	33	0	0
Endosperm	23	24	41	2	10	0	0

Chemical components were extracted from the tissues of rice with methanol and the solution extracted with ether. The ether soluble fraction was further divided into *n*-hexane soluble and insoluble fractions.

Since IR and NMR spectra of the *n*-hexane soluble fraction indicated the presence of a mixture of esters of fatty acids as the major components, a portion was hydrolyzed with methanolic 2 N KOH and the acidic fraction methylated with diazomethane to afford the

[18] K. TANAKA, *J. Biochem., Japan* **17,** 483 (1932).
[19] K. TANAKA and T. TANAKA, *J. Biochem., Japan* **18,** 1, 15 (1933).
[20] RIANG-HA KIMM., *Sci. Papers Inst. Phys. Chem. Res., Tokyo* **34,** 637 (1938).
[21] T. TADOKORO and T. SAITO, *J. Chem. Soc., Japan* **62,** 417 (1941).
[22] G. OHTA and M. SHIMIZU, *Chem. Pharm. Bull.* **5,** 36, 40 (1957); *ibid* **6,** 325 (1958); *ibid.* **8,** 5, 9, 108 (1960).
[23] T. OHMOTO, T. NIKAIDO, K. NAKADAI and E. TOHYAMA, *Yakugaku Zasshi* **90,** 390 (1970).
[24] R. KANEKO and T. TSUCHIYA, *J. Chem. Soc.*, Japan **54,** 737 (1951).
[25] J. POMINSHI and P. H. EAVES, *J. Am. Oil. Chem. Soc.* **31,** 451 (1954).
[26] K. HAYASHI, *Acta Phytochem.* **14,** 155 (1944).
[27] T. MINAMIKAWA, *Agric. Biol. Chem.* **29,** 428 (1965).
[28] S. KUWAZUKA and Y. OHSHIMA, *Nippon Nogeikagaku Kaishi* **35,** 67 (1961).
[29] J. R. HUNTER, *Cereal. Chem.* **28,** 232 (1951).
[30] S. TSUCHIYA, *J. Japan Soc. Food Nutrition,* **6,** 120 (1953).
[31] K. HURUHASHI and M. YATAZAWA, *Science, Japan* **34,** 623 (1964).
[32] E. MAEDA, *Proc. Crop Sci. Soc., Japan* **34,** 139 (1965).
[33] Y. YAMADA, T. NISHI, T. YASUDA and E. TAKAHASHI, *The International Symposium on Germfree Life Research,* Japan (1967).

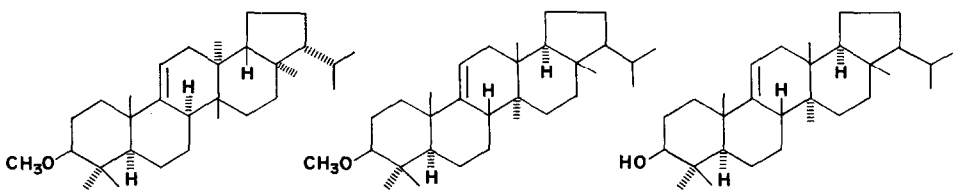
mixture of methyl esters which were identified by comparison with authentic samples by GLC. The results are shown in Table 1.

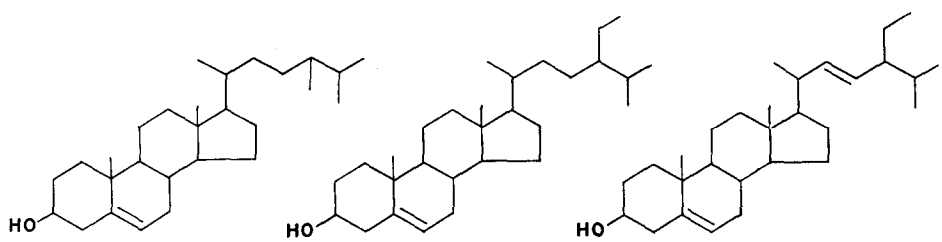
TABLE 2. DISTRIBUTION OF CHEMICAL COMPONENTS IN DIFFERENT TISSUES OF RICE

Component	Tissue*				
	Callus	Root	Leaf	Seed Embryo	Seed Endosperm
Squalene (I)	−	−	−	+	±
Cylindrin (III)	−	+	+	−	−
Arundoin (II)	+	+	+	−	−
Isoarborinol (V)	−	+	+	−	−
Campesterol (VII)	3	14	7	8	4
Sitosterol (VIII)	29	41	34	59	65
Stigmasterol (IX)	68	45	59	33	31
Higher fatty alcohol (IV)	+	−	−	−	−
Chlorophyll†	0	0	52	0	0

* For compounds VII–IX, the values are given as percentage distribution.
† In mg/100 g fr. wt.

The neutral fraction from the hydrolysis of the *n*-hexane soluble fraction was chromatographed on a column of silica gel impregnated with silver nitrate eluting successively with *n*-hexane, *n*-hexane–benzene (10:1), benzene, and benzene–isopropyl ether (10:1). The hexane fraction yielded an oily compound I, *n*-hexane–benzene gave compounds II and III, benzene afforded compound IV, and benzene–isopropyl ether yielded compounds V, VII, VIII and IX. The relative amount of these compounds are shown in Table 2.





Compound I was identified as *trans*-squalene by mass spectrum (M^+ 410, MW of $C_{30}H_{50}$=410·70) and direct comparison of IR, NMR and GLC with those of an authentic sample. Compound II, m.p. 239–240°, colorless needles, has a molecular formula of $C_{31}H_{52}O$

(M^+, 440, MW =440·73) contains a hydroxy group, and shown to be identical with an authentic sample of arundoin (II). Compound III, colorless plates, m.p. 261–262°, M^+ 440 MW of $C_{31}H_{52}O$ = 440·73, was identified as cylindrin (III), by comparison of IR, GLC and m.m.p. Compound IV has a primary alcoholic group (ν_{max} 3300 and 1065 cm^{-1} in IR) and its NMR showed a singlet at 1·30 ppm indicating a long chain-methylene group, and a multiplet at 3·75 ppm (—C<u>H</u>$_2$—OH). From the simple pattern of the NMR spectrum, compound IV was thought to be a higher fatty alcohol. High resolution mass spectrum exhibited a fragment ion $(M-18)^+$ at 336·3750 (fragment ion of $C_{24}H_{48}$ = 336·3755) and its pattern of cleavage suggested the presence of the straight chain-methylene group. From these results, compound IV, m.p. 73–75° was identified as fatty alcohol, $C_{24}H_{49}OH$. Compound V, after chromatography, was acetylated to give a mono acetate (VI), m.p. 302–304°, colorless plates; M^+ 468, MW of $C_{32}H_{52}O_2$ = 468·74, which was identical with an authentic sample of isoarborinol acetate. Compound VII, m.p. 155–157° (lit,[34] 157–158°), M^+ 400 (MW of $C_{28}H_{48}O$ = 400·66) was identified as campesterol by comparison of IR, GLC, NMR, m.p. and MS. Compound VIII, colorless needles, m.p. 140–141° (lit,[35] 140°), M^+ 414 (MW of $C_{29}H_{50}O$ = 414·69) was identical with an authentic sample of sitosterol. Compound IX, colorless plates, m.p. 168–169° (lit,[36] 170°), M^+ 412 (MW of $C_{29}H_{48}O$ = 412·67, was identified as a stigmasterol by comparison of IR, m.p., NMR and GLC. Chlorophyll was extracted with 80% aqueous acetone.

The relative amounts of fatty acids are quite different in each tissue, lauric acid, for example, was in much larger quantity in the callus as compared with that of leaf and root (Table 1). Linoleic acid was only present in leaf, whereas linolenic acid was both in leaf and callus.

As shown in Table 2, squalene was only detected in seeds, especially in the embryo, and the amount in endosperm was quite low and other tissues (callus, root and leaf) contained no detectable amounts. This is the same as observed in pumpkin.[17]

On the other hand, the relative abundance of compounds II–IX are different in each tissue. For example, no detectable amounts of cylindrin (III) and isoarborinol (V) were recognized in the callus, whereas the higher fatty alcohol (IV) was only isolated from this tissue. The amount of sitosterol in the callus was somewhat less than that of the whole plant. It is worthy to note that chlorophyll is not formed in the callus grown in the light. These results suggest that the callus (undifferentiated tissue) is clearly different chemically from other organs (differentiated tissue) and can produce specific metabolites such as the higher fatty alcohol.

EXPERIMENTAL

Tissue cultures from rice. The seeds were sterilized in 70% EtOH for 5 min, followed by immersion in 0·2% $HgCl_2$ solution for 10 min and then washed 3× sterilized H_2O. The seeds were placed in Murashige and Skoog's medium supplemented with 0·8% agar, 3 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D), yeast extract (0·2%, w/v) and coconut milk (10%, v/v). The cultures were grown under continuous illumination with white light at 25°. After 2 weeks, a yellowish white callus appeared from the roots and cultures were continued for further 2 months. The solid cultures were transferred to freshly prepared media every 4 weeks and had been maintained continuously for 2 months prior to their use in these studies. The cultivation of whole plant was done by the same procedure in a medium without 2,4-D.

Extraction and separation of materials. The callus tissues (144 g of fr. wt) were continuously extracted with 1 l. of MeOH for 2 days. This operation was repeated twice, its MeOH extract was concentrated under reduced pressure and extracted with Et_2O. The Et_2O extracts were evaporated to dryness and the residued oil were extracted with *n*-hexane. 100 mg of *n*-hexane soluble fraction in 5 ml of methanolic 2 N KOH, was left 18 hr at room temp., extracted with Et_2O; the extract dried ($MgSO_4$) and evaporated to afford a neutral fraction. The alkaline solution was acidified with 2 N HCl and extracted with Et_2O as above to give the acidic fraction.

Separation of the neutral fraction. The neutral fraction was taken up in benzene and passed through a column of SiO_2 impregnated with 5% $AgNO_3$, eluted successively with *n*-hexane, *n*-hexane–benzene (10:1), benzene, and benzene–*iso*propyl ether (10:1). The *n*-hexane eluate afforded an oily compound I, which showed IR ν_{max}^{film} cm^{-1} 2980, 2945, 2880, 1670, 992, 838, and 745. NMR ($CDCl_3$)δ 1·46 (s, C=C—Me × 6), 1·50 (s, C=C—Me × 2), 1·85–1·87 (broad s), 5·02 (m, C=C—H × 6). Mass *m/e* 410 (M^+), MW $C_{30}H_{50}$ = 410·70.

The *n*-hexane–benzene eluate gave compounds II and III. Compound II was recrystallized from *n*-hexane, colorless needles, m.p. 239–240°. IR ν_{max}^{KBr} cm^{-1} 2950, 2880, 1475, 1460, 1450, 1382, 1380, 1178, 1100. Mass *m/e* 440 (M^+), 425 $(M-15)^+$, 273 (base peak), MW $C_{31}H_{52}O$=440·73. Compound III was recrystallized from benzene, colorless plates, m.p. 261–262°. IR ν_{max}^{KBr} cm^{-1} 2930, 2850, 1477, 1455, 1442, 1382, 1368, 1190, 1108, 1096. Mass *m/e* 440 (M^+), MW $C_{31}H_{52}O$=440·73. Compound IV, obtained from the benzene eluate, gave an amorphous solid, m.p. 73–75°. IR ν_{max}^{KBr} cm^{-1} 3300, 2930, 2860, 1470, 1065, 743, 725. NMR ($CDCl_3$)δ 0·90 (broad s), 1·30 (broad s), 3·75 (m). High resolution mass *m/e* 336·3750 $(M-18)^+$, fragment ion of $C_{24}H_{48}$=336·3755. Compound V, after being eluted with benzene–*iso*propyl ether, was acetylated (Ac_2O–NaOAc) to afford the acetate (VI). Compound VI was recrystallized from benzene, colorless plates, m.p. 302–304°. IR ν_{max}^{KBr} cm^{-1} 2950, 2870, 1728, 1465, 1380, 1245, 1030, 1015, 972. Mass *m/e* 468 (M^+), 453 $(M-15)^+$, 393, 301 (base peak), MW $C_{32}H_{52}O_2$=468·74. Compound VII was recrystallized from benzene, colorless needles, m.p. 155–157°. IR ν_{max}^{KBr} cm^{-1} 3350, 2925, 2872, 1060. Mass *m/e* 400, MW $C_{28}H_{48}O$=400·66. Compound VIII was recrystallized from MeOH–EtOH to give colorless needles, m.p. 140–141°. IR ν_{max}^{KBr} cm^{-1} 3400, 2925, 2870, 1465, 1380, 1060. NMR ($CDCl_3$)δ 3·21–3·83 (broad m C$\underline{H}$—OH), 5·28–5·53 (m, =C=C$\underline{H}$—CH_2). Mass *m/e* 414 (M^+), 399 $(M-15)^+$, 396 (M-18), 381 $(M-15-18)^+$, 273 $(M-141)^+$, 131 $(M-283)^+$, MW $C_{29}H_{50}O$=414·69. Compound IX was recrystallized from MeOH–EtOH, colorless needles, m.p. 168–169°. IR ν_{max}^{KBr} cm^{-1} 3325, 2927, 2850, 1465, 1385, 1370, 1060, 975, 965. NMR ($CDCl_3$)δ 3·18–3·80 (broad m), 5·00–5·40 (m, CH—C$\underline{H}$=C$\underline{H}$—CH). Mass *m/e* 412 (M^+), MW $C_{29}H_{48}O$=412·67.

GLC. Determination of the methyl esters of fatty acids and sterols were carried out by GLC using 15% PEGS on Diasolid (column temp. 165°) and 1·5% SE-30 on Diasolid (column temp. 240°) respectively. The relative amounts of compound were determined by peak area.

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Key Word Index—Oryza sativa; Gramineae; rice; callus culture; sterols; triterpenes.