Regulation of lycopene formation in cell suspension culture of VFNT tomato (*Lycopersicon esculentum*) by CPTA, growth regulators, sucrose, and temperature

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Abstract

The onium compound 2-(4-chlorophenylthio)-triethylamine (CPTA) was used to increase lycopene formation to levels approximating those in field-or glasshouse-grown fruit, and then growth regulators, sucrose and temperature were used to regulate lycopene accumulation. It was found that the native auxin indole-3-acetic acid (IAA) was substantially more effective than 2,4-dichlorophenoxyacetic acid (2,4-D) in promoting lycopene formation, sucrose inhibited lycopene formation (cell basis), and temperature produced a pattern similar to that observed in the field with a temperature optimum between 18 and 26 °C. Suggestions for further improvements in technique are included.

Key words: Suspension culture, tomato, lycopene.

Introduction

This research addresses the need for improved tools for the analysis of the regulation of aspects of ripening of tomato and other fruits. Ripening in tomato includes many processes, such as flavour development, colour formation, and softening. Regulation of ripening initiation and its progress has been attributed to the influence of many chemical substances. These substances include ethylene, auxins, cytokinins, calcium, copper, ammonia, abscisic acid, polyamines, and oligosaccharides (McGlasson and Adato, 1976; McGlasson, 1978; Brady et al., 1987). Only the role of ethylene as a 'ripening hormone' or initiator of the chemistry of ripening is known in detail (Yang and Hoffman, 1984).

Analysis of plant biochemistry has made use of both cultured or stored, attached or detached fruit and fruit slices. In these experimental systems, cause-and-effect relations between applied treatments and the resultant modification of the biochemical processes are frequently difficult to effect and interpret. This difficulty occurs because the treating agent(s) may decay, react with other than the desired substrates, be inadequately assimilated by the experimental tissue, or be translocated to and sequestered in tissues or organs not under study (Campbell et al., 1990; Adams and Holder, 1992; Lipinski, 1989).

This research describes the utilization and development of cell suspension cultures for the simulation and analysis of aspects of the cellular regulation of the tomato ripening processes. Cell culture is extremely attractive as a tool for identifying regulating factors because of the simplicity of the system, the potentially high degree of definition, the accessibility to analysis of both the cell and its environment, and the greater homogeneity of cells and the cellular environment compared to *in vivo* tissues.

Tissue-culture technologies used for the analysis of aspects of tomato ripening include suspension, 'callus' and organ cultures. Suspension cultures were applied to observe the development of the ethylene-forming capacity in pear-cell suspensions maintained in a resting or stationary state by an auxin-free mannitol osmoticum (Pech and Romani, 1979; Puschmann and Romani, 1983; Puschmann et al., 1985; Romani, 1987; Brady and Romani, 1988). Suspension cultures were also used to observe the enhancement of lycopene in tomato cells using 2-(4-chlorophenylthio)-triethylamine (CPTA) and picloram as the auxin to confirm the role of CPTA as an enhancer of protein synthesis (Fosket and Radin, 1983) and as a stimulator of the disappearance of chloroplasts.

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Tissues were from glasshouse plants of the cherry-type tomato colour and flavour development, sugar accumulation and ethylene forming capacity. Callus cultures were manipulated through light and hormone programmes to develop 'pseudo'-fruits from callus-like tissue (Gresshoff and Doy, 1973; Krzyzko and Rogozinska, 1975).

Cell-culture methods are reported here for the in vitro observation of in vivo lycopene formation. Fruit pericarp tissue or calyx tissue of the cherry-type variety VFNT was the source of cells. The experimental strategy was to use established suspension cultures in test situations with native and synthetic plant growth regulators, known enhancers of lycopene formation, sucrose, and temperature as the independent or potential regulating variables. The dependent response to changes in these variables included changes in biomass accumulation (rate and yield) and lycopene formation (rate and yield). The experiments used the batch mode in flasks with intermittent monitoring of nutritional, physical, and chemical parameters.

Materials and methods

Media

Media were prepared by dilution of liquid concentrates of cell-culture tested reagents (Huang and Murashige, 1976) or commercial pretested premixes. One basal medium, DB, was for maintenance subculturing of callus on semi-rigid medium or in liquid suspension. DB medium included the major and minor Murashige and Skoog salts (Huang and Murashige, 1976), sucrose (30 g dm$^{-3}$ or 0.0876 M dm$^{-3}$), the auxin 2,4-dichlorophenoxyacetic acid (2.0 mg dm$^{-3}$ or 0.0091 mM), the cytokinin zeatin (2.0 mg dm$^{-3}$ or 0.0091 mM), 2,4-dichlorophenoxyacetic acid (2.0 mg dm$^{-3}$ or 0.0091 mM), the cytokinin 6-benzylaminopurine, BA, (0.1 mg dm$^{-3}$ or 0.0091 mM), myo-inositol (100 mg dm$^{-3}$ or 0.0091 mM), 2,4-dichlorophenoxyacetic acid (2.0 mg dm$^{-3}$ or 0.0091 mM), and Nitsch's vitamins (Nitsch and Nitsch, 1969). A gellan gum, 'Gelrite'™ or 'Phytage'™ (Sigma Chemical Co.), was used in the medium to initiate and maintain callus.

A second basal medium, IZ, contained the auxin indole-3-acetic acid (IAA) (5.0 mg dm$^{-3}$ or 0.0285 mM) and the cytokinin zeatin (2.0 mg dm$^{-3}$ or 0.0091 mM), but was otherwise the same as DB. Media were prepared not more than 24 h prior to use. The carotenoid inducer 2-(4-chlorophenyl-thio)-triethylamine (CPTA) was synthesized at this laboratory (Schultz and Baldwin, 1958). If not specified below, CPTA was included at 75 mg dm$^{-3}$ (0.2676 M dm$^{-3}$).

Media supplemented with CPTA are indicated below as IZ + CPTA or DB + CPTA. Variations from a basal medium (IZ or DB) are indicated as, for example, I$_{1-Z}$ to designate a series of IZ-type basal media in each of which the concentration of indole-3-acetic acid differed and IZ/sucrose$_{var}$ a series of IZ-type basal media in each of which the concentration of sucrose differed.

Tomato tissues

Tissues were from glasshouse plants of the cherry-type tomato Lycopersicon esculentum var. VFNT (Dr R. Fisher, University of California, Berkeley).

Subculture methods

Slices of immature fruit pericarp and calyx tissues from green tomato fruit produced rapidly proliferating cells when supported on DB gel medium. These cells were repeatedly subcultured to fresh media using an interval of 2 weeks. After several subcultures, cells were transferred to liquid DB-type, gel-free medium maintained in a 24°C culture room in the dark or under very low levels of indirect 'Gro-Lux'™ illumination. Subsequent subcultures were at 2-week intervals. The dilution for each subculture was 15 cm$^3$ of cells and spent medium to each 100 cm$^3$ of fresh medium. Cells were subcultured to both 500 and 1000 cm$^3$ DeLong™ flasks, respectively, with 100 cm$^3$ and 200 cm$^3$ of medium. Each flask had a polyurethane plug (Baxter Healthcare Corp. diSPo™ plugs) and a stainless steel closure with spacers (Belco Biotechnology).

Sampling and analysis

Flasks were sampled at weekly intervals beginning on day 11 and ending at day 18 or later. The cells were collected by filtration on Miracloth™ from a weighed sample of medium and cells, washed with a volume of distilled water equal to the same volume of frozen, lyophilized, and weighed. Dry cells were extracted in 25 cm$^3$ CH$_2$Cl$_2$ with an ultrasonic probe (Branson) and filtered. The extract was dried under reduced pressure and rediluted in CH$_2$Cl$_2$ to 500 μl (extracts with very low lycopene concentrations were diluted to 20 μl). Injections of 5 μl were analysed by HPLC using a 5 μ Microsorb™ C18 column (4.6 x 250 mm with a 50 mm guard column) (Rainin instruments), and a mobile phase of CH$_3$CN:CH$_2$Cl$_2$:hexane (16:4:1, by vol.) at 0.2 cm$^3$ min$^{-1}$, and detection at 450 nm. Under these conditions, lycopene retention time was 5.4 min and β-carotene 9.7 min. Lycopene concentrations were quantified by comparison with standards prepared from pure lycopene isolated from tomato paste (0.25–10.0 mg cm$^{-3}$) (Ikan, 1969).

In general, the comparative data series reported in the figures are based on subculture on the same day from the same source culture. Cell mass measurements (3 determinations) had a standard deviation of not more than 3.7% (expressed as a percentage of the mean value) and chromatography measurements not more than 5%. Therefore, the standard deviation in the calculated lycopene concentration values shown in the table and figures is not more than 8.7%.

Results

The experimental programme described below relied on two maintenance cultures: employing both the IZ type medium and the DB medium. This was necessary because the cultures were slow to adapt to the new phytohormonal environment when transferring cells from DB to IZ medium and, at the first two or three subcultures, cells in the parent culture were allowed to settle and were collected at higher than fully-suspended concentrations for the inoculation. Cells transferred from IZ to DB adapted readily.

Cell mass accumulation in maintenance medium

Tomato cell dry mass accumulated at about the same rate and to the same total yield in either DB or IZ medium. Cells maintained in IZ medium maintained peak mass from day 14 to day 20 whereas cells maintained in DB...
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Culture age (days)

Fig. 1. Time-course of the dry mass accumulation by calyx-derived cells of tomato suspended at 24°C by reciprocal shaking in IZ-type medium (○) containing IAA and zeatin, or DB-type medium (□) containing 2,4-D and BA as the growth regulators.

medium showed about a 10% decline from peak mass after day 11. The similarities, especially in the rate of growth, shown in Fig. 1, reflect the adaptive response of the cells to the strict 14 d subculture interval. It is apparent from the data of Fig. 1 that inocula for subcultures and experiments (day 14) included cells not actively accumulating dry matter.

Cell colour and lycopene accumulation with CPTA and growth regulators

The colour of cells grown in DB and IZ maintenance media was normally creamy or light-tan, and after 18 to 25 d changed to black in DB or brown in IZ. There were only trace quantities (<0.1 μg g⁻¹ dry mass) of the tomato red pigment lycopene and β-carotene.

Visually pink cells were obtained in media supplemented with CPTA. The pink colour intensity correlated with lycopene content and was also strongly affected by the media phytohormones and the concentration of CPTA. Figure 2 illustrates that there was nearly 2.5 times higher concentrations of lycopene after growth for 11 d and 5 times higher concentrations after 18 d in IZ+CPTA做强 medium than in a DB+CPTA做强 medium. There was little or no additional accumulation of lycopene after the 11th day in DB+CPTA做强 medium. Lycopene concentration in the cells was proportional to media CPTA at low CPTA concentrations, but the enhancement was saturated for CPTA concentrations above 50–75 mg dm⁻³ (0.18–0.27 mM dm⁻³) for cells in IZ or DB medium. By contrast, as shown in Fig. 3, CPTA had only small effects on cell dry mass accumulation. At the lowest levels of CPTA there was a very small increase (~10–15%) in cell dry mass with IZ-type media and a small decrease (~10–15%) in cell mass in DB-type media. The cell mass in either media after 18 d was approximately constant at these changed levels up to the highest CPTA concentrations evaluated (1.5 mM dm⁻³).

Cells originating from IZ medium and transferred to DB+CPTA medium immediately took on the lower lycopene-forming character of cells originating in DB medium and inoculated to DB+CPTA medium. This is shown in Table 1.

The range of effect of the auxin 2,4-D on cells originating from DB medium and transferred to DB+B+CPTA medium is shown in Fig. 4 (left). Cell mass accumulation increased very slightly as 2,4-D concentrations increased from 0 to 0.02 mM dm⁻³ and then in response to increasingly toxic levels of 2,4-D (0.04 to 0.2 mM dm⁻³) decreased to cell mass inoculation levels. No growth was observed at or above 0.2 mM dm⁻³ of 2,4-D. Generally low (<100 μg g⁻¹ dry mass), but very gradually increasing

Table 1. Accommodation after 11 d of CPTA-stimulated response to change of phytohormones

<table>
<thead>
<tr>
<th>Source medium</th>
<th>First subculture medium</th>
<th>Lycopene concentration (μg g⁻¹ cell dry mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB</td>
<td>DB+CPTA</td>
<td>105</td>
</tr>
<tr>
<td>IZ</td>
<td>DB+CPTA</td>
<td>159</td>
</tr>
<tr>
<td>IZ</td>
<td>IZ+CPTA</td>
<td>280</td>
</tr>
</tbody>
</table>
lycopene cell concentrations were observed over the whole range.

The range of effect of the auxin IAA on cells originating from IZ medium and transferred to IZ + CPTA medium is shown in Fig. 4 (centre). As in the case of 2,4-D, there was a small increase in cell mass up to 0.02 mM dm\(^{-3}\) of IAA. In contrast, however, this peak was followed by a long plateau of slightly reduced, but approximately constant mass, up to 0.25 mM dm\(^{-3}\) and then a decline to inoculation cell mass levels in response to increasingly toxic levels of IAA (0.25 mM dm\(^{-3}\) to 0.42 mM dm\(^{-3}\)). Lycopene was produced even without added IAA and the production increased from 150 to 400 \(\mu\)g g\(^{-1}\)dry mass as the IAA concentration increased from 0.0 to 1.0 mM dm\(^{-3}\). Above this IAA concentration, the effect of IAA on lycopene concentration was saturated at a constant level up to the range of cell-toxic concentrations where the lycopene concentration declined in parallel to the cell mass concentration.

The cytokinin zeatin had no effect (0.0 to 0.12 mM dm\(^{-3}\)) for cells originating in IZ media and transferred to IZ\(_{\text{var.}}\) + CPTA media with an IAA concentration of 0.12 mM dm\(^{-3}\).

Lycopene accumulated at a constant rate up to at least day 32 in IZ + CPTA media where a concentration of lycopene of about 750 mg g\(^{-1}\)dry mass was reached. Lycopene concentration in the pericarp of vine-ripened fruit was about 800 mg g\(^{-1}\)dry mass. As seen by comparing Fig. 1 and Fig. 5, most of the CPTA-stimulated lycopene accumulates after mass accumulation has ceased.

**Temperature**

Temperature exhibited very strong regulatory controls on both cell dry mass accumulation and mass-specific lycopene formation in cells stimulated by CPTA in IZ medium. Data for 11-d-old cells cultured from 14–32°C are shown in Fig. 6. At temperatures below common warehousing temperatures (14°C), little or no dry mass accumulated. Mass accumulation increased with temperature to a
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**Fig. 5.** Lycopene mass accumulation in cells maintained in IZ + CPTA\textsubscript{alt}-type media for up to 32 d. Data from various CPTA concentrations (50–100 mg dm\textsuperscript{-3}) in the range of saturation (see Fig. 2).

![Image of graph showing lycopene mass accumulation over time]

**Fig. 6.** Temperature regulation of dry mass accumulation and lycopene formation in CPTA-stimulated tomato suspension cultures after 11 d in IZ medium. Data include cell mass (○) and lycopene on a culture basis (■) and 'specific' lycopene on a cell dry mass basis (●).

![Image of graph showing temperature regulation of dry mass accumulation and lycopene formation]

The sucrose in IZ/sucrose\textsubscript{var} + CPTA media showed differential regulation of growth and CPTA-stimulated lycopene synthesis in 11-d-old cells as shown in Fig. 7. Growth was limited by sucrose concentration up to a sucrose molar concentration of 0.1 M and then became increasingly inhibited. At 0.35 M sucrose, cell growth was reduced to one-half of the maximum. Lycopene, by contrast, was found at the highest cell concentration in the cells with little or no sucrose and at progressively lower concentrations as the concentrations of sucrose increased.

**Discussion**

The lycopene stimulator CPTA was employed as a guide to characterize lycopene regulation by other media components. No conditions were found which supported measurable lycopene formation without CPTA. The failure of CPTA-free cells to produce lycopene suggests that CPTA removes a metabolic block, raises protein synthesis above a critical threshold level, or enables an alternate pathway to lycopene. Further evidence of the complex narrow plateau of 12 g\textsubscript{dry mass} kg\textsuperscript{-1} culture between 26°C and 29°C and then declined to nearly one-half this value at 32°C. Specific lycopene exhibited a broader plateau (18–26°C) for maximum concentration. However, the lycopene forming activity was sharply reduced at 14°C and was completely inhibited at 32°C even though mass accumulation continued.

**Fig. 7.** Sucrose regulation of dry mass accumulation and lycopene formation in CPTA-stimulated tomato suspension cultures after 11 d at 24°C in IZ/sucrose\textsubscript{var} + CPTA medium. Data include cell mass (○) and lycopene on a culture basis (■) and 'specific' lycopene on a cell dry mass basis (●).
role of CPTA has been reported in the study of normal, red whole tomato fruits treated with CPTA, where lycopene concentrations were enhanced at 21 °C and suppressed at 32 °C (Chang et al., 1977). Neither CPTA/IAA nor CPTA/DB media stimulated more than a trace of β-carotene, and this was consistent with observations employing CPTA/picloram media (Fosket and Radin, 1983; Radin, 1986). The absence of carotenes was expected, because of the reported suppression of isoprene cyclization by onium compounds like CPTA (Chang et al., 1977).

The lycopene colour response was stronger and quantitatively closer to that observed in whole fruits with IAA as the auxin rather than 2,4-D. This auxin choice confirms the initial assumption that the plant growth regulators normally observed in vivo would be more successful than synthetics such as 2,4-D. Analogous increases have been reported for the pigment shikonin in the culture of *Lithospermum erythrorhizon* when IAA was substituted for p-chlorophenoxyacetic (Kim and Chang, 1990). These observations are consistent with prior research in which 2,4-D was reported to be unsuitable for expression of secondary metabolites in cell culture (Zenk et al., 1985) and was shown to be a much more aggressive than IAA in promoting undifferentiated callus from seeds of *Lithospermum* (Branca et al., 1991). The observation of greater cell toxicity of 2,4-D relative to IAA (Fig. 4) suggests further limitation on the usefulness of 2,4-D for the study of secondary metabolic processes.

The pattern of lycopene formation dependency on temperature is similar to that for whole fruit where lycopene does not develop below 10 °C and is inhibited above 30 °C (Sayre et al., 1953; Yang et al., 1990). This contrasts to observations of secondary metabolism in other cell culture systems employing *Catharanthus roseus* where alkaloid production declines uniformly and rapidly from 16 to 38 °C (Courtois and Guern, 1980).

The accumulation of cell mass observed here for *Lycopersicon* follows a similar pattern to that observed for *Dioscorea deltoidea* and *Catharanthus roseus*, except that the range (5 °C) over which maximum cell mass accumulation is observed is smaller and the maximum temperature is lower (30 °C). A 6 °C range has been reported for *Dioscorea deltoidea* (26–32 °C) (Lipskii and Chernyak, 1983) and a 12 °C for *Catharanthus roseus* (23–35 °C) (Courtois and Guern, 1980).

The dependency of lycopene formation on sucrose parallels that reported for vacular anthocyanin accumulation by *Daucus carota* cell suspension cultures. However, the sucrose concentration at which peak concentrations of *Daucus carota* anthocyanins are obtained was found to be nearly three times higher (greater than 0.29 M dm⁻³) than that for *Lycopersicon* and dependent on inoculation levels (Ozeki and Komamine, 1985) and, in *Vitis* cell cultures, also, on the concentration and type of soluble nitrogen (Yamakawa et al., 1983). Other researches with *Vitis* suggests the importance of maintaining low division rates and residual reducing sugar concentrations as a means of augmenting the anthocyanin production (Cormier et al., 1990).

These data, and that reported by others, suggest strategies that would enable the cell culture system to more closely enable in vivo cell chemistries, in vitro. One strategy would be to employ perfusive feeding to maintain low culture concentrations in order to eliminate nutrient inhibition and reduce suppression of secondary metabolic events (see Fig. 6). Nutrient limiting conditions ought to favour cell enlargement over cell division and mimic in vivo tomato growth where there is very little division after the first 7–10 d of growth (Monselise et al., 1978; Bünger-Kibler and Bangert, 1982/1983). These conditions would also approach those which have been applied to 'ripen' in vitro isolated calyx tissues where the nutrient limitation occurs, in part, by transport through the cut end of the calyx.

Programming of other nutrients and light might also be applied to simulate known changes in the in vivo nutrient supply. This could include management of IAA concentrations which have been reported in vivo to undergo a 10-fold increase in the pericarp during early development, a 33% decline during intermediate development, and a doubling just prior to the onset of ripening (McGlasson, 1978) Management of IAA would also account for media-catalysed depletion (Dunlap et al., 1986; Dunlap and Robacker, 1988). The positive response of IAA-depleted cell cultures, reported here, to an IAA increase may be taken as partial evidence in support of this approach. Management of other constituents would require more detailed knowledge of the concentrations and flow rates of phloem transports (water, sucrose, potassium salts of organic acids, amino acids, and nitrogen (Walker and Ho, 1977)) and xylemic transportates (water and calcium (Ho et al., 1987; Adams and Holder, 1992)). However, the inability to obtain exudates from the peduncle of tomatoes has inhibited these measurements in the past (Ho et al., 1987). Finally, the success of 'pseudo-fruit' development from callus by management of hormones and light (Gresshoff and Doy, 1973; Krzyzko and Rogozinska, 1975) also suggest support for this managed approach.

**References**


Brady CJ, Romani RJ. 1988. Respiration and protein synthesis...


Bünger-Kibler S, Bangerth F. 1982/1983. Relationship between cell number, cell size, and fruit size of seeded fruits of tomato (Lycopersicon esculentum Mill.), and those induced parthenocarpically by the application of plant growth regulators. Plant Growth Regulation 1, 143–54.


