Isolation and Identification of the Phenols of Paul's Scarlet Rose Stems and Stem-Derived Suspension Cultures

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ABSTRACT

The phenols of Paul's Scarlet rose stems and stem-derived cell cultures have been analyzed using C18-reversed-phase high performance liquid chromatography.

Rose stems were found to contain gallic acid, (+)-catechin, (-)-epicatechin, the dimers (-)-epicatechin(+)-catechin and (+)-catechin(+)-catechin, a polymeric procyanidin, ferulic acid, and several gallotannins. In contrast, a cell suspension of Paul's Scarlet rose which has been maintained in culture for over 25 years contained only low levels of gallic acid and (-)-epicatechin(+)-catechin. The phenol content of a second rose cell line which was started from the same initial isolate in 1957, but which was maintained in a laboratory other than our own was quantitatively and qualitatively similar to the cell line kept in our laboratory for the last 20 years. A third cell line which we started 6 months ago contained a wide variety of phenols, most of which were in common with those of rose stems.

Selective subculturing of smaller cell clumps of our oldest cell line failed to enhance either the quantities or the diversity of phenols which accumulated in these cultured cells. Possible reasons for the failure of selective subculturing to enhance phenol levels in this long-established cell line are discussed.

Several investigators have studied the synthesis of phenols in cultures of Paul's Scarlet rose (1, 6, 7, 15, 16, 18); however, only tentative identifications were made of a few of the phenols, and no effort was made to compare the phenol content of cultured cells with that of intact rose plants. In our present research, we have separated and identified the major phenols present in cultured rose cells. Furthermore, we have made both a qualitative and quantitative comparison between the phenol content of our oldest cell line (maintained in this laboratory since 1965) with the phenol content of two other rose cell lines and rose stem tissue from which the cultures were started. We have also conducted studies with our oldest cell line to evaluate the influence of clump size on phenol accumulation.

MATERIALS AND METHODS

Plant Materials and Culture Conditions. Suspension cultures of Rosa sp. L., cv. Paul's Scarlet were grown in MPR media and harvested as described previously (21). Three separate cell lines were used in this study. Two of the cell lines (PSR No. 1 and PSR No. 2) are from the same original isolate, started by Dr. Walter Tuleecke in 1957 (19). PSR No. 1 has been maintained in our laboratory since 1965. PSR No. 2 was obtained from the laboratory of Dr. Abraham Marcus. This cell line was cultured under our conditions (21) for four successive transfer periods (a total of 8 weeks) before its phenolic content was characterized. PSR No. 3 was a newly established culture which was initiated in our laboratory in August 1982 from stem explants.

Sieved cultures were obtained by inoculating new media with 14-d-old cells which passed through either 250- or 500-μm screens, a procedure which has previously been used by Kinningsly and Dougall (13) and Ozeki and Komamine (20). The sieve pore sizes used in the study were determined empirically by microscopic examination of those cell clumps which passed through sieves of differing porosity. The porosity of the sieves used in this study (250 and 500 μm) were larger than those used in previous studies conducted with carrot cells, 63 μm by Kinningsly and Dougall (13) and 81 μm by Ozeki and Komamine (20). This difference is a reflection of the large size of rose cells in comparison to carrot cells.

Stem tissue used for phenol analysis was removed from a 14-year-old Paul's Scarlet rose bush. Tissue designated as 'young stem' in this report represents new spring growth removed from the tips of older canes, whereas 'old stem' tissue represents 0.5- to 1.5-cm diameter sections of stem, removed from lower portions of older canes which had experienced considerable secondary growth.

Isolation of Phenols. Phenols from either rose stem or suspension cultures were extracted into 70% (v/v) acetone (grinding ratio 1 g:2 ml) using a mortar and pestle. The grindings were centrifuged at 20,000 g for 10 min and the resulting pellets were discarded. The grinds were centrifuged at 20,000 g for 10 min and the resulting pellets were discarded. The extract was collected and evaporated to dryness. The extract was then analyzed by high performance liquid chromatography (HPLC).

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Phenol Analysis. Rose phenols were analyzed by reverse-phase liquid chromatography using a Waters Associates M-273 microprocessor controlled gradient HPLC system, equipped with a Waters M-480 variable wavelength detector and a Waters MicroBondapak C-18 column (30 cm x 4 mm i.d.). Separation was accomplished using the gradient protocol of Lea (17), with 5% acetic acid as solvent A, and methanol as solvent B. The flow rate was 1.5 ml/min, sample sizes were between 10 to 100 μl, and the chromatography was monitored by measuring the A at 280 nm of the column effluent. Quantitative chromatography was performed using small aliquots of rose phenol extracts in order to avoid any potential concentration effects (22).

Phenol Identification. Total phenols were quantified using the Folin-Ciocalteau reagent as described by Amorim, et al. (1). Gallic acid served as the standard.

Gallic acid, (+) catechin, and (-) epicatechin were identified in rose extracts by cochromatography with commercially available standards during HPLC. The procyanidin dimers epicatechin and cat-cat were identified in the same manner, using authentic standards kindly supplied by Dr. Edwin Haslam. ECGG and ECG were not available from commercial sources; therefore, these compounds were extracted from green (unfermented) tea leaves and separated and identified by paper chromatography (24). ECGG and ECG were then eluted from the paper chromatograms and cochromatographed with extracts of rose using paper chromatography (24) and HPLC (12). Vanillin/HCl (3) and ethylene diamine (4) were used as diagnostic reagents to detect procyanidins and gallotannins, respectively. A rose procyanidin polymer with (+) catechin as its terminal unit (23) was isolated using Sephadex LH-20 (5).

RESULTS AND DISCUSSION

Phenols of Paul’s Scarlet Rose Stem. Analysis of the phenols extracted from young stems of PSR showed the predominate constituents to be: gallic acid, the procyanidin dimer epicatechin, the gallotannin ECGG, ferulic acid, and an unidentified compound (Fig. 1a).

Compound I exhibited a positive reaction to vanillin/HCl and the ethylene diamine reagent which suggests that I is a gallotannin. Mass spectral analysis performed on the trimethylsilyl derivative of compound I yielded data which is consistent with our contention that this compound is a substituted flavan-3-ol.

In addition to the phenols found in younger stems, older stems contained the flavan-3-ol (-) epicatechin, a second procyanidin dimer (cat-cat), the gallotannin ECG, and a polymeric procyanidin (Fig. 1b). Although we were not able to make positive identifications of several of the peaks which elute near the void volume, it was shown that they yielded gallic acid upon hydrolysis.

Several of the compounds which we found present in stem tissue have been previously shown to be present in other tissues of rose. Epicatechin was identified as a major procyanidin component of rose leaves (23). Cat-cat (11) and a procyanidin polymer (10) have been isolated from rose hips. The presence of gallotannins in rose cells was suggested but not proven by Davies (6) when he showed that compounds extracted from cultured rose cells gave a positive reaction to ethylene diamine. It is also noteworthy that several phenols that had previously been shown to be present in rose leaves (14) were not present in rose stems. Phenolic acids which were not present included: ellagic, gentisic, caffeic, protocatechuic, p-hydroxybenzoic, p-coumaric, syringic, vanillic, and salicylic.

Phenols of PSR Cell Suspension Cultures. In sharp contrast to the diversity of phenols found in rose stems, only two compounds (gallic acid and the procyanidin dimer epicatechin) were present in extracts of PSR No. 1 cells harvested during early stationary phase (14-d-old, Fig. 2a). Also, the quantities of these two phenols in PSR No. 1 were much less than those found in other tissues (Fig. 2).

In addition to the analysis of PSR No. 1 cultures, the cell line which has been used extensively in this laboratory, we also analyzed two other rose cell lines (PSR No. 2 and PSR No. 3) for their phenol contents. PSR No. 2, was started from the same original isolate as PSR No. 1 but has been maintained in a
different laboratory than our own. PSR No. 3 was initiated in our laboratory from rose stem tissue 6 months prior to the analysis of its phenol content. HPLC profiles of these cell lines revealed that PSR No. 2 contained only gallic acid and epi-cat (data not shown), while PSR No. 3 contained a wide variety of phenolic compounds (Fig. 2b). The level of total phenols in the young cell line (PSR No. 3) is intermediate between that found in stems and in the established cell lines PSR No. 1 and No. 2 (Fig. 3). These results are consistent with the common report that most cultured plant cells have lower levels of secondary products than the plant organs from which they were started and that the levels of these compounds in cultured plant cells tends to diminish with increasing numbers of transfers (8). The phenolic content of PSR No. 2 is essentially identical with its ‘sister’ culture PSR No. 1 (Fig. 3) even though these two cell lines were maintained in different laboratories for over 20 years. It is interesting to note that all three cell lines have similar gallic acid and epi-cat levels, perhaps suggesting an upper limit for the quantity of these particular compounds which can be accumulated by cultured rose cells.

**Effects of Selective Subculturing on the Phenol Levels in PSR No. 1.** We have made the casual observation in our laboratory over a 15-year period of an apparent decline in the phenol levels of PSR No. 1, since this culture no longer becomes brown upon entering the stationary phase of growth. This apparent decline in the phenol levels of our cell cultures may have come about by an unconscious selection pressure against phenol-producing cells (13). It has been shown that the ability of carrot cell cultures to accumulate anthocyanin is reversible (9) and that a positive correlation exists between decreased clump size and anthocyanin production (13, 20). Recognizing that the early metabolic steps leading to the biosynthesis of phenols in rose cells are the same as those giving rise to the anthocyanins in carrot cultures, we have attempted to increase the phenol level of our old established line (PSR No. 1) to that of a newly started cell line such as PSR No. 3 by employing a selective subculturing technique. Four sublines of PSR No. 1 were started; two subcultured by sieving through 250-μm screens (one subline for four transfer periods and a second subline for five transfer periods), and two by sieving through 500-μm screens (one subline for four subculturing periods and another subline for 12 subculturing periods). Sieving failed to enhance, in either a quantitative (Fig. 3) or a qualitative manner (data not shown), the phenol content of the PSR No. 1 cultures. Thus, our results are in direct contrast to those of Kinnersey and Dougall (13) and Ozeki and Komamine (20) where sieving significantly enhanced the anthocyanin content of carrot suspension cultures. In their research with carrot cultures, Kinnersey and Dougall (13) have speculated that small cell aggregates produce more anthocyanins because they contain lower endogenous levels of cytokinins, which inhibit anthocyanin synthesis. If this is true, then the failure of rose cells to behave in a similar manner suggests that the endogenous cytokinin levels of sieved rose cells is still high enough to inhibit increased phenolic synthesis. Alternatively, the biosynthesis of tannins may not be regulated by cytokinins. A third possibility is that the PSR No. 1 cell line, which has been in culture for so many years, may have lost the genetic capability to produce the diversity and quantities of phenols which are found in stem tissues and in newly initiated cell lines (e.g. PSR No. 3).

The expression of desired genes by plant tissue cultures is a major problem which must be overcome to make tissue culture systems economically important as a source of plant secondary products (2). Our positive identification for the first time of the individual phenols in rose cultures permits this system to be used as a model for studying the metabolic regulation of the enzymes associated with secondary product formation in cultured cells.

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**LITERATURE CITED**

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