

***Short Communication***

# Relationship between Vital Staining and Subculture Growth during the Senescence of Plant Tissue Cultures<sup>1</sup>

Received for publication June 22, 1982 and in revised form August 14, 1982

BETH A. SMITH, MARY L. REIDER, AND JOHN S. FLETCHER

*Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma, 73019*

## ABSTRACT

The vital staining properties of rose cultures (*Rosa* cv Paul's Scarlet) of increasing age were compared with their ability to be subcultured. At 4-day intervals beginning on day 14, after cell division and expansion had stopped, cells were stained separately with Evans blue, fluorescein diacetate, and phenosafranin. The degree to which parent cultures stained with each of these dyes was compared to the dry weight of their subcultures harvested after 9 and 21 days of growth.

Staining with either Evans blue or fluorescein diacetate was demonstrated to be a good means of establishing when senescing cells died. However, the staining properties of aging cultures did not correlate well with their ability to be subcultured, because an increasing proportion of the living cells appeared to lose their ability to divide as senescence progressed.

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In work with tissue cultures and isolated protoplasts, three vital stains—FDA<sup>2</sup> (3, 12, 17), EB (13), and PS (14)—have been used extensively to ascertain the viability of cells and protoplasts. Several investigators have reported chemical or heat-kill experiments to establish that these stains distinguish living from nonliving cells (7, 9, 16). A quantitative comparison of these stains on senescing tissue has not been reported, and only limited information is available relating the staining properties of cells with their potential to divide and grow (16).

The present investigation examined the usefulness of vital stains to follow cell senescence in plant tissue cultures. The staining properties of cells of increasing age were compared with the ability of the cells to be subcultured. An additional feature of the study was to determine if staining techniques, used previously with dispersed cell lines (13, 16), were also useful in studying the viability of clumped cells as present in rose cultures (5, 6, 10, 15).

## MATERIALS AND METHODS

Suspension cultures of Paul's Scarlet rose were grown in 80 ml of MPR medium as described previously (11). Starting on day 14, the normal transfer age when cell division has stopped (6), five cultures were harvested every 4 d until day 38. Each harvested culture served as the parent culture for eight subcultures. Carefully weighed 500-mg fresh weight samples were used to inoculate the

subcultures. Four subcultures were harvested after 9 and 21 d of growth, and dry weights were determined. The average weights of the subcultures were compared with the vital staining properties of the respective parent cultures. Dry weights were determined after cultures had been kept at 80°C for 48 h.

The cells used in this study grew as clumps, a normal feature of this cell line (5, 6, 10, 15). Extensive efforts on our part to grow dispersed cells or separate the clumped cells present in mature cultures failed. Procedures which were tested to grow dispersed cells included: variations in the kind and concentration of hormones, different salt concentrations, and enzyme (pectinase, protease, and cellulase) additions to cell media at the beginning and during growth. Experiments to separate cell-clumps recovered from mature cultures were performed by treating the clumps with pectinase, protease, cellulase, or various combinations of these enzymes at different concentrations. Two difficulties were encountered in the latter experiments: (a) we were not able to achieve reproducible separation of cell-clumps recovered from older cultures (22–38 d); (b) the enzyme preparations appeared to kill the cells as others have reported (4), and partial purification by us of the commercial enzymes nullified their ability to separate clumped cells.

Cells removed from parent cultures were stained with FDA and PS in the manner described by Widholm (16). EB was used according to the procedure of Gaff and Okong'O-ogola (7). FDA is absorbed by living cells (16), whereas EB and PS are excluded from living cells (7, 16). The clumped nature of the cultures made it impossible to quantify staining on an individual cell basis. As an alternative, the degree to which individual clumps stained was recorded and relative values were assigned. Values were assigned according to the percent of cells within a clump which stained. Assigned values were as follows: no stained cells, 0; less than 25% stained, 1; 25 to 50% stained, 2; over 50% stained, 3. Two hundred clumps ranging in size from 50 to 200 cells were recovered from each of five parent cultures and evaluated for their staining properties. The relative staining values assigned to each clump were totaled giving a total additive value for 200 clumps. The total additive values for each of five parent cultures were used to determine the average additive values shown in Figures 1 and 2.

Preliminary studies conducted with acridine orange, brilliant cresyl blue, neutral red, Nile blue, malachite green, thionine, trypan blue, and 2,3,5-triphenyltetrazolium chloride proved unsatisfactory for our work.

## RESULTS AND DISCUSSION

Similar results were obtained when cultures of increasing age were stained with either FDS or EB (Fig. 1). Between days 14 and 22, the staining properties did not change. Thereafter, the degree of staining changed, and by day 34 minimal staining was observed for FDA and maximal staining for EB. Both of these results

<sup>1</sup> Supported by the National Institutes of Health Grant 5R01 AG01709-01.

<sup>2</sup> Abbreviations: FDA, fluorescein diacetate; EB, Evans blue; PS, phenosafranin.

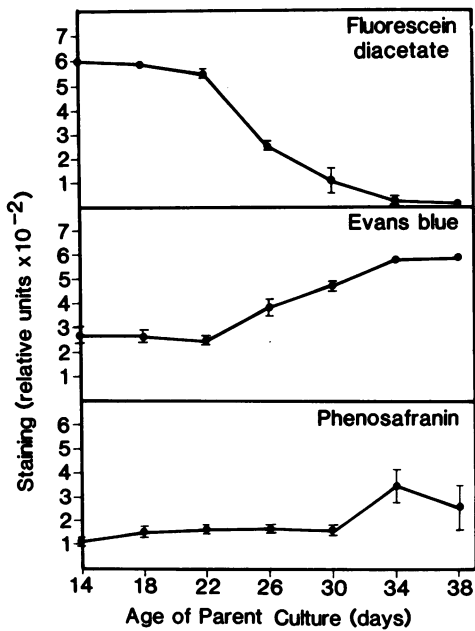


FIG. 1. Vital staining of cultures of increasing age. Each datum point is the total of relative staining values assigned to 200 clumps. Details of the analysis and computations are provided in "Materials and Methods." Vertical bars, SE.

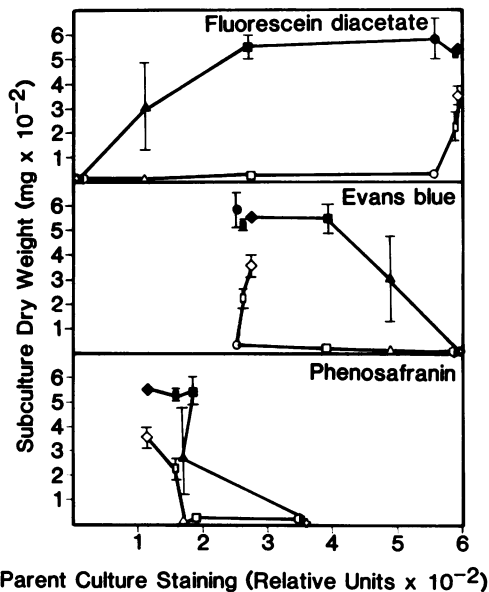


FIG. 2. Relationship between vital staining of parent cultures of increasing age and the dry weight of subcultures started from them. Dry weight values are the average of 20 subcultures; four from each of five parent cultures. Measurements were made after 9 d (open symbols) and 21 d (closed symbols) of subculture growth. Staining values and the vertical bars are described in Figure 1. Parent cultures are identified as: 14 d (◆), 18 d (■), 22 d (●), 26 d (■), 30 d (▲), 34 d (●), 38 d (⊕).

indicated that the cultures were dead since living cells absorb FDA and exclude EB (16). Staining with PS did not agree closely with the other stains. This was most apparent between days 22 and 30 when there was no change in the staining properties of PS-treated cells as compared to those treated with either FDA or EB. The decline in the PS staining between days 34 and 38 was also not in agreement with the other stains.

With all of the stains examined, the intensity of staining of individual cells within a clump diminished with increasing cell

age. This was an acute problem with PS. At the older ages, accurate judgments of stained *versus* nonstained cells could not be made with the PS-treated cells, and as a result large SE were observed on days 34 and 38 (Fig. 1). Thus, the staining discrepancies noted between PS and the other stains (Fig. 1) can be attributed to the difficulties we experienced with PS. For this reason, no further attention will be given in this paper to the PS results.

The staining properties of aging cultures did not correlate well with their ability to be subcultured. Parent cultures ranging from 14 to 22 d old stained approximately the same, but their subcultures grew at different rates during the first 9 d of subculture growth (Fig. 2). In contrast to this, parent cultures ranging from 22 to 30 d old stained differently, but subcultures started from them experienced approximately equal amounts of dry weight increase after 9 d of growth.

After 21 d of growth, subcultures started from four parent cultures (14-, 18-, 22-, and 26-d-old cultures) had all grown to about the same extent. The staining of these cultures with FDA and EB was similar except for the 26-d-old culture whose relative staining was only half that of the other three cultures. Vital staining of the 30-d-old cultures indicated that a large proportion of the cells were dead, and the poor growth of subcultures started from them supported this contention. Staining of the 34- and 38-d-old parent cultures indicated that these cultures were essentially dead, and the inability to subculture these cultures confirmed this observation.

A major point of interest in this study was that parent cultures with similar staining properties did not possess equal potentials for subculturing. Thus, cells which appeared to be viable, by virtue of their staining properties with FDA or EB, were not necessarily capable of dividing. Furthermore, this population of living-but-nondividing cells within a culture appeared to increase during senescence. Cells in this physiological condition have been observed in animal tissue cultures (2). Blomquist *et al.* (1) showed that the percentage of non-dividers rose from 18 to 73% between passages 11 and 40. After 40 passages, further subcultivation of the mass culture was impossible because the cells had lost their capacity to divide. Hayflick and Moorhead (8) have termed this condition as phase III of cell growth in animal tissue cultures. Additional work by Blomquist *et al.* (2) has shown that cells in the phase III condition remain alive for extended periods of time as nondividing cells.

We conclude that the vital stains EB and FDA can be used to determine when senescing cells die. However, during the course of senescence it appears that an increasing proportion of the cell population becomes living-but-nondividing cells. The stains do not distinguish cells capable of dividing from those which have lost their potential to divide. Therefore, caution should be exercised by individuals who use vital stains in evaluating the potential of protoplasts, cells, excised organs, etc., to undergo division and growth.

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