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A HIGH-YIELDING CALLUS CULTURE OF *RHAMNUS PURSHIANA* BY VISUAL SELECTION

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Physiological and morphological characteristics of individual cells in a callus or suspension culture are not always uniform. Callus cultures often show heterogeneity in consistency or color; on the other hand, homogeneous-looking callus parts sometimes segregate with different morphological appearance or different pigmentation.

Several cell lines that accumulate high levels of colored substances have been established by visual selection methods. Cells showing the desired coloration are picked out for further subcultivation until a pure line is obtained. In 1951, a *Daucus carota* callus line was isolated with a high production of *beta*-carotene (1). This high level was maintained for more than 10 years (2). High shikonin-containing strains of *Lithospermum erythrorhizon* were obtained by selecting intensively pigmented sectors of colonies (3). The commercial production of this red naphthoquinone from suspension cultures of *L. erythrorhizon* has been started by Mitsui Petrochemical Industries Ltd. (Japan) (4). It also seems that Japanese investigators are approaching commercialization of the yellow alkaloid berberine from plant cells (5). More reports on high-producing variant strains obtained by visual selection have been reviewed by Widholm (6) and Misawa (7).

The dried bark of *Rhamnus purshiana* DC. (Rhamnaceae), stored at least one year before use, is applied medicinally as a purgative. The main cathartic principles of the bark are known to be glycosides of 1,8-dihydroxyanthracene derivatives (8). The content of anthracene derivatives in callus cultures of *R. purshiana*, established and investigated at our laboratory (9), was rather low compared to the medicinal bark. An approach to enhance the production of anthra-derivatives by a visual selection procedure is described in this paper.

Initially, callus cultures of *R. purshiana* consisted of fragile tissue in which structure and color showed slight variations. Upon subcultivation, color and consistency of the callus changed gradually. After subcultivation for 300 days, the callus consisted of soft, friable tissue in which a green color was dominant. After 940 days, however, a yellow tissue with a somewhat more compact consistency was obtained. This yellow intensified from 940 to 1320 days. During the subcultivation period, the total content of anthracene derivatives fluctuated from 0.09 to 0.24% (w/w, dry w) (Figure 1).

After 1070 days, the yellowest parts of the source callus were picked for further selective subcultivation, and by repeating this every 5 weeks for the separately grown tissues, a callus was obtained with a markedly increased anthra-derivative content. High-yielding callus cultures obtained after eight passages (250 days) from the moment the selection procedure was started produced five times more anthracene derivatives (1.16% w/w, dry w) than did the source callus (Figure 1). Mainly glycosides of anthracene derivatives occurred in the high-yielding callus obtained after ten passages (310 days) of subcultivation. Only 5% of the anthra-derivatives occurred in the free state. The total content of anthra-derivatives in high-yielding cultures was about 50% of that present in the medicinal bark.

Figure 2 shows the contents of indi-

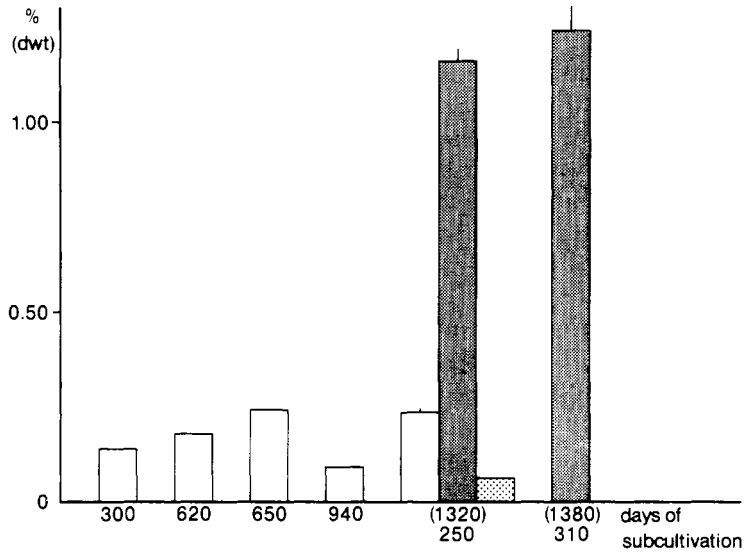

FIGURE 1. Total contents of anthracene derivatives in source callus, ▒ = high-yielding callus; ▒ = low-yielding callus

vidual anthraquinones determined by hplc in source callus and high yielding callus after 250 (1320) days. For both cultures the same qualitative pattern of anthraquinones was observed. More than 50% of the anthra-derivatives were present as glycosides of physcion and/or its reduced forms.

In the MeOH extract of the freeze dried, high-yielding callus a variety of glycosides was found. Physcion monoglucoside, chrysophanol monoglucoside, emodin monoglucoside (located at Rf 0.46) and aloe-emodin monoglucoside (Rf 0.38) were identified by combined two-dimensional tlc-enzymatic hydrolysis (9,12). Similarly, more polar glycosides of chrysophanol, physcion, and emodin located at Rf 0.20 were tentatively assigned as diglucosides.

Glycosides that could not be hydrolyzed with *beta*-glucosidase, including

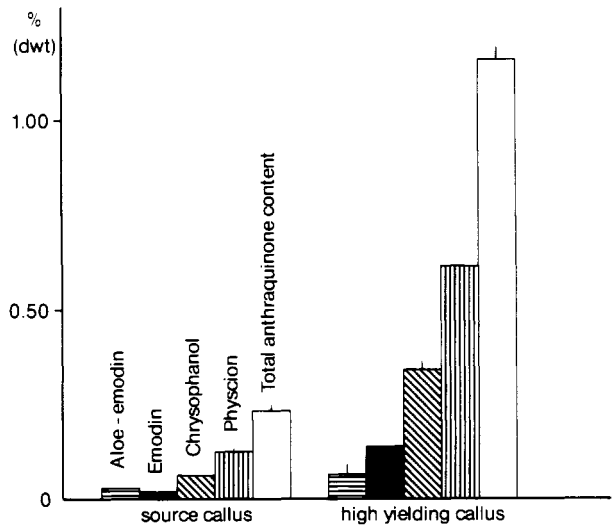

FIGURE 2. Contents of individual and total anthraquinones in source callus and high-yielding callus after 250 (1320) days of selective subcultivation

O-glycosides of chrysophanol, physcion, and emodin (located at Rf 0.12 and 0.28) and of chrysophanol and physcion (Rf 0.34) were found. *O*-Glycosides of chrysophanol dianthrone, chrysophanol physcion dianthrone, and physcion dianthrone were also found (located at Rf 0.15) and identified using a different tlc system for the separation of these glycosides as described in the experimental part. The *O*-glycosides were characterized by combination of two dimensional tlc with acid hydrolysis on the thin-layer chromatogram. Aloin, 11-desoxyaloin, and cascarosides, major constituents of the medicinal bark, could not be detected in the MeOH extract of the high-yielding callus. Also, in the source callus these *C*- and combined *C*- and *O*-glycosides had not been found (9).

Parallel to the high-yielding callus, low-yielding tissue cultures were also obtained. After eight passages (250 days) the total content of anthra-derivatives was determined to be 0.06% (w/w, dry weight) (Figure 1).

High-yielding callus showed a greater heterogeneity in color and structure than did the source callus and was also more compact. It could well be possible that further selective subcultivation would result in still higher yields. The dry-weight content of source callus and high-yielding callus was 3.6% and 4.1%, respectively. The low-yielding cultures consisted of faint yellow tissue that was homogeneous in color and structure and also more friable than was observed for the source callus. The dry-weight content was 3.5%. During a single passage of 5 weeks, a tenfold increase in fresh weight of the inoculum was observed for high- as well as low-yielding cultures. It was observed by fluorescence microscopy that in the high-yielding callus most of the cells accumulated anthracene derivatives located as droplets within the central vacuole. The droplets emitted a strong yellow or yellow-orange auto-fluorescence under uv excitation. In the low-yielding cultures, however, only part of the individual cells accumulated anthra-derivatives observed as yellow fluorescent droplets. Comparison of high- and low-yielding callus by light microscopy showed that in the high-yielding callus the individual cells were generally smaller and also more subcellular particles were present. Apparently, a higher level of subcellular organization and differentiation is correlated to an increased biosynthetic activity.

EXPERIMENTAL

PLANT CELL CULTURE METHODS AND SELECTION PROCEDURE.—Callus cultures of *R. purshiana* were obtained and subcultured as previously described (9). The amount of 1,8-dihydroxyanthracene derivatives in these callus cultures was determined by spectrophotometry (9) after 300 days and by hplc after 620, 650, 940, and 1320 days of subcultivation.

Selection of high-yielding callus was described in the text. High-yielding callus cultures obtained after 250 and 310 days of their selective subcultivation (and, respectively, 1320 and 1380 days of total subculturing) were phytochemically examined.

Low-yielding callus was obtained by a similar procedure, picking the most colorless parts for subcultivation. Low-yielding callus was subjected to quantitative hplc analysis after 250 days (1320 days of total subcultivation).

EXTRACTION PROCEDURE.—Freeze-dried, high-yielding callus (0.2 g) was extracted with 100 ml of MeOH under reflux for 1 h. The extract was concentrated to 5 ml under reduced pressure and subjected to tlc analysis.

IDENTIFICATION AND CHARACTERIZATION OF FRACTION CONSTITUENTS.—Identification of 1,8-dihydroxyanthracene aglycones and monoglucosides by tlc and tlc-spectrophotometry was performed as previously described (9,10).

Anthracene *O*-glycosides were characterized by combination of two dimensional tlc with acid hydrolysis on the thin layer plate. An extract of glycosides was chromatographed in the first direction as described for glycosides. After development, the zone containing glycosides was moistened with a solution of 25% HCl using a capillary glass tube. The tlc plate was then covered with a glass plate and heated at 110° for 15 min. The plate was dried and developed in the second direction by the tlc system as described for hydroxyanthracene aglycones. This method of analysis allows a characterization of the *O*-glycosides

of hydroxyanthracene derivatives by correlated chromatographic and chemical properties of each glycoside and its corresponding aglycone. A different tlc system was used, however, for the separation of *O*-glycosides of chrysophanol dianthrone, chrysophanol physcion dianthrone, and physcion dianthrone. To characterize these compounds the plates (Si gel 60) were developed in the first direction in a saturated chamber over a distance of 10 cm using a solvent system consisting of EtOAc-PrOH-H_2O (40:40:30).

QUANTITATIVE DETERMINATION.—Total anthracene derivatives in callus cultures (source callus) investigated after 620, 650, 940, and 1320 days and in high-yielding and low-yielding callus examined after 250 (1320) days of subcultivation, were determined quantitatively by the extraction and hplc procedure that were previously reported (11). For high-yielding callus investigated after 310 (1380) days free aglycone and glycoside content were determined separately. Freeze-dried, high-yielding callus (0.1 g) was extracted with MeOH. From the extract the solvent was evaporated, and the residue was dissolved in 7.5 ml of 70% MeOH. After addition of 75 ml of H_2O and 0.1 ml of 37% HCl, free aglycones were exhaustively extracted from the aqueous solution with Et_2O. The Et_2O solution was washed with H_2O, and the aqueous layers were added to the H_2O solution. The Et_2O solution was kept for the quantitative determination of free aglycones. The H_2O solution was neutralized with Na_2CO_3 (5% w/v). $FeCl_3$ was added at a concentration of 2.4% w/v, and the mixture was refluxed for 30 min. The mixture was cooled, concentrated H_2SO_4 was added at 1 M, and refluxing was performed for 1 h. After cooling, the mixture was exhaustively extracted with $CHCl_3$. The $CHCl_3$ solution was washed with H_2O and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure at 50°. The solution of the residue in 12.0 ml of MeOH-$CHCl_3$ (1:1) was used for hplc (glycosidic bound anthraquinones, anthrones, and dianthrones, obtained as anthraquinone aglycones). From the solution containing free aglycones, the Et_2O was evaporated. To the residue 50 ml of H_2O and 1.2 g of $FeCl_3$ were added, and the mixture was refluxed for 30 min. The mixture was cooled, 5 ml of concentrated H_2SO_4 was added, and refluxing was performed for 1 h. Aglycones were extracted with $CHCl_3$ as described above. The aglycone fraction was dissolved in 1.0 ml MeOH-$CHCl_3$ (1:1) for hplc analysis (free anthracene derivatives, obtained as anthraquinone aglycones).

Quantitative determinations were performed in duplicate. The determined values are expressed as a mean±S.D. and were evaluated by the Students *t*-test.

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