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INFLUENCE OF CULTURE CONDITIONS ON POLYPHENOL PRODUCTION BY *FAGOPYRUM ESCULENTUM* TISSUE CULTURES

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ABSTRACT.—Tissue cultures of buckwheat, selected for their anthocyanin content (red in color), were grown in vitro on supplemented Gamborg's medium. Optimal growth was obtained with 2 mg/liter 2,4-D and 30 g/liter sucrose, over a 20-day culture period in light or a 30-day period in darkness; the mass production was similar in both cases (80 mg dry wt per callus). Flavonoids and anthocyanins were mainly synthesized under light, with a correlation between amount produced and growth. Synthesis of catechins and procyanidins was less dependent on light conditions. The flavonoids, isoquercitrin, rutin, and hyperin, existed a low levels regardless of culture age. Levels of quercetin-3-rhamnogalactoside were higher, with an optimum reached on the 16th day (according to their low flavonoid synthesis capacity). Buckwheat tissue cultures are characterized by good catechin and procyanidin production.

Buckwheat leaves (*Fagopyrum esculentum* Moench and *Fagopyrum tataricum* Gaertn., Polygonaceae) are a potential source for the industrial extraction of rutin (1,2), which is used mostly in the form of H_2O-soluble hemisynthetic derivatives to prevent capillary fragility.

Cell cultures derived from hypocotyls, cotyledons, and leaves of these two species accumulate flavonoids such as rutin in much lesser amounts than the plant organs (3). In vitro cultures also produce anthocyanins, catechins, and procyanidins, whose pharmacological properties are similar (4,5). The two last classes of substances are either absent or not abundant in the plant. Tissue cultures derived from *F. esculentum* hypocotyls were selected on the basis of their red pigmentation and used to study polyphenol production. Preliminary trials on the efficiency of a few hormones (auxins, cytokinins) and sugars have prompted us to a more specific study on the effects of 2,4-D and sucrose in light and in darkness.

EXPERIMENTAL

TISSUE CULTURE.—Red calli were initiated from *F. esculentum* hypocotyl fragments (3). The culturing was performed in sterile plastic Petri dishes (90 mm diameter) containing 25 ml nutrient medium solidified by 0.7% agar (Biokar type E). The culture medium contained Gamborg mineral solution elements (6) per liter: sucrose (30 g), myoinositol (100 mg), casein hydrolysate (100 mg), pyridoxine (0.5 mg), nicotinic acid (0.5 mg), thiamine (0.1 mg), kinetin (0.5 mg), and 2,4-D (2, mg). The pH was adjusted to 5.6 before autoclaving. Five-year-old calli were transferred every 3 weeks (4 calli per dish; each 150 ± 20 mg fresh wt) and maintained under permanent light (16 $W \cdot m^{-2}$) at 22°.

These basic conditions were used to test a range of 2,4-D and sucrose concentrations, except for the light conditions (permanent light or darkness). Each experiment was made on 20 calli with 3 measurements. Mean values and standard deviations are given.

QUANTITATIVE ANALYSIS OF CATECHINS AND FLAVONOIDS.—Harvested calli were stored frozen. Calli (5 g) were washed and extracted twice with MeOH (2 × 50 ml) (24 h at 5°). Filtrates were concentrated under reduced pressure to obtain a fresh wt g/vol ml ratio of 1 to 10. An aliquot (20 ml) was adsorbed on 2 g of polyamide powder (Macherey Nagel MN CC6) washed before use by MeOH followed by distilled H_2O. After drying, this sample was poured on an MN CC6 polyamide column (2 × 22 cm) washed in the

same manner. Phenolic acids and anthocyanins were eluted by H_2O, then catechins and flavonoids by MeOH.

Reversed-phase lc was performed on Si gel Lichrosorb RP18 Merck, 5 μm (column dimensions 4.6 × 150 mm) with 20-μl samples (Figure 1). Solvent A: MeOH-H_2O-HOAc (5:90:5). Solvent B: MeOH-H_2O-HOAc (90:5:5). Elution at flow rate of 0.8 ml/min was performed with a linear gradient of 0 to 35% B in A. Detection = 280 nm from 0 to 20 min, then 360 nm.

Internal standards (Extrasynthese) were used for quantitation: dihydrofisetin and naringenin were used for catechin and flavonoid evaluation, respectively. Aborbances were corrected according to the absorbance values of (+)-catechin at 280 nm and rutin at 360 nm. The global values were the total of 3 catechin and 4 flavonoid peaks. The results are given as mg/g dry wt of (+)-catechin and rutin.

IDENTIFICATION OF CATECHINS AND FLAVONOIDS.—Catechins and flavonoids were identified by comparison with authentic samples (Extrasynthese): (+)-catechin, (−)-epicatechin, rutin, hyperin (quercetin-3-galactoside), and isoquercitrin (quercetin-3-glucoside). Quercetin-3-rhamnogalactoside was identified by comparison with literature data (7).

The MeOH eluate from a polyamide column as described above was obtained from 2500 g calli. Flavonoid bands were initially separated by pc (Whatman 3 MM) using 15% HOAc and *n*-BuOH–HOAC–H_2O (4:1:5). Products were finally isolated by preparative tlc on Merck Si gel using EtOAc-HOAc (10:4) or on Merck cellulose using 75% phenol. Residual phenol was eliminated by H_2O on pc. All the MeOH eluates were then purified by elution on a Sephadex LH20 column, giving the following substances: quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoglucoside, and quercetin-3-rhamnogalactoside.

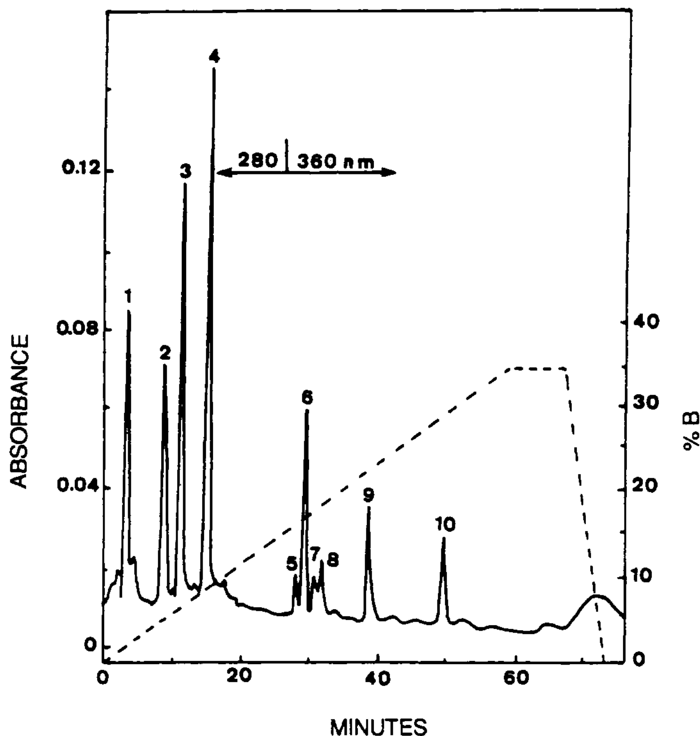

FIGURE 1. Separation of flavonoids and catechins from *Fagopyrum* callus cultures by gradient elution on Lichrosorb RP 18 with solvent B [MeOH-H_2O-HOAc (90:5:5)] in solvent A [MeOH-H_2O-HOAc (5:90:5)]. Flow rate 0.8 ml/min. Gradient is shown by dotted line. Peaks: 1 = (+) catechin; 2 = (−) epicatechin; 3 = dihydrofisetin (internal standard); 4 = unknown catechin; 5 = hyperin; 6 = quercetin-3-rhamnogalactoside; 7 = isoquercitrin; 8 = rutin; 9 = unknown; 10 = naringenin (internal standard).

Characterization was then made by means of uv spectrophotometry in MeOH solution with usual reagents (8): $AlCl_3$, $AlCl_3 + HCl$. NaOAc, NaOAc + H_3BO_3, NaOH.

Determination of aglycones and sugars of the four compounds was performed using a direct acid hydrolysis method on Si gel thin layer plates by HCl vapors at 105°, followed by 2D chromatography according Kartnig and Wegschaider (9), using quercetin, glucose, galactose, and rhamnose as standards (Extrasynthese).

Pure eluates of quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rhamnoglucoside were compared to commercial standards. Quercetin-3-rhamnogalactoside was not available.

QUANTITATIVE ANALYSIS OF ANTHOCYANINS AND PROCYANIDINS.—Colorimetry was used, according to Troyer (10).

The crushed frozen calli were extracted 24 h at 5° by MeOH-12 N HCl (5:1). The filtrate was adjusted with solvent to a 1:10 ratio (fresh wt g/vol ml). A first aliquot was taken and absorbance measured at 535 nm, using a cyanidin-chloride solution as a reference (Extrasynthese) (=anthocyanins). A second aliquot was heated in an H_2O bath, 15 min at 100 °, cooled, and adjusted to the initial volume by MeOH/HCl. Absorbance was measured at 535 nm, against the same standard solution (=anthocyanins + procyanidins). The amount of anthocyanins and of procyanidins is given in cyanidin-chloride equivalents (mg/g dry wt).

RESULTS AND DISCUSSION

EFFECTS OF 2,4-D.—Without 2,4-D, there was no cell proliferation (see Figure 1). Optimal growth occurred within a range of 2 to 7 mg/liter concentrations, without any noticeable effect on the different phenolic contents. In order to reduce 2,4-D concentration to a minimum while preserving appropriate growth, we chose a concentration of 2 mg/liter.

SUCROSE EFFECTS.—Sugar was necessary for growth. The optimal concentration was between 30 and 40 g/liter (Figure 2). With higher concentrations, growth decreased and browning occurred. Sucrose did not stimulate polyphenol synthesis but did inhibit it at concentrations higher than 30 g/liter. Sucrose at 30 g/liter was chosen as a standard condition.

GROWTH KINETICS.—Standard medium was supplemented with 2 mg/liter 2,4-D and 30 g/liter sucrose. Growth of calli cultured in light increased regularly until the

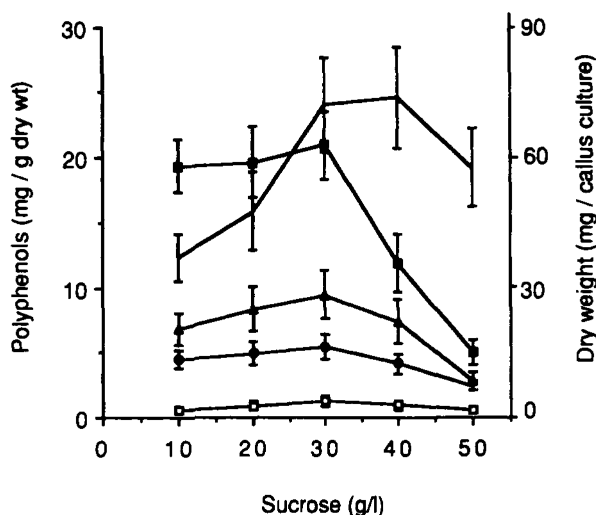

FIGURE 2. Effect of sucrose concentrations on growth (—) and polyphenol formation by *Fagopyrum* callus cultures cultivated for 21 days in continuous light on a standard B_5 medium containing 2 mg/liter 2,4-D: flavonoids (-⊟-), catechins (-▲-), procyanidins (-■-) and anthocyanins (-●-).

20th day, then decreased (Figure 3). A similar curve was obtained in darkness but with an optimum reached 10 days later. Maximum dry wt was similar in both cases.

POLYPHENOL KINETIC ANALYSIS.—Polyphenol amounts in calli were determined every 4th day up to the 36th day, either in light or in darkness. Results were variable according to the phenolic class.

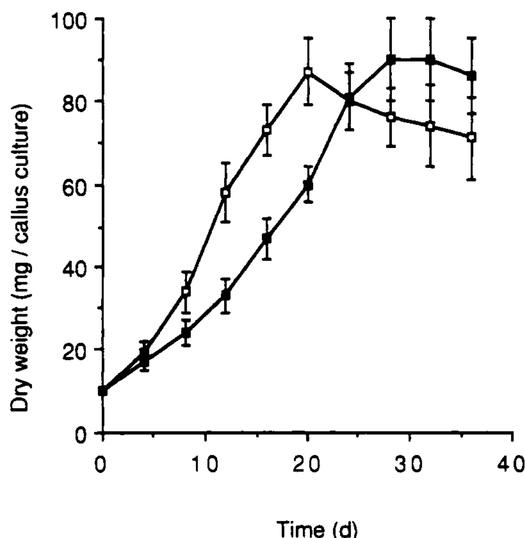

FIGURE 3. Growth of *Fagopyrum* callus cultures grown in continuous light (-□-) or in darkness (-■-) on a standard B_5 medium containing 30 g/liter sucrose and 2 mg/liter 2,4-D.

Anthocyanins were nearly absent in the calli grown under darkness (Figure 4B), and their amount increased in light between the 4th and 8th day of culture 3.3 and 6.5 mg/g dry wt, respectively, and stabilized until the 24th day before decreasing.

The flavonoid content was low in calli grown in darkness (Figure 4A). In light-grown tissues, the content increased from the 4th to the 16th day 0.42 and 0.80 mg/g dry wt, respectively, stabilized until the 24th day, and then decreased.

Catechins were synthesized at significant amounts either in light or in darkness. Optimum production was obtained between days 12 and 28, with more fluctuations in production in light (Figure 4C).

Procyanidins were the most abundant phenolics in buckwheat tissue cultures, in light or in darkness (Figure 4D). Content changes were slight in darkness and did not depend on the culture time. The optimum accumulation for light-grown calli reached 24 mg/g dry wt at day 24. It was shown in vivo by Troyer (10) and Margna *et al.* (11) that procyanidin synthesis is little influenced by light.

FLAVONOID KINETIC ANALYSIS.—Independent of individual compounds, flavonoid content was low in dark-grown tissues (Figure 5). In light, the isoquercitrin

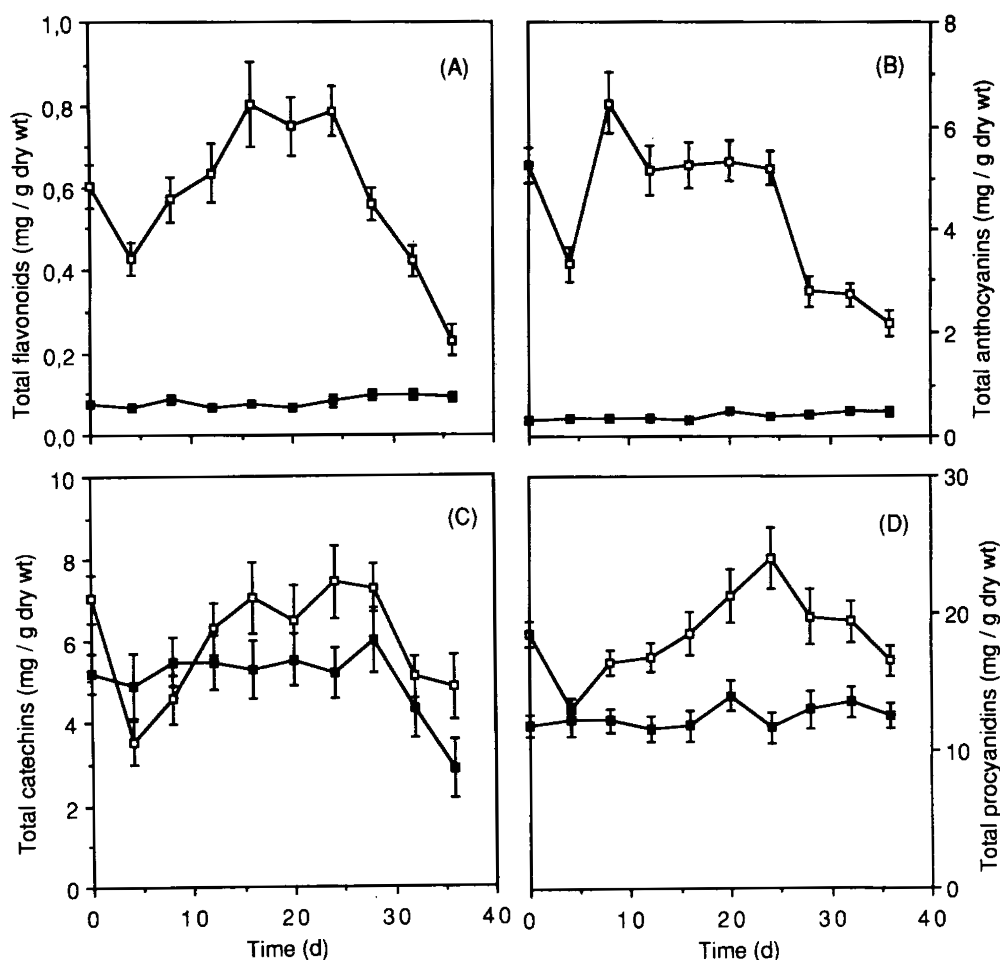

FIGURE 4. Flavonoid (A), anthocyanin (B), catechin (C), and procyanidin (D) formation in *Fagopyrum* callus cultures grown in continuous light (–□–) or in darkness (–■–) on a standard B_5 medium containing 30 g/liter sucrose and 2 mg/liter 2,4-D.

values were similar to values observed in darkness; the rutin and hyperin contents, though superior, were low. Quercetin-3-rhamnogalactoside, detected in vivo by Krause (7), was the major flavonoid in calli, with optimum accumulation at the 16th day (0.5 mg/g dry wt). Light stimulation on flavonoid synthesis was already observed in vivo in buckwheat seedlings by Margna (12).

In conclusion, tissue cultures derived from *F. esculentum* hypocotyls grew in continuous light on a medium containing 2 mg/liter 2,4-D and 30 g/liter sucrose. Phenolic contents generally increased during the active phase of growth, the anthocyanin synthesis beginning sooner. In comparison to the in vivo tissues, our experiments showed that the in vitro flavonoid synthesis was weak. This was also observed for other plant species by Arens *et al.* (13). Absence of light did not seem to be a limiting factor for catechin and procyanidin production. The procyanidins are found in some medicinal plants and are used in vascular protective drugs (4, 5). Their presence in buckwheat tissue culture suggests further study.

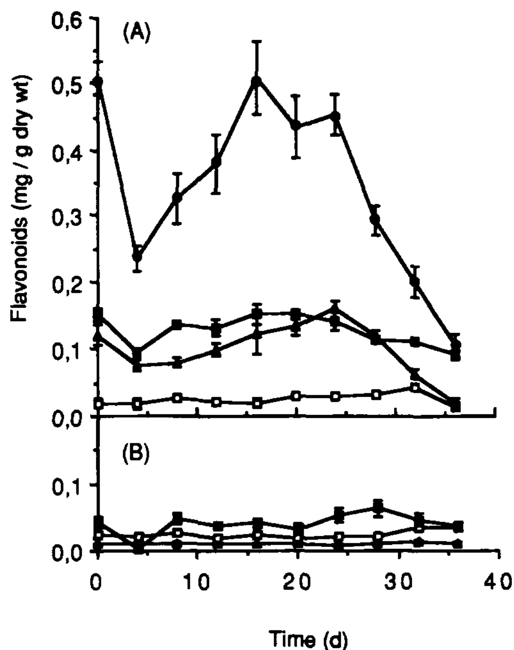

FIGURE 5. Changes in the formation of various flavonoids: isoquercitrin (-⊟-), hyperin (-▲-), rutin (-■-), and quercetin-3-rhamnogalactoside (-●-) by *Fagopyrum* callus cultures during the growth cycle in continuous light (A) or in darkness (B) on a standard B_5 medium containing 30 g/liter sucrose and 2 mg/liter 2,4-D.

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