Plant Cell, Tissue and Organ Culture **38:** 31–37, 1994.

Variation in the growth and biosynthetic activity of cloned cell cultures of *Capsicum frutescens* and their response to an exogenously supplied elicitor

Peter R. Holden[1] & Michael M. Yeoman

Institute of Cell and Molecular Biology, Darwin and Rutherford Building, University of Edinburgh Edinburgh EH9 3JR. ([1]present address: The Beatson Institute for Cancer Research, Wolfson Laboratory for Molecular Pathology, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD)

Received 20 April 1993; accepted in revised form 4 May 1994

Key words: Capsicum frutescens, clones, elicitation, variation

Abstract

Twenty clones established from single cells of a suspension culture of *Capsicum frutescens* were maintained as callus and in suspension over a sixteen week culture period. These clones exhibited marked differences in growth, chlorophyll and chloroform-soluble phenolic content which became more apparent with increasing time in culture. Clones in suspension exhibited a more rapid change in morphology and biosynthetic activity than those cultured as callus. Elicitation increased PAL activity, reduced the incorporation of L-[U-^{14}C] phenylalanine into the chloroform-soluble fraction of the culture medium and increased incorporation into the methanol-soluble fraction of the cells in ten suspension clones. Differences to elicitation were observed among clones; in particular the faster growing isolates incorporated more radioactive label into soluble phenolics that remain in the cells than those that are released into the medium. The implications of these results are discussed.

Abbreviations: SH – Schenk & Hildebrandt, PAL – phenylalanine ammonia-lyase, RGR – relative growth rate, TCC – total chlorophyll content, HPLC – high performance liquid chromatography

Introduction

The initiation of a callus culture from an explant leads to the formation of a heterogeneous population of cells that can differ in size, structure, DNA content and metabolism (Mantell & Smith 1983; Yeoman & Forche 1980). The proportion of the different cell types varies with time both within and between cultures making it impossible to rely on the stability of cells in culture. Various procedures have been used to illustrate the intrinsic variation of cell cultures in attempts to isolate morphologically stable cultures or biosynthetically-high yielding cell lines (Dix 1990). As a consequence, individual clones derived from isolated cells of callus or suspension cultures have been shown to exhibit marked differences in their biosynthetic activity (Zenk et al. 1977; Creche et al. 1987; Dougall et al. 1980) and morphology (Davey et al. 1971; Selby & Collin 1976; Dougall & Vogelien 1990).

Cell cultures are known to differ in their response to culture conditions which include the nature and concentration of growth regulators, pH and temperature (Mantell & Smith 1983). Therefore it is to be expected that the biosynthetic activity of clones will vary in response to added fungal elicitors; which may promote or inhibit the activities of specific enzymes and the accumulation of secondary products (Dixon & Harrison 1990). By these means it may be possible to select clones which respond to elicitors and lead to the production of secondary products at levels exceeding those accumulated in non-selected cultures.

Capsicum frutescens is a plant that has been the subject of numerous studies on the regulation, manipulation and commercial exploitation of the secondary metabolite capsaicin in tissue culture (Lindsey & Yeoman 1984; Holden et al. 1987; Mavituna et al. 1987; Salgado-Garciglia & Ochoa-Alejo 1990; Hall & Yeoman 1991; Sucrasno & Yeoman 1993). Results are

presented here on the differences in selected morphological and biochemical features within clones and how these change during subculture in both callus and suspended cells. The response of suspension clones to a fungal elicitor, *Gliocladium deliquescens* is also described in which the incorporation of L-[U-^{14}C]phenylalanine by the cells and the activity of phenylalanine ammonia lyase (PAL) were measured and compared in clones.

Materials and methods

Cell culture

Callus was obtained from internode tissue of greenhouse grown plants of *Capsicum frutescens* Mill (McNair, Edinburgh, UK) using the medium of Schenk & Hildebrandt (1972) supplemented with 0.5 mg l^{-1} (2.5×10^{-6} M) 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg l^{-1} (0.5×10^{-6} M) kinetin, 30 g l^{-1} sucrose and 0.6% Oxoid No. 3 agar, pH 5.8. Cell suspension cultures (50 ml in a 250 ml Erlenmeyer flask) were initiated from callus and maintained in the same medium without agar. Cultures were incubated at 25 ± 2°C under continuous fluorescent illumination (25 µmol photons m^{-2} s^{-1} warm white fluorescent tubes; Osram, Munchen, FRG). Suspension cultures were agitated on a rotary shaker (90 rpm, 0.7 cm amplitude). Unless stated otherwise the cell material was routinely subcultured to fresh medium every 28 days.

Cloning procedure

A cell plating technique was used to isolate single cells from a uniform suspension culture. Cells were sieved through a series of nylon mesh filters down to 100 µM in size, centrifuged at 2000 x *g* for 5 min and diluted with SH medium to achieve a volume of approx. 50,000 cells ml^{-1}. These were then mixed with 2 ml of SH medium with 1% low temperature agarose (Sigma Ltd) to give a dilution of 25,000 cells ml^{-1}, then plated out on a 9 cm diameter Petri dish (Sterilin, UK). The plates were examined microscopically and the positions of single cells were marked. When the single cells had developed to 1 mm diameter colonies they were removed and placed individually on SH medium where they developed into callus cultures. These cultures were subcultured every four weeks (approx. 1.5 g inoculum).

Determination of relative growth rate and total chlorophyll content

The material that remained after subculture was used to determine the relative growth rate (RGR) and the total chlorophyll content (TCC). At each subculture the weight of cells transferred to fresh medium was recorded. The RGR was determined by subtracting the log value of the initial wet weight from the log value of the final wet weight of callus or suspension cells. This value was divided by the length of time (28 days) that the cells were in culture. The results were expressed as $day^{-1} \times 10^{-2}$. Using approximately lg of cells (FW) a measurement of TCC was made using the method described by Harborne (1976) and expressed as µg DW^{-1}.

Elicitor treatment and determination of phenylalanine ammonia-lyase (PAL) activity

Cultures of *Gliocladium deliquescens* the source of fungal elicitor, were maintained on solid SH medium and subcultured routinely onto fresh medium every four weeks. After each subculture the remaining spores were harvested from the medium with ethanol, dried down in a 60°C oven and stored at -20°C. Each suspension culture was incubated for 6 h at 25°C with an aliqout of both spores (30 mg ml^{-1} (w/v distilled water)) and radiolabel. Control cultures received only distilled water and radiolabel. After 6 h the cells and medium were removed for analysis. The determination of PAL activity was carried out using the method of Ozeki & Komamine (1985) with the modification that the reaction time was 2 h. The specific activity of the enzyme is expressed as µkat kg^{-1} protein (Tanaka et al. 1974). Total protein content was calculated using the method described by Bradford (1976).

Radiolabelling

L-[U-^{14}C]phenylalanine (18.49 GBq/mmol) was obtained from Amersham International (Amersham, Bucks., UK) in a sterile solution of 2% (v/v) ethanol in water. Aliquots (40 µl; 74 kBq) of this solution were added to each flask and left for 6 h. For the radioassay of the extracts a liquid scintillation counter (SL3000; Intertechniques, Portslade, Sussex, UK) was used and calculated in Bq $culture^{-1}$. The scintillation cocktail was as described in Lindsey & Yeoman (1984).

Extraction of soluble phenolics from medium and cells

Free, non-polar phenolic compounds (including capsaicin and its pathway intermediates) were extracted from the medium and from the cells with chloroform and methanol respectively using the protocol described by Hall et al. (1987). In addition 50 ml chloroform was added to callus agar medium and mixed with a magnetic flea for 30 min then treated in the same way as suspension medium. The residues were taken up in methanol and filtered prior to liquid scintillation counting or analysis by high performance liquid chromatography (HPLC). The extracts were analysed by HPLC using a Hewlett-Packard chromatographic system (Hewlett-Packard, Reading, UK) with a diode array detector as previously described (Hall et al. 1987).

Results

Variation among clones over a sixteen week subculture period

Differences in growth and total chlorophyll content
The twenty callus clones selected at random from two hundred were established from single cells of a finely divided suspension culture. The material from each clone was used to prepare both a suspension and a callus culture which were maintained for a sixteen week period with subculture every four weeks. Measurements of the relative growth rate (RGR), total chlorophyll content (TCC) and chloroform-soluble phenolic content of the medium were made on the residual material after the first and last subculture, four and sixteen weeks respectively.

There are considerable differences between the twenty callus clones in their RGRs and TCCs after four and sixteen weeks (Table 1). The differences between slow growing (eg: BZ4) and fast growing (eg: AE2) clones were paralleled by differences in their morphology. Slow growing callus clones were aggregated and hard, fast growing clones were friable. The differences in TCC were reflected in the colour of the callus material, those with low values (eg: BQ3 and BV7) were yellow, intermediates (eg: Z1, Q5) were yellow/green and high values (eg: AG2 and N3) were vivid green.

Suspension cultures showed similar variation in growth and pigmentation (Table 1). Visible differences in RGRs and TCCs of the twenty suspension clones were measured at four and sixteen weeks. It would appear that some clones that maintained a high RGR and TCC (eg: AE2, AG2) as callus cultures throughout this study also maintained these characteristics as suspensions (Table 1). In general, RGR and TCC values were lower at sixteen weeks than at four and this was more marked in suspensions where only three clones (AE2, AG2 and AB9) had measurable amounts of chlorophyll by the end of the experimental period (Table 1). By this stage suspension culture values were generally lower than callus values which suggests that liquid culture provided conditions less favourable for growth and chlorophyll biosythesis (Table 1).

However, despite the effect of culture condition it is important to note the existence of variation among clones. Three clones (AE2, AG2 and AB9) differed from the rest in that they maintained a high TCC and RGR in suspension, and AG2 and AE2 did so in callus also, and appeared to be less susceptible to the influences of the culture medium (Table 1).

Differences in soluble phenolic content of the medium
The HPLC chromatograms illustrate the presence and abundance of chloroform-soluble phenolic compounds in the medium of the callus and suspension clones after four and sixteen weeks (Fig. 1). They show differences in phenolic synthesis among clones, between regimes and over time (Fig. 1).

Phenolic production was greatest in AE2 and least in Z1 in both regimes at week four and sixteen (Fig. 1). Clones in suspension appeared to produce marginally more phenolics than in callus and there was a slight drop in the number and height of phenolic peaks at sixteen weeks (Fig. 1). It was interesting to note that AE2 which had the highest phenolic activity among these clones also showed the greatest RGR and high levels of chlorophyll production (Fig. 1, Table 1).

Variation among suspension clones in response to elicitation

Ten suspension cultures were selected from the twenty remaining after subculture at sixteen weeks. Each of these was used to prepare two suspension cultures which were incubated for 21-days until the downturn of growth. At this point each flask received the elicitor and L-[U-^{14}C]phenylalanine (74kBq) and were subsequently harvested after 6 h. PAL activity and the total incorporation of label into the chloroform-soluble fraction of the medium and the methanol-soluble fraction of the cells was measured.

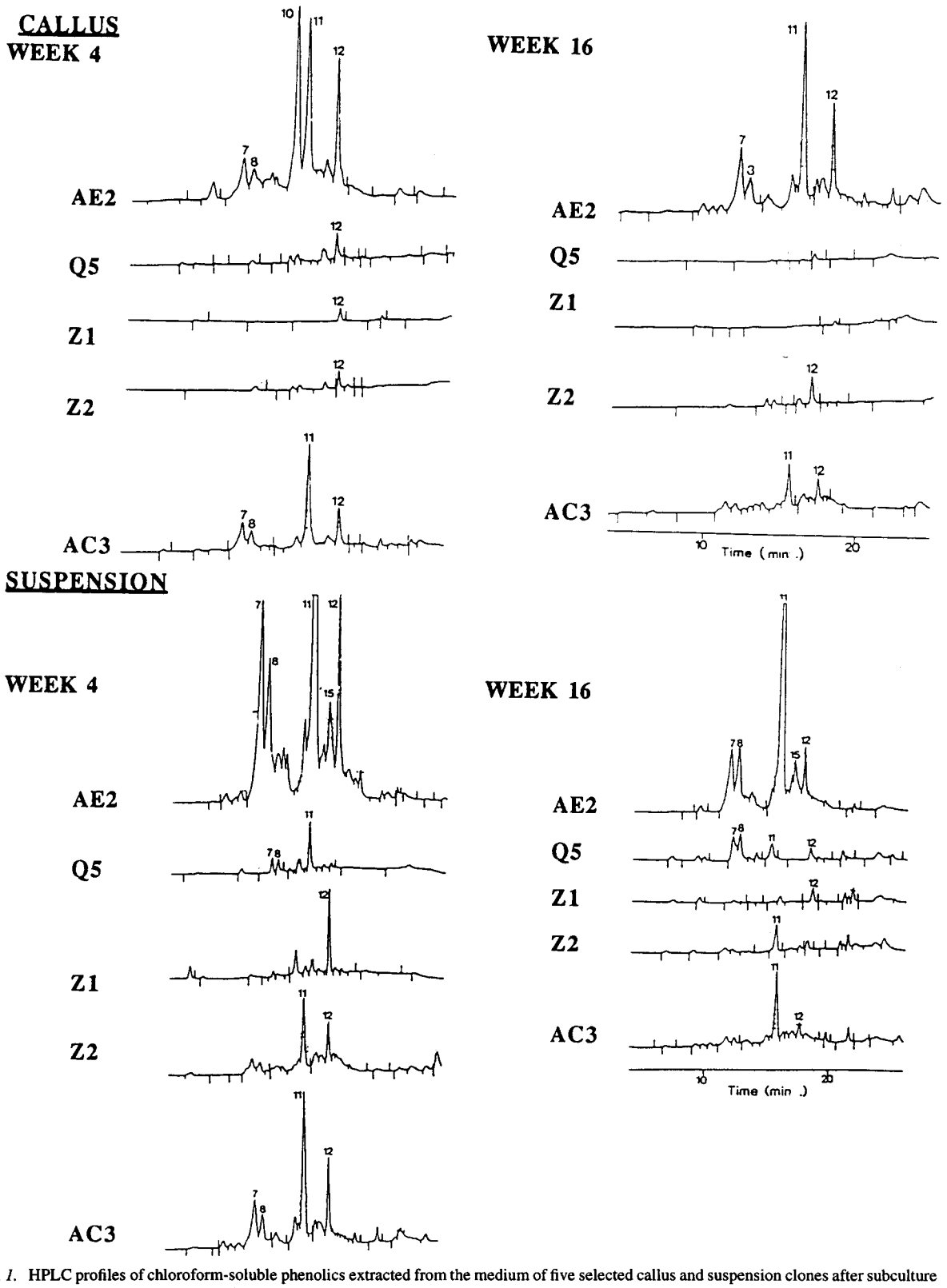

Fig. 1. HPLC profiles of chloroform-soluble phenolics extracted from the medium of five selected callus and suspension clones after subculture at four and sixteeen weeks. Numbers denote individual phenolic compounds characterised by retention time and absorption spectrum.

Table 1. The relative growth rate (RGR) ($day^{-1} \times 10^{-2}$) and total chlorophyll content (TCC) ($\mu g\ DW^{-1}$) of twenty callus and suspension clones after 4 and 16 weeks.

Clone	RGR		TCC	
	Week 4	Week 16	Week 4	Week 16
Callus				
BZ4	0.78	0.56	N.D.	N.D.
AGZ	1.83	2.16	10.6	8.3
CD8	1.23	1.11	N.D.	N.D.
N4	0.94	0.33	N.D.	N.D.
AC2	2.73	2.56	4.5	3.4
Q5	1.48	1.23	7.8	2.7
Y3	0.73	0.34	9.2	2.9
BZ4	0.96	0.45	N.D.	N.D.
AE2	4.28	6.12	4.2	4.6
CD2	2.11	2.00	N.D.	N.D.
EF4	1.76	2.13	N.D.	N.D.
AC1	0.12	0.11	N.D.	N.D.
BV7	3.45	2.67	1.3	1.2
CB6	1.93	1.56	2.7	1.7
N3	1.11	1.88	14.2	10.5
AB9	2.76	1.49	N.D.	N.D.
AC3	3.56	2.11	N.D.	N.D.
Z1	2.59	2.12	6.5	5.3
Z2	2.12	1.67	N.D	N.D.
BQ3	1.78	1.70	1.3	1.2
Suspension				
BZ4	1.78	0.46	N.D.	N.D.
AG2	1.54	2.54	9.2	5.6
CD8	1.03	0.56	N.D.	N.D.
N4	1.16	0.23	N.D.	N.D.
AC2	2.50	1.99	5.3	N.D.
Q5	1.23	1.24	6.5	N.D.
Y3	0.54	0.14	10.2	N.D.
BZ4	1.41	0.23	N.D.	N.D.
AE2	4.20	4.76	6.2	5.2
CD2	1.23	1.00	N.D.	N.D.
EF4	1.72	1.76	3.4	N.D.
AC1	0.23	0.16	N.D.	N.D.
BV7	3.00	1.18	N.D.	N.D.
CB6	1.63	1.32	N.D.	N.D.
N3	1.48	1.92	5.9	N.D.
AB9	2.67	2.54	4.2	3.2
AC3	3.65	1.64	N.D.	N.D.
Z1	2.11	2.22	N.D.	N.D.
Z2	1.59	0.78	N.D.	N.D.
BQ3	1.27	0.91	2.6	N.D.

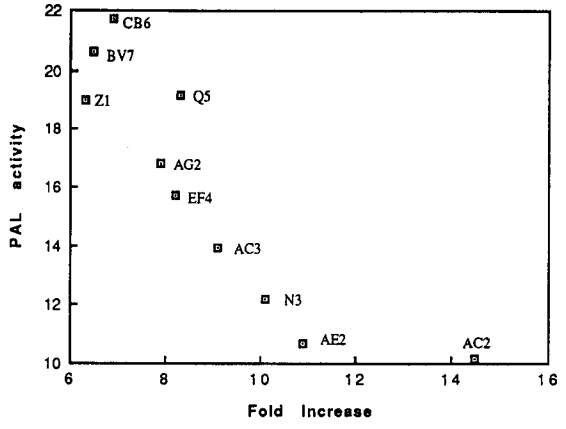

Fig. 2. The relationship between PAL activity ($\mu kat\ kg^{-1}$) in unelicited cultures and the fold increase for PAL after elicitation in each of the ten suspension clones.

Differences in PAL activity following elicitation

The specific activity of the enzyme PAL in the non-elicited (control) clones showed a 2-fold variation between AC2 and CB6 with the remaining clones falling between these extremes (Fig 2). Elicitation increased the specific activity of PAL in all ten suspension clones from over 6-times (Z1) to almost 15-fold (AC2) and the greatest increase was detected in clones with the lowest unelicited values (Fig. 2). In fact despite this variation in 'fold increase' the actual elicited values were relatively similar for each clone (values ranging between 120 and 160 $\mu kat\ kg\ FW^{-1}$), suggesting the presence of a controlling factor imposing a ceiling on PAL activity.

Differences in the incorporation of L-[U-^{14}C]phenylalanine following elicitation

Elicitation markedly reduced the total incorporation of L-[U-^{14}C]phenylalanine into the chloroform-soluble (C-Fraction) fraction of the medium (mean recovery of 0.5%) and increased the incorporation of label into the methanol-soluble (M-Fraction) fraction of the cells (mean recovery of 15%) in all ten suspension clones (Table 2). The diversion of label from medium to cell bound soluble phenolics is strongly affected by elicitation and maybe caused by the elicitor-induced increase in PAL activity.

Differences among the clones were apparent in the C-Fraction despite suppression of activity by elicitation (Table 2). A tenfold difference can be seen between AG2 and N3. Differences also occur among the clones with respect to incorporation into the M-Fraction; non-elicited Q5 has a value three fold greater than CB6,

Table 2. Incorporation of L-[U-^{14}C] phenylalaline into the chloroform (C-Fraction) soluble fraction of the culture medium and the methanol (M-Fraction) soluble fraction of the cells in elicited and non-elicited suspension clones. All values in Bq/culture × 10^3.

Clone	AG2	AC2	Q5	AE2	EF4	BV7	CB6	N3	AC3	Z1
C-Fraction										
Elicited	0.13	0.33	0.17	0.23	0.11	0.23	0.73	1.4	0.44	0.16
Non Elicited	10.5	15.2	21.3	9.6	15.1	23.6	21.9	14.0	18.0	9.5
M-Fraction										
Elicited	25.1	13.2	10.6	24.3	8.3	7.9	7.4	11.6	10.4	23.1
Non Elicited	3.6	1.9	3.9	1.6	1.5	3.1	1.3	2.0	2.4	3.0

and elicited AG2, AE2 and Z1 three times that of CB6 (Table 2). It is also interesting to note that the levels of incorporation into the M-Fraction were markedly lower in seven clones although the elicitor-induced PAL activity remained similar in all ten clones (Table 2, Fig. 3). The correlation between PAL activity and incorporation in elicited and unelicited cells suggests that these seven clones were utilising the label less efficiently under elicited conditions than Z1, AG2 and AE2 (Fig. 3). The reason for this differential response is unclear. It is possible that more phenylalanine was required by the cells of AE2 and AG2 to support cell division and maintain the high RGRs observed throughout the experimental period (Table 1).

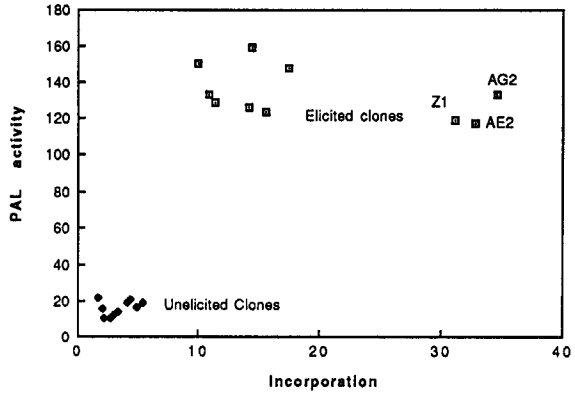

Fig. 3. The relationship between PAL activity (μkat kg^{-1}) and the incorporation of L-[U-^{14}C] phenylalanine (Bq/culture × 10^3) into the methanol (M-Fraction) soluble fraction of the cells in elicited and elicited and unelicited suspension clones.

Discussion

Differences among clonal culture lines have already been described in a wide range of plant species (Dougall et al. 1980; Creche et al. 1987; Dougall & Vogelien 1990). The differences in growth, chlorophyll content and biosynthetic activity observed and recorded among the clones in this paper support these findings and reflect the heterogeneous nature of the parent culture. The causes of these changes are unclear although the close intercellular relationships that are intrinsic to the parental source material, and which are impaired in callus and suspension culture may well disturb the genetic and epigenetic stability of the component cells (Yeoman & Forche 1980). It is also important to recognise the possibility of the changes occurring during the cloning and culture period and we cannot ignore the possibility that these differences could be attributed to the individual growth patterns of the clones.

The culture regime and time in culture had an influence on the clones particularly in suspension where values dropped more rapidly than in callus causing greater variation among and between the two regimes. Time and frequency of subculture have long been recognised as a cause of variation and instability but the effect and cause of suspension culture-induced variation has been too often overlooked (Mantell & Smith 1983). The cell-to-cell contact in callus cultures, though less than that in the plant internode from which the explants were taken, permits chemical and physical gradients to exist between cells (Yeoman 1987). In transferring the callus to liquid medium the enhanced disturbance of cell-to-cell contact may have further effects on cell function.

The treatment of plant tissue cultures with elicitors often leads to an increase in PAL activity and a concomitant rise in the production of phytoalexin and secondary compounds (Hahlbrock et al. 1981; Bailey & Mansfield 1982; Dixon, & Harrison 1990). We found a marked increase in PAL activity after elici-

tation of all ten clones demonstrating the ease with which the enzyme can be stimulated in pepper cultures. This was accompanied by a decrease in incorporation of L-[U-^{14}C]phenylalanine into the chloroform-soluble phenolic compounds released into the medium and an increase into the methanol-soluble fraction of the cells. As yet it is not known whether capsidiol, the major phytoalexin found in *Capsicum*, is produced in response to the elicitor in these suspension cultures.

We found that the faster and more stable growing cultures incorporated more phenylalanine, irrespective of PAL activity, into the cells. It is possible that the downturn in growth recorded among the other clones could have resulted in the diversion of phenylalanine into compounds related to growth retardation. These results show the possibility of selecting stable variants which is as significant to continuing research as is the recognition of the culture components that are responsible for inducing variability. These results have also shown that pre-existing, culture and metabolic variation is commonplace among and within cultures derived from cloned cells. The understanding and manipulation of this phenomenon is important for both the maintenance of long-term cultures and the exploitation of morphologically and, more importantly, metabolically useful tissue cultures.

Acknowledgement

Some of the work on this project was funded by Glaxo Group Research Limited.

References

Bailey JA & Mansfield JW (1982) Phytoalexins. Blackie, Glasgow

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye-binding. Anal. Biochem. 72: 248–254

Creche J, Guiller J, Andreu F, Gras M, Chenieux JC & Rideau M (1987) Variability in tissue cultures of *Choisya ternata* originating from a single tree. Phytochemistry. 26: 1947–1953

Davey MR, Fowler MW & Street HE (1971) Cell clones contasted in growth, morphology and pigmentation isolated from a callus culture of *Atropa belladona* var. *lutea*. Phytochemistry. 10: 2559–2575

Dix PJ (1990) Plant Cell Line Selection: Procedures and Applications. VCH Weinhein, New York: Basel: Cambridge

Dixon RA & Harrison MJ (1990) Activation, structure and organisation of genes involved in microbial defense in plants. Adv. Genet. 28: 165–234

Dougall DK & Vogelien DL (1990) Anthocyanin yield of clonal wild carrot cultures. Plant Cell Tiss. Org. Cult. 23: 79–91

Dougall DK, Johnson JM & Whitten GH (1980) A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot. Planta 149: 292–297

Hahlbrock K, Lamb CL, Purwin C, Ebel J, Fautz E & Schafer E (1981) Rapid response of suspension-cultured parsley cells to elicitors from *Phytophthora megasperma* var. *soyae*. Plant Physiol. 67: 768–777

Hall RD & Yeoman MM (1991) The influence of intracellular pools of phenylalanine derivatives upon the synthesis of capsaicin by immobilized cell cultures of the chilli pepper, *Capsicum frutescens*. Planta 185: 72–80

Hall RD, Holden MA & Yeoman MM (1987) The accumulation of phenylpropanoid and capsaicinoid compounds in cell cultures and whole fruit of the chilli pepper, *Capsicum frutescens* Mill. Plant Cell Tiss. Org. Cult. 8: 163–176

Harborne JB (1976) Phytochemical methods: A guide to modern techniques in plant analysis. Chapman and Hall, London

Holden MA, Hall RD, Lindsey K & Yeoman MM (1987) Capsaicin biosynthesis in cell cultures of *Capsicum frutescens*. In: Webb C & Mavituna F (Eds) Plant and Animal Cells, Process Possibilities (pp 45–63). Ellis Horwood Books in Biochemistry & Biotechnology, Ellis Horwood Limited, Chichester

Lindsey K & Yeoman MM (1984) The synthetic potential of immobilized cells of *Capsicum frutescens* Mill. cv. annuum. Planta 162: 495–501

Mantell SH & Smith H (1983) Cultural factors that influence secondary metabolite accumulations in plant cell and tissue cultures. In: Mantell SH & Smith H (Eds) Plant Biotechnology (pp 75–110). Cambridge University Press, Cambridge

Mavituna F, Park JM, Wilkinson AK & Williams PD (1987) Characteristics of immobilized plant cell reactors. In: Webb C & Mavituna F (Eds) Plant and Animal Cells, Process Possibilities (pp 92–115). Ellis Horwood Books in Biochemistry & Biotechnology, Ellis Horwood Limited, Chichester

Ozeki Y & Komamine A (1985) Changes in activities of enzymes involved in general phenylpropanoid metabolism during the induction and reduction of anthocyanin synthesis in a carrot suspension culture as regulated by 2,4-D. Plant Cell Physiol. 26: 903–911

Salgado-Garciglia R & Ochoa-Alejo N (1990) Increased capsaicin content in PFP-resistant cells of the chilli pepper (*Capsicum annuum*). Plant Cell Rep. 8: 617–620

Schenk RU & Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199–204

Selby C & Collin HA (1976) Clonal variation in growth and flavour production in tissue cultures of *Allium cepa* L. Ann. Bot. 40: 911–918

Sucrasno N & Yeoman MM (1993) Phenylpropanoid metabolism during growth and development of *Capsicum frutescens* fruits. Phytochemistry. 32: 839–844

Tanaka Y, Kojima M & Uritani I (1974) Properties, development and cellular localisation of cinnamic acid 4-hydroxylase in cut injured sweet potato. Plant Cell Physiol. 15: 843–854

Yeoman MM (1987) Bypassing the plant. Ann. Bot. 60: 157–174

Yeoman MM & Forche E (1980) Cell proliferation and growth in callus cultures. Int. Rev. Cytol. Suppl. 11A: 1–24

Zenk MH, El-Shagi H, Arens H, Stockigt J, Weiler EW & Deus B (1977) Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In: Barz W, Reinhard E & Zenk MH (Eds) Plant Tissue Culture and its Biotechnological Application (pp 27–43). Springer-Verlag, Berlin and New York