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EFFECT OF 2,4,5-TRICHLOROPHENOXYACETIC ACID ON THE YIELD OF DIOSGENIN AND YAMOGENIN FROM SEEDS OF *BALANITES ORBICULARIS* DURING GERMINATION

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Abstract—Germination of the seed of *Balanites orbicularis* Sprague (Balanitaceae) was accompanied by a marked increase in the yield of diosgenin and yamogenin and a reduction in the oil content of the embryo. Incorporations of acetate-2-^{14}C and cholest-4-en-3-one-4-^{14}C indicated that the increases were caused by the biosynthesis of sapogenin. 2,4,5-Trichlorophenoxyacetic acid inhibited germination and prevented the incorporation of the labelled precursors into the sapogenin.

INTRODUCTION

THE FRUITS of *Balanites orbicularis* Sprague (Balanitaceae) have been shown to contain commercially attractive quantities of diosgenin and yamogenin in the seed and in the fruit wall.[1] Aqueous incubation of the powdered, partially defatted seed afforded an additional sapogenin yield which was further increased when low concentrations of chlorinated-phenoxy acids were included in the incubation mixture.[2] It was considered that the increased yields could be attributed to the enzymic release of bound sapogenin.[2,3]

Differences in the nature and content of steroid in the dormant seed and the mature plant of some species have prompted investigations to determine if these originate during germination. It has been shown that biosynthesis of both sterol[4,5] and sapogenin[6,7] occurs soon after germination. Hardman *et al.*[8,9] have demonstrated an increase in the yield of total sapogenin from the seed of the closely related species *B. aegyptiaca* after only five days germination. The present work investigates the effect of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) on the germination of the ripe seeds of *B. orbicularis* with special reference to the metabolism of the sapogenin.

RESULTS AND DISCUSSION

Ripe seeds of *B. orbicularis* were germinated at 25° in the dark for periods up to 12 days in the presence of a small excess of distilled water. The results obtained in these preliminary

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[1] R. HARDMAN and C. N. WOOD, *Phytochem.* **10,** 887 (1971).
[2] R. HARDMAN and C. N. WOOD, *Phytochem.* **10,** 757 (1971).
[3] C. N. WOOD, Ph.D. thesis, Bath (1970).
[4] D. J. BAISTED, *Phytochem.* **6,** 93 (1967).
[5] D. S. INGRAM, B. A. KNIGHTS, I. J. MCEVOY and P. MCKAY, *Phytochem.* **7,** 1241 (1968).
[6] A. AKAHORI, F. YASUDA, I. OKUNO, M. TAGANI and T. OKANISHI, *Phytochem.* **8,** 45 (1969).
[7] R. HARDMAN and F. R. Y. FAZLI, *Planta Medica*, in press (1971).
[8] R. HARDMAN and E. A. SOFOWORA, *Phytochem.* **9,** 791 (1970).
[9] R. HARDMAN and E. A. SOFOWORA, *Planta Med.* **20,** 193 (1971).

experiments were identical to those obtained in the presence of either acetate-2-^{14}C or cholestenone-4-^{14}C (cholest-4-en-3-one-4-^{14}C). Accordingly the results reported are those obtained with the labelled compounds in the presence of 2,4,5-T or in its absence (control experiments). After 3 days germination the radicle had started to emerge in 60% of the seeds and there was little apparent difference between those treated with phenoxy acid and those in the control experiments. After 8 days germination the radicle had emerged in all of the seeds of the control experiments and measured greater than 5 mm in 50% of the seeds (Table 1). In contrast only 10% of the auxin treated seeds had a radicle length greater than 5 mm (the remainder appeared discoloured and stunted) and after 12 days the average radicle length was 9 mm in the control experiments but only 3 mm in the phenoxy acid treated seeds.

TABLE 1. THE EFFECT OF 2,4,5-TRICHLOROPHENOXYACETIC ACID ON THE GERMINATION OF THE SEEDS OF *Balanites orbicularis* IN THE PRESENCE OF LABELLED COMPOUNDS

Germination period (days)	Av. radicle length (mm)*	% Increase in wt.*	Light petroleum extractive		
			% of seed*	% of label recovered	
				Acetate-2-^{14}C	Cholestenone-4-^{14}C
Controls (without 2,4,5-T)					
0			39·0		
3	4	180	36·5	4·73	60·3
8	7 (3–10)†	230	36·0	4·64	64·7
12	9 (5–20)†	335	34·3	5·56	
With 2,4,5-T					
3	3	155	39·0	6·20	60·9
8	3	240	34·3	4·65	62·3
12	3 (1–6)†	290	35·4	6·29	

* Fifty whole seeds for each result.
† Range of measurements.

The increase in seed weight during germination, an indication of the water uptake, was very similar in the control experiments and for the phenoxy acid treated seeds (Table 1). Germination was accompanied by a fall in the petroleum soluble extractive value (largely fixed oil[1]), reflecting the metabolism of the seed food reserves during germination. This fall was delayed in the phenoxy acid treated seeds (Table 1).

The yield of total sapogenin, i.e. diosgenin plus yamogenin,[1] from the seeds in the control experiments rose by 46% to a maximum of 1·3% after 8 days germination but after 12 days germination the yield fell back to 1·08%. In contrast, those seeds treated with 2,4,5-T gave a reduced total sapogenin yield (Fig. 1a). The increase in sapogenin yield of the control experiments was accompanied by an increase in the proportion of yamogenin (25β epimer) (Fig. 1b). TLC examination of the crude sapogenin extracts showed that, both in the presence and absence of 2,4,5-T, no change occurred in the nature of the sapogenin.

Examination of the residual liquors remaining after germination showed that over 96% of the acetate-2-^{14}C had been taken up by the seed and between 90 and 95% of the choleste-

none-4-^{14}C was similarly absorbed. These high uptakes were in agreement with the increase in weight of the whole seed with water imbibition (Table 1). The recovery of tracer in the petroleum soluble oils from the seeds was similar in the control and auxin experiments (Table 1). Autoradiographs prepared from TLC plates showed that most of the acetate-2-^{14}C had been incorporated into the glycerides of the oil whereas the cholestenone-4-^{14}C was largely unchanged. No free sapogenin was detected in any of the oils.

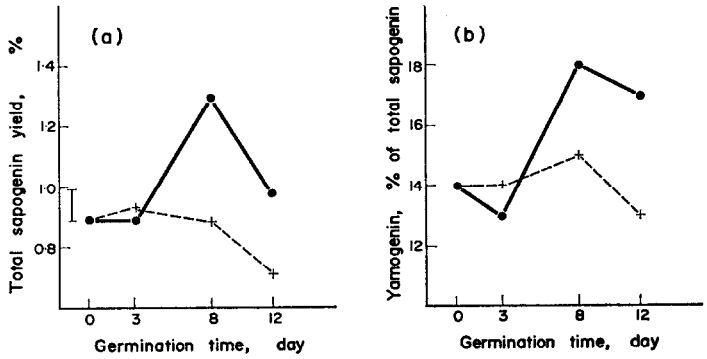

FIG. 1. THE EFFECT OF GERMINATION WITH 2,4,5-TRICHLOROPHENOXYACETIC ACID ON THE SAPOGENIN YIELD FROM THE SEEDS OF *B. orbicularis*.
Seeds were germinated with water at 25° in the dark. Yields of total sapogenin and yamogenin were estimated by IR spectrometry. ●—● Controls, 2,4,5-T absent; +----+ With 2,4,5-T (2×10^{-4} M); I Minimum significant range.

The unsaponifiable matter obtained from the crude sapogenin (isolated from the seeds after acid hydrolysis) was acetylated, fractionated by preparative TLC and the mixtures of acetates of diosgenin and yamogenin so obtained were recrystallized to constant activity (Table 2). In the acetate-2-^{14}C feed the specific activities of the sapogenin acetates isolated from the 8-day and the 12-day control experiments were high. In both cases approximately 0·025% of the tracer fed was incorporated into the sapogenin. Such incorporations compare favourably with those of other workers using both *in vivo* and *in vitro* plant systems.[10,11] In contrast the incorporation of label from acetate into the sapogenin obtained from the 2,4,5-T treated seeds was negligible, being below 0·0005% of that fed. Thus biosynthesis of diosgenin and yamogenin contributed to the observed increase in sapogenin yield noted at the end of 8 days of germination in the absence of phenoxy acid.

No significant incorporation of cholestenone-4-^{14}C was obtained until after 8 days germination (without phenoxy acid) when 0·21% of the label was recovered in the sapogenin fraction (Table 2). Once again the 2,4,5-T treated seeds yielded inactive sapogenin. Tschesche *et al.*[12] fed cholestenone-4-^{14}C to *Digitalis lanata* plants and obtained 3·3% of the label in the isolated sapogenin after 21 days. Our inability to incorporate a greater proportion of the cholestenone suggests that this compound is not readily metabolized by the *Balanites* system or alternative natural metabolites are utilized preferentially.

[10] R. D. BENNETT and E. HEFTMANN, *Archs Biochem. Biophys.* **92**, 13 (1961).
[11] R. D. BENNET, E. HEFTMANN, W. H. PRESTON, JR. and J. R. HAUN, *Archs Biochem. Biophys.* **103**, 74 (1963).
[12] R. TSCHESCHE, R. HULPKE and R. FRITZ, *Phytochem.* **7**, 2021 (1968).

TABLE 2. THE EFFECT OF 2,4,5-TRICHLOROPHENOXYACETIC ACID ON THE METABOLISM OF LABELLED PRECURSORS BY THE GERMINATING SEED OF *B. orbicularis*. RECRYSTALLIZATION OF THE SAPOGENIN ACETATE TO CONSTANT ACTIVITY

Solvent	Activity of the sapogenin, counts/min/μmole			
	Acetate-2-^{14}C feed		Cholestenone-4-^{14}C feed	
	Control	With phenoxy acid	Control	With phenoxy acid
3 days germination				
n-Hexane	6·48	3·19	4·64	1·76
Methanol	1·41	1·05	3·28	0·0
Acetone	1·10 ± 0·07	0·73 ± 0·04	2·81 ± 0·14	0·0
8 days germination				
n-Hexane	55·7	14·0	15·4	4·23
Methanol	49·2	0·0	16·0	0·75
Acetone	54·9 ± 2·2	0·0	15·8 ± 0·8	0·64 ± 0·03
12 days germination				
n-Hexane	65·0	6·17		
Methanol	69·7	1·87		
Acetone	68·1 ± 2·8	1·57 ± 0·8		

The inhibition of germination by 2,4,5-T was not achieved by the blocking of transport through the cell membrane because the phenoxy acid did not significantly reduce the uptake of water and labelled compounds into the seed. Similarly, incorporation of tracer into the glycerides of the oil occurred in the presence of phenoxy acid but incorporation of tracer into sapogenin was greatly reduced. Whilst 2,4,5-T could have directly influenced the biosynthetic pathway leading to steroids, the non-production of sapogenin could have been due to a general disruption of the metabolism of the seed by this phenoxy acid which has strong auxin-like properties.[13] It has been proposed that during the germination of the seed of *B. aegyptiaca*, sapogenin is synthesized in the newly formed root[8] and this may be related to the reported growth stimulatory action of saponin.[14,15] In the present work treatment with 2,4,5-T would have prevented the participation of the normal radicle.

Aqueous incubation of the powdered, partially defatted seed of *B. orbicularis*, has been shown to cause a rise in the total sapogenin yield similar to that now observed during germination.[2] It is unlikely that the same mechanism of sapogenin increase operates in the two systems because incubation affords a further yield of mainly diosgenin (25α sapogenin) which can be explained by the release of pre-existent bound material.[3] In contrast, it was found that germination involved the synthesis of predominantly yamogenin (25β) and this synthesis was susceptible to phenoxy acid. The additional yamogenin may have been synthesised *de novo* or may have been formed from diosgenin via the $\Delta^{25(27)}$-sapogenin, such genins having been isolated from *Hosta* species.[16]

EXPERIMENTAL

The seeds of *B. orbicularis* (wt. 0·7–0·9 g) were freshly removed from undamaged dried ripe fruits obtained from the Conservator of Forests, Entebbe, Uganda and authenticated after consultation with the East

[13] B. ÅBERG, in *Plant Growth Regulators*, p. 219, Iowa State University Press, Iowa (1963).
[14] E. HEFTMANN, *Lloydia* **30**, 209 (1967).
[15] E. C. NORD and G. R. VAN ATTA, *Forest Sci.* **6**, 350 (1960).
[16] K. TAKEDA, T. OKANISHI, H. MINATO and A. SHIMAOKA, *Tetrahedron* **21**, 2089 (1965).

African Herbarium in Nairobi. (A reference sample, RH.1999/1, has been deposited with the Museum of the Pharmaceutical Society of Great Britain at Bradford.) All yields are expressed on a moisture free basis.

Germination with acetate-2-^{14}C. Tared groups of 5 seeds were surface sterilized with bromine water and hydrogen peroxide solution, as previously described,[8] and germinated at 25° in 100 ml conical flasks under aseptic conditions. To each flask was added 1 ml of an aqueous solution containing 10 μc of acetate-2-^{14}C. This solution was filtered into the flask through a bacteriological membrane with, in the case of the control experiments, 3 ml distilled water/flask and, in the case of the phenoxy acid treated seeds, 3 ml of a solution of 2,4,5-T, at a final concentration of 2×10^{-4} M. After 1, 3, 5, 7 and 9 days of germination 1 ml sterile water was added to each flask of seeds in the control experiment and 1 ml of a 2,4,5-T solution (2×10^{-4} M) was added to each flask of the phenoxy acid treated seeds. Five groups of seeds were harvested at each time interval (3, 8 and 12 days germination).

After harvesting, the groups of seeds were blotted dry and reweighed. The 25 seeds in each experiment were bulked, sliced and dried for 16 hr in a vacuum oven at 60°. After powdering in a water-cooled mill the dried seed was extracted for 24 hr in a Soxhlet with light petroleum (b.p. 40–60°). The oil obtained on removal of the petrol was dissolved in toluene (Analar grade) prior to scintillation counting. Diosgenin and yamogenin were isolated from the defatted seed in the usual way following acid hydrolysis.[17] The sapogenin yield of the crude extract so obtained was estimated by IR spectroscopy (S.D. = 2·3%).[3,18] An aliquot of the crude sapogenin was used for scintillation counting and the remainder was refluxed for 1 hr with 25 ml 0·5 N alcoholic KOH. The unsaponifiable matter was recovered with ether, acetylated and fractionated by preparative TLC on 1 mm silica gel plates using hexane–EtOAc, 4:1 as the solvent system. Mixtures of acetates of diosgenin and yamogenin so obtained were recrystallized to constant activity as shown in Table 2. Sapogenin samples, 8–10 mg, were counted in toluene containing 41 mg P.P.O. and 1 mg P.O.P.O.P. The efficiency of ^{14}C counting was 94·5% ± 1%. Pure sapogenin did not cause quenching but the quenching of oily samples was corrected after counting the samples with and without a known quantity of reference hexadecane-1-^{14}C. All extracts were examined by TLC as previously described[19] and autoradiographs were prepared from the chromatographs.

Germination with cholestenone-4-^{14}C. In a similar experiment an ethanolic solution of cholestenone-4-^{14}C (4 μc) was measured into each flask and the solvent was removed *in vacuo*. The flasks were then sterilized by autoclaving at 110° for 15 min. Tared groups of 5 seeds were placed in each flask with either 4 ml of sterile water (control experiments) or 4 ml of a solution of 2,4,5-T (2×10^{-4} M). The experiments were completed as described above. Seeds were harvested after 3 and 8 days germination.

In assessing the uptake of tracers into the seed the residues remaining in the flask, after removal of the seeds, were evaporated to a small volume, dissolved in ethanol and an aliquot of the ethanolic solution was used for scintillation counting.

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[17] G. Blunden and R. Hardman, *J. Pharm. Pharmacol.* **15,** 273 (1963).
[18] K. R. Brain, F. R. Y. Fazli, R. Hardman and A. B. Wood, *Phytochem.* **7,** 1815 (1968).
[19] G. Blunden and R. Hardman, *J. Chromatogr.* **15,** 273 (1964).

Key Word Index—Balanites orbicularis; Balamitaceae; sapogenins; diosgenin; yamogenin; 2,4,5-trichlorophenoxyacetic acid.