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ISOLATION OF TOMATINE FROM CULTURED EXCISED ROOTS AND CALLUS TISSUES OF TOMATO

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Abstract—Tomatine was present in cultured excised tomato roots but in lower concentrations than in seedling radicles of the same age. The alkaloid was not detected in 'spent' root medium. Newly-initiated callus cultures of hypocotyl, radicle and cotyledon origin produced roots, and tomatine was isolated from both roots and callus. Roots contained more tomatine than callus, but neither contained as much as the organ explants from which the cultures were initiated. The number of roots produced decreased with time, as did also the tomatine content of the callus tissues. After 447 days, when no organized structures were produced by callus cultures, tomatine was not detected. An established hypocotyl callus contained small amounts of tomatine when grown on certain nutrient media, but a chlorophyllous sub-isolate of this callus did not produce detectable quantities of the alkaloid. Tomatine was not detected in an established root callus isolate or in suspension cultures initiated from established, tomatine-containing hypocotyl callus.

INTRODUCTION

TOMATINE, a glycoside of the steroidal alkaloid tomatidine, has been isolated from a number of species of *Lycopersicum* and *Solanum*.[1] Tomatine synthesis occurs in both the shoot and the root but the former is the main site of accumulation of the alkaloid.[2] Highest levels are found in fully-expanded flowers,[3] and young green fruits are rich in tomatine,[4] but as fruit development proceeds, tomatine degradation occurs.[2,5] Little, however, is known of the sites of, or factors regulating tomatine synthesis in the different organs of the plant. Tomatine has been isolated from cultured excised tomato roots[2] and from crown-gall tumours of tomato,[6] but in neither case was it reported how levels of the alkaloid compared with those in the intact plant. The object of this work, therefore, was to investigate the abilities of cultured excised roots, callus tissues and cell suspensions to accumulate tomatine.

RESULTS

Analysis of Cultured Excised Roots and Seedling Radicles

Growth and tomatine analyses were made of 10-day-old cultured roots and seedling radicles. The excised root clone used had been maintained in culture for 3 yr. Extension growth of the main axis was similar in both cultured and seedling roots but, whereas the former produced large numbers of lateral roots, the latter produced none (Table 1). In appearance, cultured roots were thicker than seedling radicles. These differences were

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reflected in the higher fresh weight (×5) and dry weight (×10) of cultured roots. There was no significant difference in the amounts of tomatine per unit of fresh weight, but, on a dry weight basis, seedling radicles contained almost 3 times as much alkaloid as cultured roots. Tomatine could not be as detected in the 'spent' root culture medium.

TABLE 1. GROWTH AND TOMATINE PRODUCTION IN 10-day-old CULTURED ROOTS AND SEEDLING RADICLES

	Growth analysis					
Callus	Length of main axis (mm)	No. of lateral roots	Total length of all lateral roots (mm)	Fresh wt per root (mg)	Dry wt per root (mg)	Total tomatine per root (μg)
Cultured roots	128 7 ± 3 4	30·4 ± 2 0	385 8 ± 39 7	27 8 ± 2·1	2 7 ± 0 2	11 97 ± 1·32
Seedling radicles	121 6 ± 4 3	0	—	5 9 ± 0 2	0 26 ± 0 01	3 64 ± 0·53

Seedlings were germinated in Petri dishes containing damp filter paper under low light at 25°. For growth analysis, 15 cultured roots and 20 seedling radicles were used. Tomatine data represent the means of 4 replicates. Each replicate of cultured roots and seedling radicles consisted of 5 roots and 20 radicles respectively. Mean values are followed by the standard error (s.e.).

Analysis of Newly-Initiated Callus Cultures

New callus cultures were initiated on chemically-defined medium D (containing 2·0 ppm NAA, 0·1 ppm kinetin, 400 ppm *myo*-inositol and supplementary salts) from the hypocotyl, radicle and cotyledon of a tomato seedling. Analyses of these organs showed tomatine to be present in similar amounts on a dry weight basis (Table 2). The callus tissues which developed from the organ explants gave rise to large numbers of roots. Before extracting callus, the degree of rooting was noted and all visible roots were removed.

TABLE 2. TOMATINE CONTENT OF 7-day-old TOMATO SEEDLING ORGANS USED TO INITIATE NEW CALLUS CULTURES

Callus	No. per replicate	Total fresh wt (mg)	Total dry wt (mg)	Tomatine (μg mg^{-1} fresh wt)
Hypocotyl	40	328·9 ± 50·0	13·6 ± 2·2	0·71 ± 0·07
Radicle	60	278·8 ± 24·7	11·2 ± 0·7	0·68 ± 0·05
Cotyledon	100 pairs	240·4 ± 15·2	68 7 ± 0·2	4·67 ± 0·20

Seedlings were germinated in Petri dishes containing damp filter paper in the dark at 25°. Data represent means of 4 replicates followed by the s.e.

After 38 days growth, only hypocotyl callus yielded sufficient material for analysis, but subsequently, all cultures were examined. Tomatine was present in this callus, although the concentration was only 2·5% of that in the original explant. With increasing age, the tomatine content of all the cultures declined, as did also the number of roots produced (Table 3). With the exception of hypocotyl callus after 176 days, tomatine was detected in callus tissues only when roots were present. Neither roots nor root primordia were produced after 447 days and none of the callus extracts contained tomatine. Growth rates at this time were the highest recorded for these cultures.

Roots which had been removed from callus cultures after 72 days were analysed for

tomatine. Roots from all three cultures contained tomatine, in higher concentrations than in the corresponding callus, but in lower concentrations than in seedling radicles (Table 3).

TABLE 3. TOMATINE CONTENT OF NEWLY-INITIATED CALLUS CULTURES OVER A PERIOD OF TIME

Callus	Age (days)	Length of last passage (days)	No. of flasks used	Total dry wt (mg)	Total tomatine (μg)	Tomatine (μg mg^{-1} dry wt)	Degree of rooting	Tomatine content of roots produced by callus (μg mg^{-1} dry wt)
Hypocotyl	38	38	5	523	235·9	0·45	+++	N.A.*
	72	34	6	485	136·1	0·28	++	1·66
	176	43	12	273	38·4	0·14	—	—
	447	64	10	1445	0	—	—	—
Radicle	72	34	5	165	19·2	0·12	++	1·83
	176	43	11	240	17·7	0·07	+	N.A.*
	447	64	10	760	0	—	—	—
Cotyledon	72	34	12	875	75·6	0·09	++	0·69
	176	43	12	490	15·3	0·03	+	N.A.*
	447	64	10	976	0	—	—	—

* N.A. = not analysed.

Analysis of Established Callus Cultures

Since it was difficult to determine if tomatine was actually synthesized in the newly initiated callus cells, analyses were made of other callus tissues which had been maintained in culture for more than 2 years and which had never produced organized structures.

Root and hypocotyl callus and a chlorophyllous sub-isolate of the latter were initially grown on complex medium A (containing 6 ppm 2,4-D, 50 ppm *myo*-inositol and 15% coconut milk). Later, sub-isolates of the root and non-chlorophyllous hypocotyl callus were established on chemically-defined media B (containing 6 ppm 2,4-D, 0·5 ppm kinetin, 50 ppm *myo*-inositol and supplementary salts) and C (containing 0·15 ppm 2,4-D, 0·1 ppm kinetin, 400 ppm *myo*-inositol and supplementary salts). Growth of these cultures, as measured by fresh and dry weights, is shown in Table 4.

On medium A, tomatine was present only in the non-chlorophyllous hypocotyl callus and in very small amounts (Table 4). Growth of root and hypocotyl callus on medium B was less than on medium A and neither culture produced detectable quantities of tomatine. Medium C, on the other hand, supported better growth (comparable with that on medium A) but again, tomatine was isolated only from the hypocotyl callus. The level of alkaloid in this callus was similar to that found on medium A.

Analysis of Suspension Cultures

Cultures were initiated by transferring hypocotyl callus from medium A and medium C into liquid media A and C respectively and agitating on a rotary shaker. At the time of analysis, the former culture had been maintained for 8 weeks (2 passages) and the latter

for 16 weeks (4 passages). Growth in medium A was poor and cells showed little dispersion whereas, in medium C, growth was vigorous and cultures grew as fine cell suspensions. Tomatine was not detected in the cells of either culture.

TABLE 4. GROWTH AND TOMATINE PRODUCTION IN ESTABLISHED CALLUS CULTURES

Nutrient medium	Callus	Growth analysis: Fresh wt per flask (g)	Growth analysis: Dry wt per flask (mg)	Tomatine analysis: Dry wt (g) of tissue extracted	Tomatine analysis: Tomatine (μg mg^{-1} dry wt)
A	Hypocotyl	6·5 ± 0·6	341·1 ± 25·1	4·61	0·013
	Hypocotyl (green)	1·0 ± 0·1	61·4 ± 6·2	4·08	0
	Root	4·6 ± 1·2	197·3 ± 46·6	4·31	0
B	Hypocotyl	7·4 ± 1·0	238·5 ± 17·2	5·67	0
	Root	3·8 ± 0·8	139·3 ± 20 6	3·15	0
C	Hypocotyl	14·5 ± 0·8	389·1 ± 15·1	2·89	0·009
	Root	6·8 ± 1·8	223·5 ± 48·4	2·32	0

Growth data represent the means of 12 replicates followed by the s e.

DISCUSSION

The finding that cultured excised roots of tomato were capable of synthesizing tomatine confirms an earlier report by Sander.[2] However, the amount of tomatine, per unit of dry weight, in excised roots was much less than in intact seedling radicles. The reason for this is not yet known but it does not appear to be a simple nutritional effect or due to release of tomatine into the medium by excised roots. The levels of tropane alkaloids in cultured roots of *Hyoscyamus niger*[7] and *Datura stramonium*[8] have also been found to be lower than in intact roots.

The higher tomatine content of roots produced by callus than of the callus itself is similar to findings from *in vitro* studies of the synthesis of nicotine[9] and tropane alkaloids.[10] The initiation of roots by the newly-initiated callus cultures complicates interpretation of the results, since it is probable that root primordia within the callus contributed to the extracted tomatine. This could explain the correlation between the number of roots produced and the tomatine content of the callus tissues, although there was also some evidence of a decline in the tomatine content of the callus cells. Working with newly-initiated tobacco callus which was devoid of organized structures, Dawson found that the level of nicotine decreased with time.[11]

The detection of tomatine in 3-year-old hypocotyl callus, which had never produced organized structures, is evidence that tomatine biosynthesis can take place in tomato callus tissues. The isolation of tomatine from the hypocotyl callus grown on medium A and C, but not medium B, suggests that certain components of the media are capable of influencing,

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either directly or indirectly, accumulation of the alkaloid. The causal factors are not yet known but there are some indications that they may be growth substances (viz. coconut milk, 2,4-D and kinetin). The type, concentration and ratio of growth substances in the culture medium have been shown to influence synthesis of other alkaloids in callus cultures.[12–16] The apparent absence of tomatine from the chlorophyllous hypocotyl callus and the various root callus sub-isolates emphasizes the unpredictable nature of secondary metabolism in callus cultures. Krikorian and Steward[13] reported that, of a number of tobacco callus isolates analysed, some produced nicotine whereas others did not.

The inability to detect tomatine in either of the suspension cultures, which showed large differences in yield of tissue, suggests that growth rate was not the critical factor influencing alkaloid biosynthesis in these tissues. Cells grown in liquid suspension often have different nutritional and hormonal requirements from those grown as callus on solidified medium,[17] and these differences may be sufficient to account for the absence of tomatine.

The small amounts or apparent absence of tomatine in callus and suspension cultures, but the higher levels in cultured roots are consistent with findings from other studies[10,18] of alkaloid biosynthesis in cultured tissues and organs.

EXPERIMENTAL

Plant material. Lycopersicum esculentum var. Suttons Best of All.

Culture media. The standard nutrient medium (SM) for excised roots was modified White's medium,[17] except that Fe was added in the form of Fe–EDTA[19] and *myo*-inositol (50 ppm) was also added. The various callus culture media consisted of the standard root medium supplemented as follows: Medium A: SM + 15% coconut milk, 6 ppm 2,4-D, 50 ppm *myo*-inositol; Medium B: SM + 845 ppm KCl, 1800 ppm $NaNO_3$, 230·7 ppm $NaH_2PO_4.2H_2O$, 790 ppm $(NH_4)_2SO_4$ (supplementary salts of Braun and Wood[20]), 6 ppm 2,4-D, 0·5 ppm kinetin, 50 ppm *myo*-inositol; Medium C: as medium B except with 2,4-D at 0·15 ppm, kinetin at 0·1 ppm, *myo*-inositol at 400 ppm; Medium D: as medium C except that 2,4-D was replaced by 2 ppm NAA. Media were solidified by addition of 0·7% Oxoid Agar No. 3. Agar was omitted from suspension culture media.

Initiation and maintenance of the cultures. The clone of excised roots was initiated from a 7-day-old sterile tomato seedling radicle and maintained as described by Street and Henshaw.[17] For experimental work, 5 roots were grown in 100 cm^3 of medium in a 250-cm^3 Erlenmeyer flask for 10 days. New callus cultures were initiated by placing on nutrient medium a small piece of the appropriate organ from a 7-day-old aseptically-grown seedling. After 8 weeks, the callus which developed was excised and transferred to fresh medium. Established hypocotyl and root callus cultures were obtained from spontaneous cell outgrowths from the hypocotyl of a 6-week-old aseptically-grown seedling and the basal end of a cultured excised root respectively. Callus cultures were grown on 50 cm^3 of medium in a 100-cm^3 Erlenmeyer flask. Suspension cultures were initiated by transferring a piece of callus (c. 2 g fr. wt) to 100 cm^3 of liquid medium in a 250-cm^3 Erlenmeryer flask and agitating on a rotary shaker at 200 rpm. Unless otherwise stated, callus and suspension cultures were subcultured at 56 and 28 day intervals respectively and harvested for growth and tomatine analyses at the end of such periods. All cultures were grown at 25°. Chlorophyllous hypocotyl callus was grown under a light intensity of 2000 lx, all other callus cultures and suspension cultures under 500 lx and excised roots in the dark.

Extraction and separation of tomatine. Roots were homogenized and extracted with 94% MeOH + 2% HOAc for 18 hr. After filtration, the residue was extracted twice more with 64% MeOH for 5 hr and 2 hr. The filtrates were combined and reduced to dryness under reduced pressure at 45°. Flask contents were

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taken up 3× in hot MeOH and applied as a band to a TLC plate (50 × 200 mm, Silica gel G). Chromatograms were developed in H_2O, dried and re-developed in *iso*PrOH–HCOOH–H_2O (IFW, 73:3:24). Tomatine was located (R_f 0·70) by spraying one edge of the plate with modified Dragendorff reagent.[21] The tomatine zone was scraped off the plate and eluted with MeOH.

Callus tissue and cell suspensions were extracted as above but the combined filtrates were reduced to *ca.* 50 cm^3 under reduced pressure at 45° and the pH adjusted to 10·0 with NH_3 solution (sp. gr. 0·88). After standing overnight at 4°, the extract was filtered, washed with 2% NH_3 and dried at 40°. The ppt was extracted with MeOH for 18 hr and after filtering, the filtrate reduced to dryness under reduced pressure at 35°. Flask contents were taken up and chromatographed as above, except that 200 × 200 mm TLC plates were used. The chromatograms to which had been applied the seedling cotyledon extract were developed in Et_2O to remove pigments before developing as stated above.

'Spent' root culture medium was combined and reduced to *ca.* 50 cm^3 under reduced pressure at 45°. Thereafter, the procedure was as for callus and suspension cultures.

Identification of tomatine. The identity of extracted tomatine was confirmed by TLC of both the glycoside and the aglycone tomatidine (obtained by refluxing tomatine in 1·0 N HCl for 1 hr) in 3 solvents, (IFW, as above; $CHCl_3$–MeOH, 94:6; *n*-BuOH–HOAc–H_2O, 4:1:5); colour reactions of tomatine and tomatidine on TLC plates after spraying with 50% (v/v) H_2SO_4 and heating at 100°; visible/UV spectroscopy of the conc. H_2SO_4 chromogen of tomatine (λ_{max} 322 nm); MS of tomatidine (*m/e* 415, [M^+].); TLC of the sugars released on hydrolysis of the glycoside (methyl ethyl ketone–HOAc–H_2O, 3·1:1); quantitative estimation of glucose (using G-6-P dehydrogenase), galactose (using gal. dehydrogenase) and xylose (by subtraction from total sugar[22]).

Tomatine assay. After elution of tomatine from the silica gel, the MeOH was evaporated off at 80°. When cool, 5 cm^3 of conc. H_2SO_4 was added and the mixture incubated at 40° for 24 hr. The absorbance of the H_2SO_4 chromogen of tomatine was measured at 325 nm and the amount of tomatine determined by reference to a calibration graph. Beer's law applied between the limits of the graph (10–100 μg).

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