Phytochemistry, 1972, Vol. 11, pp. 2991 to 2997. Pergamon Press. Printed in England.

TOMATINE PRODUCTION IN CULTURED EXCISED TOMATO ROOTS

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(*Received* 29 *March* 1972. *Accepted* 18 *May* 1972)

Key Word Index—*Lycopersicum esculentum*; Solanaceae; tomato; tomatine production and root growth; steroidal alkaloid.

Abstract—The amount and concentration of tomatine in cultured tomato roots were unaffected by addition of sodium acetate or cholesterol to the nutrient medium but reduced by addition of mevalonic acid lactone. The steroid inhibitor SKF 7997-A_3 caused decreases in root growth and total tomatine, but the concentration of tomatine remained constant. Ammonium sulphate, urea and casamino acids did not influence tomatine concentration, although higher levels of ammonium sulphate reduced both total alkaloid and root weight. By following root growth and changes in total tomatine over a period of time, the close quantitative association between these two factors was confirmed.

INTRODUCTION

PREVIOUS work has established that cultured tomato roots synthesize the steroidal alkaloid, tomatine.[1,2] However, the concentration of alkaloid was lower than in seedling radicles of the same age.[2] Stienstra[3] reported that the level of tropane alkaloids in cultured roots of *Datura stramonium* was lower than in intact roots and attributed this to a lack of suitable tropane alkaloid precursors in the culture medium. The amount of tropane alkaloids in cultured *Atropa belladonna* roots has, in fact, been increased by supplementing the medium with arginine or ornithine.[4] On the other hand, nicotine levels in cultured tobacco roots were unaffected by addition of nicotinic acid, ornithine or lysine,[5] and nicotinic acid and succinic acid did not increase the yield of ricinine from cultured roots of *Ricinus communis*.[6] The present studies are concerned with the effects of steroid precursors, a steroid inhibitor and nitrogenous compounds on tomatine synthesis in cultured tomato roots, and with the relationship between root growth and tomatine production.

RESULTS

Effects of Steroid Precursors on Root Growth and Tomatine Production

The precursors used were acetate (as the sodium salt), mevalonic acid lactone (MVA) and cholesterol. ^{14}C-labelled acetate[7] and cholesterol[8,9] have been shown to be incor-

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[1] H. SANDER, *Planta* **47**, 374 (1956).
[2] J. G. RODDICK and D. N. BUTCHER, *Phytochem.* **11**, 2019 (1972).
[3] T. M. STIENSTRA, *Proc. K. ned. Akad. Wet.* **57**, 584 (1954).
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[5] M. L. SOLT, R. F. DAWSON and D. R. CHRISTMAN, *Plant Physiol.* **35**, 887 (1960).
[6] L. A. HADWIGER and G. R. WALLER, *Plant Physiol.* **39**, 244 (1964).
[7] H. SANDER and H. GRISEBACH, *Z. Naturf.* **13**, 755 (1958).
[8] R. TSCHESCHE and H. HULPKE, *Z. Naturf.* **21**, 893 (1966).
[9] E. HEFTMANN, E. R. LIEBER and R. D. BENNETT, *Phytochem.* **6**, 225 (1967).

porated into tomatine in the intact plant, and ^{14}C-labelled MVA into the alkaloid in cultured roots.[10] Acetate, MVA and cholesterol were added to root medium at concentrations up to 20, 50 and 25 ppm respectively. In these and subsequent experiments, root fresh weight (FW) was measured before extracting the alkaloid, and parallel experiments were conducted to determine the length of the main root axis (LMA), number of visible lateral roots (LN) and total length of all the visible lateral roots (TLL). Unless otherwise stated, roots were harvested for growth and tomatine analyses after 10 days.

TABLE 1. EFFECTS OF SODIUM ACETATE, MVA AND CHOLESTEROL ON GROWTH AND TOMATINE PRODUCTION IN CULTURED TOMATO ROOTS

Compound	Concn (ppm)	Length of the main axis (mm)	Number of lateral roots	Total length of all lateral roots (mm)	Fr. wt per flask (mg)	Total tomatine per flask (μg)	Tomatine concn (μg mg^{-1} fr. wt)
	Control	143	57	276	129	62	0·48
	1	141	60	298	138	55	0·40
Acetate	5	135	55	237	123	55	0·45
	10	119†	49	202	107	44	0·41
	20	107‡	29‡	115‡	104	43	0·39
	Control	121	36	216	138	69	0·51
	5	109	30	179	140	59	0·42
MVA	10	116	36	247	129	50*	0·39*
	25	121	35	200	129	46*	0·36†
	50	107	29	180	145	51*	0·35†
	Control	129	38	122	74	23	0·32
	5	116	35	109	78	29	0·37
Cholesterol	10	100‡	18‡	60‡	105	31	0·30
	25	84‡	12‡	34‡	88	32	0·36

Measurements of elongation growth and lateral root number were made on at least 10 replicate roots grown singly in 50 cm^3 of medium. For fr. wt and tomatine measurements, 5 roots were grown in each flask (containing 100 cm^3) of medium and 4 replicate flasks were used. Results were recorded after 10 days' growth in the dark at 25°. * Significantly different from control at 5% level; † at 1% level; ‡ at 0·1% level.

High concentrations of sodium acetate caused decreases in LMA, LN and TLL, but FW, total tomatine and tomatine concentration were not affected by up to 20 ppm acetate (Table 1). Growth, as measured by FW, LMA, LN and TLL, was not influenced by addition of MVA, but total tomatine and tomatine concentration were reduced by about 25% at concentrations of MVA equal to, or greater than, 10 ppm. The effects of added cholesterol were similar to those of acetate. Decreases in LMA, LN and TLL were caused by higher concentrations of the sterol, but FW, total tomatine and tomatine concentration did not differ significantly from the control values (Table 1).

Effect of the Steroid Inhibitor SKF 7997-A_3 on Root Growth and Tomatine Production

SKF 7997-A_3 [tris-(2-diethylaminoethyl)-phosphate trihydrochloride], an inhibitor of the conversion of lanosterol to zymosterol,[11] was added to root culture medium at concen-

[10] J. G. RODDICK, unpublished data.
[11] W. L. HOLMES and N. W. DI TULLIO, *Am. J. Clin. Nutr.* **10**, 310 (1962).

trations up to 5 ppm. LMA, LN and TLL were all reduced in the presence of SKF, the respective reductions at 5 ppm inhibitor being 46, 93 and 93% (Fig. 1a). Both FW and total tomatine decreased with increasing levels of SKF but the quantitative changes in these two factors were so similar that the concentration of tomatine remained constant throughout the experiment (Fig. 1b).

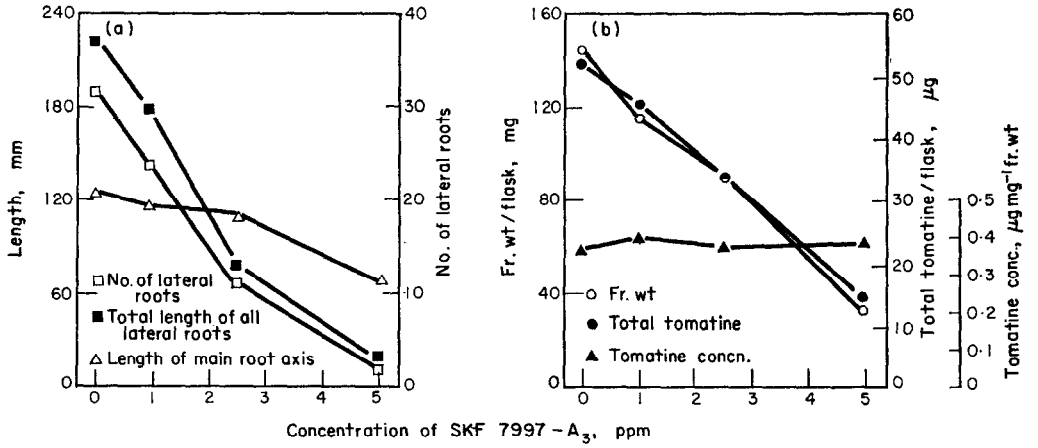

FIG. 1. EFFECT OF SKF 7997-A_3 ON (a) ELONGATION GROWTH AND LATERAL ROOT NUMBER AND (b) FR. WT AND TOMATINE PRODUCTION IN CULTURED TOMATO ROOTS.

Measurements of elongation growth and lateral root number were made on 12 replicate roots grown singly in 50 cm^3 of medium. For fr. wt and tomatine measurements, 5 roots were grown in each flask (containing 100 cm^3 of medium) and 4 replicate flasks were used. Results were recorded after 10 days growth in the dark at 25°.

Effects of Nitrogenous Compounds on Root Growth and Tomatine Production

Since the above intermediates of steroid biosynthesis failed to increase the concentration of tomatine in cultured tomato roots, it was decided to study the effects of the nitrogenous compounds $(NH_4)_2SO_4$, urea and casamino acids which, although not directly involved in tomatine biosynthesis, would be expected to influence nitrogen metabolism in the roots.

$(NH_4)_2SO_4$ did not affect LMA or LN, but at all tested concentrations up to 106·8 ppm, reduced TLL by about 30%. FW and total tomatine were unaffected by lower concentrations of the salt but were depressed by higher concentrations. The reductions, however, were so similar that the concentration of tomatine did not differ significantly from that in any other treatment, including the control. No changes in any of the growth parameters or in the level of tomatine were observed in roots grown in the presence of up to 200 ppm urea (Table 2).

Increasing concentrations of casamino acids (casein hydrolysate) up to 100 ppm caused increases in LMA and TLL but higher concentrations proved supra-optimal. LN was reduced at the highest concentration (500 ppm) but was not influenced by lower concentrations of the hydrolysate. At the concentrations tested, casamino acids had no effect on root weight or tomatine levels (Table 2).

TABLE 2. EFFECTS OF $(NH_4)_2SO_4$, UREA AND CASAMINO ACIDS ON GROWTH AND TOMATINE PRODUCTION IN CULTURED TOMATO ROOTS

Compound	Concn (ppm)	Length of the main axis (mm)	Number of lateral roots	Total length of all lateral roots (mm)	Fr. wt per flask (mg)	Total tomatine per flask (μg)	Tomatine concn ($\mu g\ mg^{-1}$ fr. w t)
	Control	127	39	262	163	65	0·40
	5·3	104	28	137‡	135	49	0·37
$(NH_4)_2SO_4$	10·7	116	35	162†	149	57	0·38
	53·4	132	41	193*	88*	33*	0·37
	106·8	121	38	163†	113*	34*	0·30
	Control	100	25	146	135	54	0·40
	25	98	30	172	127	58	0·46
Urea	50	106	28	172	132	55	0·42
	100	106	24	184	155	65	0·42
	200	121	29	208	164	66	0·40
	Control	84	22	109	75	34	0·45
	50	101	26	152	93	38	0·40
Casamino	100	115*	26	182*	83	28	0·34
acids	250	95	18	96	99	39	0·39
	500	70	12*	72	93	38	0·41

Measurements of elongation growth and lateral root number were made on at least 10 replicate roots grown singly in 50 cm^3 of medium. For fr. wt and tomatine measurements, 5 roots were grown in each flask (containing 100 cm^3 of medium) and 4 replicate flasks were used. Results were recorded after 10 days' growth in the dark at 25°. * Significantly different from control at 5% level; † at 1% level; ‡ at 0·1% level.

Growth of Cultured Tomato Roots and Production of Tomatine

In some of the above experiments, changes in FW of roots were accompanied by similar changes in total tomatine so that the concentration of the alkaloid remained constant. This relationship was examined in more detail by following increases in root weight and total tomatine during a period of growth.

FW and total alkaloid were determined at 2 day intervals for 10 days and also after 17 and 24 days. Growth, as measured by LMA, LN and TLL, was recorded at 2 day intervals for the first 10 days only. Data for LMA and TLL were summed to give an estimate of total elongation growth in the root system.

When results are plotted on a semi-logarithmic scale, it is apparent (Fig. 2) that total elongation, FW and total tomatine increase logarithmically, and at very similar rates, for about the first 8 days, the increases after 8 days being 3920, 3191 and 3984% respectively. LN increases logarithmically for the first 6 days but at a different rate from that of the other factors. Declines in the rate of lateral root initiation and elongation growth become evident after about 6 and 8 days respectively, although changes in these factors subsequent to 10 days are not recorded. FW increases and rate of tomatine biosynthesis also begin to decline after about 8 days and no further accumulation of the alkaloid was observed after about 12 days growth. Analysis of 'spent' root medium after 30 days did not reveal the presence of tomatine.

DISCUSSION

The failure of added precursors to increase the concentration of tomatine in cultured tomato roots is comparable with findings of some other *in vitro* studies of alkaloid biosynthesis.[5,6] Assuming that the acetate and cholesterol are taken up by roots, our results

suggest that tomatine synthesis in cultured roots is not limited by levels of intermediates prior to, and including, cholesterol. However, the possibility of deficiencies in enzyme systems associated with the biosynthetic pathway subsequent to cholesterol cannot be excluded.

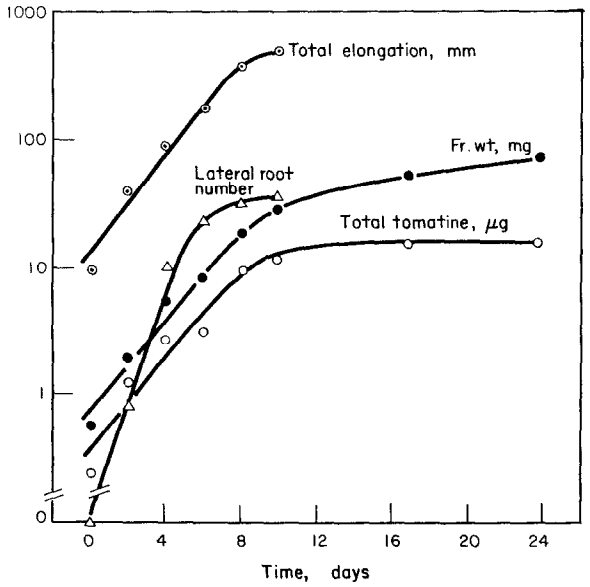

FIG. 2. GROWTH AND TOMATINE PRODUCTION IN CULTURED TOMATO ROOTS OVER A PERIOD OF TIME.

Five roots were grown in each flask (containing 100 cm^3 of medium)in the dark at 25° for the times shown. Fr. wt and total tomatine were measured in 4 replicates each consisting of 5 roots and mean values per root were calculated. Measurements of elongation growth and lateral root number were made on 12 individual roots, 3 being randomly selected from each of 4 flasks.

The fact that SKF 7997-A_3 caused similar reductions in both FW and total tomatine without altering the concentration of the alkaloid, suggests that the step inhibited may be common to certain growth processes and tomatine biosynthesis. The point at which certain steroids are diverted into tomatine biosynthesis would appear to occur after the synthesis of zymosterol. The lack of effect of nitrogenous compounds on the concentration of tomatine in cultured roots is similar to the finding that the concentration of nicotine in cultured tobacco roots was not influenced by changes in the nitrogen supply.[12]

The close quantitative association between root growth and tomatine production, especially during the first 8–10 days of the culture period, is in keeping with the observation by Sander[1] that tomatine production in the intact plant is related to the growth of the plant. Similar relationships have also been found between ricinine production and growth of cultured *R. communis* roots,[6] and between nicotine production and growth of cultured tobacco roots.[5,13] In the latter case, time course experiments showed that changes in root weight, elongation, lateral root number and total nicotine were all of a similar order. Although our data indicate close correspondences between changes in root weight, total

[12] R. F. DAWSON, *Am. Sci.* **48,** 321 (1960).
[13] M. L. SOLT, *Plant Physiol.* **32,** 480 (1957).

elongation, lateral root number (to a lesser extent) and total tomatine, experiments with nitrogenous compounds and steroid precursors showed that tomatine synthesis is more closely related to weight increases than to elongation growth and lateral root initiation. Furthermore, the association between root weight and tomatine production is remarkably consistent and only with MVA were differential changes in these two factors observed. Since MVA has been shown to be incorporated into tomatine in cultured tomato roots, its effects on tomatine production were unexpected and, as yet, inexplicable. Attention has also been drawn to the fact that the proportionality between nicotine production and weight of cultured tobacco roots was not easily altered by changes in the root environment.[12] It is interesting that the cultured root species (viz. castor-oil plant, tobacco and tomato) whose endogenous alkaloid levels could not be increased by addition of precursors all exhibited close relationships between growth and alkaloid production. It would appear, therefore, that in such cases there is a common rate-limiting step (or steps) for both root growth and alkaloid biosynthesis. In the case of tomato, this is supported by experiments with SKF 7997-A_3.

The close and consistent association between growth of cultured tomato roots and accumulation of tomatine suggests that actively growing tissues may be the principal sites of tomatine biosynthesis, at least in the root. In cultured tobacco roots, nicotine synthesis, which was also related to growth, was found to be confined to growing root apices,[5,13] whereas anabasine synthesis, which was not directly associated with root growth, occurred in mature, non-growing tissues.[5]

Since tomatine does not appear to be released into the culture medium, the lack of further alkaloid accumulation after about 12 days may then be due to cessation of cell division and enlargement, the subsequent weight increases being the result of accumulation of dry matter such as starch, cell wall material, etc. Alternatively, cell division, cell enlargement and tomatine synthesis may still be taking place (albeit at a lower rate) as may also degradation of the alkaloid in senescent root tissues, so that there is no net accumulation of tomatine. Tomatine is, in fact, known to be degraded in ripening fruits[1,14] and a tomatine-degrading enzyme has been isolated from tomato leaves.[15]

EXPERIMENTAL

Initiation and maintenance of excised root cultures. The clone of excised roots was initiated from the radicle of a 7-day-old aseptically-grown tomato seedling (*Lycopersicum esculentum* cv. Suttons Best of All) and maintained as described by Street and Henshaw.[16] The standard nutrient medium was modified White's medium,[16] except that iron was added as Fe–EDTA[17] and *myo*-inositol was added at 50 ppm. The stock clone was maintained by growing roots singly in 50 cm^3 of medium in a 100 cm^3 Erlenmeyer flask. For FW and tomatine determinations, 5 roots were grown in 100 cm^3 of medium in a 250 cm^3 Erlenmeyer flask for 10 days. Measurements of LMA, LN and TLL were conducted on roots grown singly, as for stock clonal roots, except in the last experiment when 5 roots were grown in each flask, as stated above. Cultures were incubated in the dark at 25°.

Addition of tested compounds to the culture medium. NaOAc (1, 5, 10 and 25 ppm) was added directly to the culture medium before autoclaving. MVA solutions were filter sterilised (Millipore filter, pore size 0·45 μm) and added to previously autoclaved medium; the final concentrations were 5, 10, 25 and 50 ppm. Cholesterol, dissolved in a small vol. of hot EtOH containing one drop of 1% Tween 80, was added to distilled water. Aliquots of this solution were added to the culture medium to give final sterol concentrations of 5, 10 and 25 ppm and an alcohol concentration of 0·1%. After autoclaving the culture medium, the

[14] H. SANDER and B. ANGERMANN, *Tagber. Dt. Akad. LandwWiss.* **27,** 163 (1961).
[15] S. M. PROKOSHEV, E. I. PETROCHENKO and V. A. PASESHNICHENKO, *Dokl. Akad. Nauk. SSSR* **106,** 313 (1956).
[16] H. E. STREET and G. G. HENSHAW, in *Cells and Tissues in Culture* (edited by E. N. WILLMER), Vol. III, p. 459, Academic Press, New York (1965).
[17] D. E. G. SHEAT, B. H. FLETCHER and H. E. STREET, *New Phytol.* **58,** 128 (1959).

alcohol could not be detected. Using this method, cholesterol was incorporated into the medium as a very fine suspension. SKF 7997-A_3 (Smith, Kline and French Laboratories, Philadelphia) was added to sterile root medium as for MVA, except that the concentrations tested were 1·0, 2·5 and 5·0 ppm

$(NH_4)_2SO_4$ was added directly to the culture medium before autoclaving at concentrations of 5·3, 10·7, 53·4 and 106·8 ppm. Stock solutions of urea were prepared by transferring sterile 40% urea from ampoules to sterile distilled H_2O. The appropriate volumes of this stock solution were added to the sterile medium to give final concentrations of 25, 50, 100 and 200 ppm. Bacto casamino acids (Difco Laboratories, Detroit, Michigan) were added as for $(NH_4)_2SO_4$ except that the concentrations used were 50, 100 and 250 and 500 ppm.

Extraction, separation and assay of tomatine. Details of procedures have been reported previously.[2] Briefly, they involved extraction of homogenised root tissues with acidified aqueous MeOH, concentration of the extracts and separation of tomatine by TLC. After elution, tomatine was assayed by referring the absorbance of its conc. H_2SO_4 chromogen at 325 nm to a calibration graph, prepared using authentic tomatine.

Statistical analysis. Data from experiments in which compounds were added to the medium were subjected to analysis of variance.

Acknowledgements—We wish to thank Professors P. W. Brian FRS and J. H. Burnett for providing the facilities necessary for this work. Our thanks also to Dr. C. G. Elliott for a supply of SKF 7997-A_3 and to Miss I. Freer for technical assistance. The award of a research studentship to one of us (J.G.R.) by the Carnegie Trust for the Universities of Scotland is gratefully acknowledged.