Plant Physiol. (1985) 78, 25–28
0032-0889/85/78/0025/04/$01.00/0

Influence of Culture Age and Spermidine Treatment on the Accumulation of Phenolic Compounds in Suspension Cultures[1]

Received for publication September 18, 1984 and in revised form December 31, 1984

MICHAEL J. MUHITCH[2] AND JOHN S. FLETCHER*
Botany and Microbiology Department, University of Oklahoma, Norman, Oklahoma 73019

ABSTRACT

The influence of cell age on phenol accumulation was examined by determining the quantity of individual phenols which accumulated in Paul's scarlet rose cultures of increasing age. During log-phase growth (days 7 and 11), only gallic acid and epicatechin-catechin were detected; whereas, during early and late stationary phase (days 14 and 35), several other phenols were present in addition to gallic acid and epicatechin-catechin. When stationary-phase cultures were provided with a supplement of sucrose and spermidine, a treatment previously shown to arrest the senescence of rose cultures (Muhitch, Edwards, Fletcher 1983 Plant Cell Rep 2: 82–84), the cells then accumulated a higher level and a wider assortment of phenols. These results suggest that extending the lifespan of mature nondividing cell cultures offers a means of increasing the yield of secondary products by cultured cells.

Plant tissue cultures have been regarded for many years as an attractive alternative to whole plants as a source of commercially important secondary products (4, 17, 20, 21). It has been speculated that cultured cells have the potential of providing a steady supply of less expensive plant chemicals providing techniques can be developed to grow high yielding strains on inexpensive media (21). Unfortunately, some of the most highly sought after compounds are not produced in cultured cells (2) while others are only produced in small amounts (20), and in these cases there is a tendency for the yield per culture to decline upon continued subculturing (5).

The disappointingly low yield of secondary products by cultured cells in comparison to that of intact plants can not be attributed to genetic incompetence of the cultured cells, since whole plants regenerated from cultured cells have been demonstrated to synthesize normal amounts of secondary products (2). Thus, the inability of cultured plant cells to produce secondary products appears to result from a failure of gene expression.

A substantial amount of data collected on both plants and microorganisms indicates that the selective expression of different portions of the genome is dependent upon cell maturation (10). Tissue culture studies on several different plant species have shown a greater accumulation of secondary products in mature nondividing (stationary phase) cultures than in actively growing (logarithmic phase) cultures (8, 16, 18, 20). Yeoman *et al.* (20) point out how this feature of some tissue cultures is also a common feature of intact plants where secondary product accumulation is most pronounced in older plant organs after their growth has stopped. Recognizing that older mature cells are frequently the site of secondary product accumulation, it is worth noting a major distinction between intact plants *versus* cultured cells. In the intact plant, it is common for mature fully expanded cells to remain alive for months or even years. In contrast to this, the limited number of studies reported on tissue culture senescence (3, 13–15) indicates that once a culture has reached its maximum biomass, the cells undergo rapid senescence unless they are induced to divide by placing them in fresh medium. Herein may lie a partial explanation for the low secondary product yield of cultured cells. The brief stationary phase which cultured cells pass through may severely curtail the accumulation of secondary products produced by enzymes which are only present or active in mature expanded cells. If this is true, it follows that extending the life span of mature cell cultures should increase the level of secondary products.

The research reported in this paper was designed to establish the influence of culture age on the amounts and kinds of phenols accumulated in mature cultures of PSR[3] and to determine if treatments administered to arrest senescence altered the accumulation of this group of compounds.

MATERIALS AND METHODS

Suspension cultures of *Rosa* sp. (L.) cv Paul's scarlet were grown in MPR medium and harvested as previously described (15). Sucrose and spermidine were added to the cultures as filter sterilized solutions on day 14, when the cultures have entered into early stationary phase (6). The isolation, separation, and identification of the phenols of Paul's scarlet rose cells have been described previously (12). Briefly, phenols were separated using reverse-phase HPLC and quantified by measuring the *A* at 280 nm of the column effluent. Total phenol levels were measured using the method of Amorim, *et al.* (1). Individual compounds have been identified by collection from HPLC and subsequent co-chromatography with authentic standards as described in Muhitch and Fletcher (12).

RESULTS

When phenol extracts recovered from cultures of increasing age were subjected to HPLC fractionation, the profile of UV absorbing compounds changed dramatically (as the cultures aged) (Fig. 1). On days 7 and 11, during log-phase growth (6), only gallic acid and epi-cat (Fig. 2) were detected; whereas during early stationary-phase (day 14), when cells had stopped growing but were still viable (11), additional compounds started to accumulate. By day 35, the cultures which were undergoing rapid

[1] Supported by National Institute of Health Grant 5 R01 AGJ01709-03.

[2] Present address: American Cyanamid Co., Agricultural Research Division, P.O. Box 400, Princeton, NJ 08540.

[3] Abbreviations: PSR, Paul's scarlet rose; epi, (−)epicatechin; epi-cat, (−)epicatechin-(+)catechin (C4-C8 linked); EGCG, (−)epigallocatechin gallate.

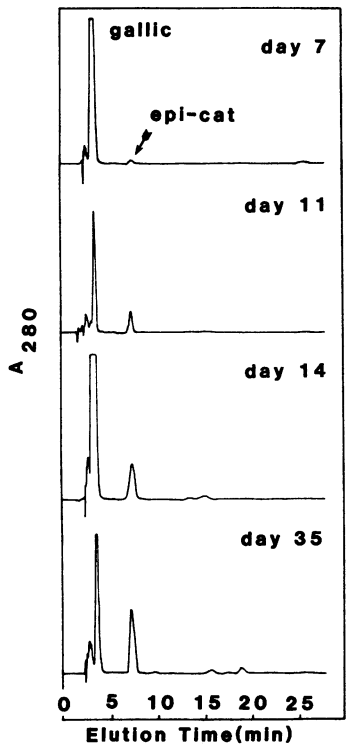

FIG. 1. HPLC elution profiles of the phenolic extracts from PSR suspension cultures of various ages.

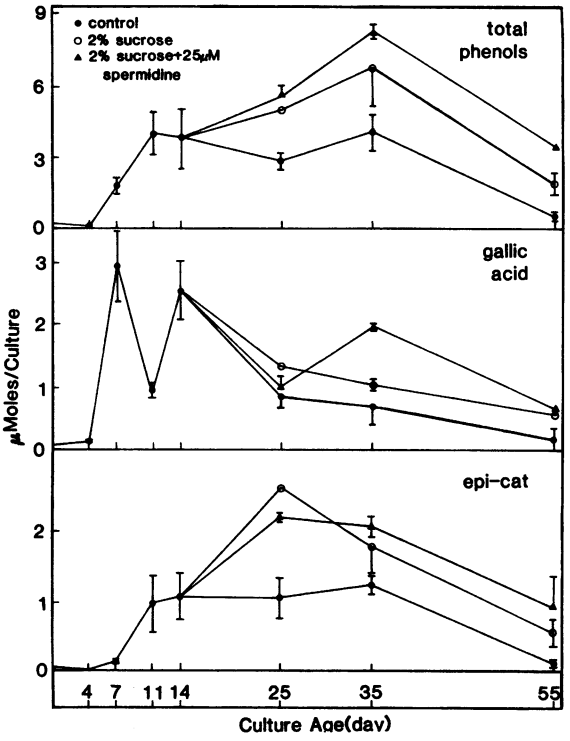

FIG. 3. Effects of sucrose or sucrose + spermidine on the total phenol, gallic acid, and epicatechin-catechin levels in suspension cultures of PSR. Verticle bars represent 1 SD (n = 3).

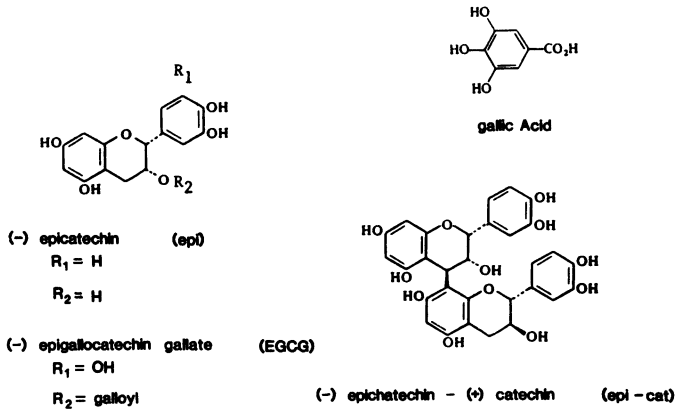

FIG. 2. Chemical structures for (−)epicatechin (epi), (−)epigallocatechin gallate (EGCG), gallic acid, and (−)epicatechin-(+)catechin (epi-cat).

senecence (11) contained a lower amount of gallic acid, an unchanged level of epi-cat, and increased levels of the trace compounds. The increased level of several phenolic compounds during senescence emphasizes the potential importance of this phase of development to secondary product synthesis and supports the contention advanced in the introduction that cultured plant cells may not remain viable long enough in the stationary and poststationary phases to allow for the full expression of their biochemical potential.

The influence of cell aging on secondary product synthesis was examined further by providing stationary-phase cultures with treatments which have previously been shown to arrest the senescence of rose cultures (11). The effects of adding sucrose or sucrose + spermidine to early stationary-phase (14-d) PSR cultures on the total and individual phenol levels are shown in Figures 3 and 4. In control cultures, the total phenol content increased until day 11, remained relatively constant through day 35, and subsequently declined. Treatment of 14-d-old PSR cultures with sucrose or sucrose + spermidine resulted in 1.6- and 2-fold increases (respectively) in the total phenol levels at day 35 when compared to control cultures (Fig. 3). Fractionation of the phenols recovered from cultures of all ages showed the primary constituent to be gallic acid. The bimodal pattern of gallic acid accumulation between days 4 and 14 which was observed in repeated experiments may reflect this compound's probable role as an intermediate in the synthesis of several different tannins (7). The addition of sucrose only slightly enhanced gallic acid levels in PSR cultures, while sucrose + spermidine treatment elevated gallic acid levels to 3-fold those of control cultures at day 35 (Fig. 3). Epi-cat, the other major phenol of PSR cultures, increased until day 14, remained relatively constant until day 35, and subsequently declined. The addition of sucrose or sucrose + spermidine to 14-d-old cultures elevated the epi-cat levels 2- to 2.5-fold over control values on day 25.

The brief stationary phase which cultured cells pass through may severely curtail the accumulation of secondary products produced by enzymes whose genes are only expressed at a particular stage during the life of mature expanded cells. If this is true it follows that extending the life span of mature cell cultures should increase the level of secondary products. Both the sucrose and the sucrose + spermidine additions enhanced the accumulation of compounds in 25-, 35-, and 55-d-old cultures which were not present in young cultures ranging from 4 to 14 d old. The accumulation was most pronounced in 35-d-old cultures; therefore, only data pertaining to that age are presented (Fig. 4). The most influential treatment was sucrose + spermidine (Fig. 4). Provision of these compounds to 14-d-old cultures either caused or enhanced the accumulation of 11 compounds (11 profile peaks) in 35-d-old cultures, only one of which was present in 14-d-old cells (Fig. 1) and only a few of which were detected in 35-d-old control cultures (Fig. 4). Two of these compounds have been positively identified as epi and EGCG (Fig. 2). The sucrose addition caused a 4- and 5-fold

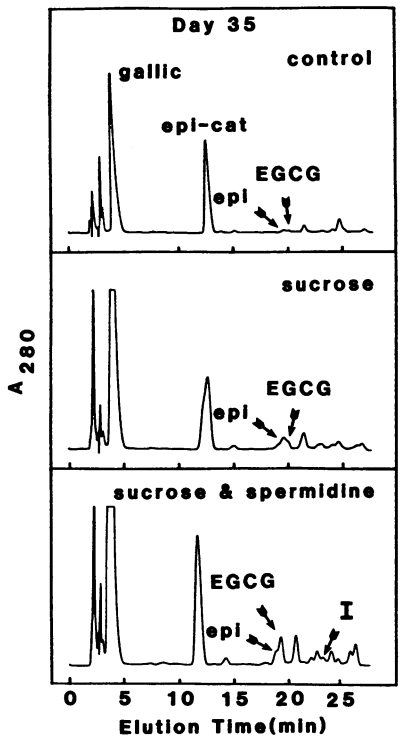

FIG. 4. HPLC elution profiles of the phenolic extracts from 35-d-old PSR suspension cultures. Control cultures received no additions, whereas the sucrose and sucrose + spermidine cultures were supplemented on day 14 with the designated compounds. Sufficient amounts of spermidine and/or sucrose were added to yield final concentrations in the media of 25 μM and 2%, respectively. Differences in the retention times for particular compounds in Figures 1 and 4 reflect the gradual column deterioration which occurs over an extended period of usage under acidic conditions.

increase in epi and EGCG, respectively; whereas, the sucrose + spermidine caused a 3- and 18-fold increase in the two compounds. Although positive identifications have not been made of the other accumulating compounds, one of these, labeled as I (Fig. 4), is of special interest. This compound has been demonstrated to be present in the intact rose plant and in cultures newly started from the plant (12), but not in the established cell line used in this study except for when spermidine treatments were provided.

DISCUSSION

Previous studies have demonstrated that an antagonistic relationship exists between primary metabolism (growth, cell division, and protein synthesis), and the production of secondary products in plant tissue cultures (20). As plant or microbial cell maturation progresses, there appears to be an ordered expression of the genome such that particular enzymes or groups of enzymes are synthesized or activated at a particular stage of cell development (10). In tulips, for example, there is an orderly, sequential rise and fall in the enzymes and intermediates of flavanol biosynthesis during another maturation (19). In plant cell cultures, the synthesis of precursor phenols and alkaloids occurs during the log phase of growth, while the synthesis of more complex products occurs predominately during the stationary phase of growth (1, 9). In a similar fashion, we observed quantitative changes in the two most abundant phenols (gallic acid and epicat) as well as qualitative changes in the overall UV absorption profile of phenols recovered from cultured rose cells of increasing age.

Of special interest in our study were those phenols which were present only on day 35 in senescing cultures (Fig. 4). Since these compounds did not accumulate in younger cultures (Fig. 1), it can be inferred that their accumulation was dependent upon metabolic features characteristic of only senescing cells. Furthermore, it can be assumed that the extent to which these compounds accumulated was a reflection of how long cells remained metabolically active at this particular stage of development. Since it has previously been shown that rose cultures die rapidly (15), it was not surprising that only limited amounts of the complex phenols accumulated. When the cultures were subjected to treatments previously shown to extend the life-span of the mature nondividing cells (11), the level of total phenols was increased 1.6-fold. A quantitative comparison of individual phenols recovered, from treated *versus* control cultures, showed only modest increases in the treated cultures of those phenols such as gallic acid and epi-cat which are normally present during early stages of culture growth. In contrast to this, phenols which were only observed in senescing (35-d-old) control cultures, experienced dramatic increases in the treated cultures (Fig. 4). There was an 1800% and 350% increase in EGCG and epi, respectively, when sucrose + spermidine-treated cultures were compared to the 35-d-old control cultures (Fig. 4). The less dramatic effect of the treatment on the level of epi may reflect the precursor/product relationship between this compound and EGCG. Knoloch *et al.* (9) reported similar results in *Catharanthus* cultures where elevated sucrose levels enhanced the accumulation of the product serpentine while decreasing the levels of is metabolic precursor ajmaline.

One of the most noteworthy observations in this study was that the phenolic compound 'I' was present in the sucrose + spermidine cultures (Fig. 4), but was absent in the control cultures. The appearance of this compound in the treated cultures, even though in small quantity, was of special interest. Although we have shown this compound, tentatively identified as a substituted flavan-3-ol (12), to be the second most abundant phenol present in older rose stems (12), we have never recovered it previously from the established line of rose cells used in this study. Thus, the treatments which we administered to extend the life span of the cell lead to an accumulation of a compound which would otherwise not have occurred. This proved that the line of PSR used in this study which had been in culture for 25 years still possessed the genes controlling the synthesis of a compound which is present in intact plants (12), but normally does not accumulate in cultured cells.

In conclusion, we have shown that by extending the lifespan of mature nondividing cells it was possible to alter both the composition and the amount of phenols which accumulated in an established cell line. Thus, expression of the biochemical potential of cultured plant cells appeared to be associated at least in part with the length of time which the cells remained in a particular phase of senescence. A better understanding of the metabolic features of senescing cells in conjunction with improved means of arresting senescence at particular points during its course may prove to be extremely valuable in future efforts to use plant tissue cultures as a commercial source of plant secondary products.

LITERATURE CITED

1. AMORIM HV, DK DOUGALL, WR SHARP 1977 The effects of carbohydrate and nitrogen concentration on phenol synthesis in Paul's Scarlet Rose cells grown in tissue culture. Physiol Plant 39: 91–95
2. BOHM H 1983 The inability of plant cells to produce secondary substances. *In* A Fujiwara, ed, Plant Tissue Culture 1982. The Japanese Association for Plant Tissue Culture, Tokyo, pp 325–328
3. CODRON H, A LATCHE, JC PECH, B NEBIE, J FALLOT 1979 Control of quiescence and viability in auxin-deprived pear cells in batch and continuous culture. Plant Sci Lett 17: 29–35
4. CROCOMO OJ, E AQUARONE, OR GOUTLIEB 1981 Biosynthesis of secondary products *in vitro*. *In* JA Thorpe, ed, Plant Tissue Culture. Methods and

Applications in Agriculture. Academic Press, New York, pp 359–372
5. DOUGALL DK 1977 Factors affecting the yields of secondary products in plant tissue cultures. *In* WR Sharp, PO Larsen, EF Paddock, V Raghaven, eds, Plant Cell and Tissue Culture. Principles and Applications. Ohio State University Press, Columbus, pp 727–743
6. FLETCHER JS, H BEEVERS 1969 Acetate metabolism in cell suspension cultures. Plant Physiol 45: 765–772
7. HASLAM E 1981 Vegetable Tannins. *In* EE Conn, ed, The Biochemistry of Plants. A Comprehensive Treatise, Vol 7. Academic Press, New York, pp 527–556
8. KNOBLOCH KH, J BERLIN 1980 Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* (L.) G. Don. Naturforsch Sect C Biosci 35: 551–556
9. KNOBLOCH KH, G BAST, J BERLIN 1982 Medium- and light-induced formation of serpentine and anthocyanins in cell suspension cultures of *Catharanthus roseus*. Phytochemistry 21: 591–594
10. LUCKNER M 1980 Expression and control of secondary metablism. *In* EA Bell, BV Charlwood, eds, Secondary Plant Products. Springer-Verlag, Heidelberg, pp 23–63
11. MUHITCH MJ, LA EDWARDS, JS FLETCHER 1983 Influence of diamines and polamines on the senescence of plant suspension cultures. Plant Cell Rep 2: 82–84
12. MUHITCH MJ, JS FLETCHER 1984 Isolation and identification of the phenols of Paul's scarlet rose stems and stem-derived suspension cultures. Plant Physiol 75: 592–595
13. OKAMURA S, K SUEKI, A NISHI 1975 Physiological changes of carrot cells in suspension culture during growth and senescence. Physiol Plant 33: 251–255
14. PECH JC, RJ ROMANI 1978 Senescence of pear fruit cells cultured in a continuously renewed, auxin-deprived medium. Plant Physiol 63: 814–817
15. REIDER ML, BA SMITH, JS FLETCHER 1982 Protein content and subculturing properties of senescing plant suspension cultures. In Vitro 18: 1004–1008
16. SASSE F, U HECKENBERG, J BERLIN 1982 Accumulation of β-carboline alkoloids and serotonin by cell cultures of *Peganum harmala* L. I. Correlation between plants and cell cultures and the influence of medium constituents. Plant Physiol 69: 400–404
17. STABA EJ (ed) 1980 Plant Tissue Culture as a Source of Biochemicals. CRC press, Boca Raton
18. TAL B, I GOLDBERG 1982 Growth and diosgenin production by *Dioscorea deltoidea* cells in batch and continuous cultures. Planta Med 44: 107–110
19. WIERMANN R 1981 Secondary plant products and cell and tissue differentiation. *In* EE Conn, ed, The Biochemistry of Plants. A Comprehensive Treatise, Vol 7. Academic Press, New York, pp 85–116
20. Yeoman MM, MB Miedzybrodzka, K Lindsey, WR McLauchlan 1980 The synthetic potential of cultured plant cells. *In* F Sala, B Parisi, R Cella, O Ciferri, eds, Plant Cell Cultures: Results and Perspectives. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 327–343
21. ZENK MH 1978 The impact of plant cell culture on industry. *In* TA Thorpe, ed, Frontiers of Plant Tissue Culture 1978. University of Calgary Press, Calgary, pp 1–13