Growth and photosynthesis of photoautotrophic callus derived from protoplasts of *Solanum tuberosum* L.

C. Bladier and P. Chavardieff

Summary. We describe a photoautotrophic culture procedure of potato (cvs Kennebec, Haig, DTO-33) callus derived from mesophyll protoplasts. The protoplast culture was initiated at very low concentration of glucose (down to 0.25 g l⁻¹). Callus was subcultured under CO₂ enriched air and glucose was suppressed by the successive dilutions with glucose free media. Regeneration was successfully obtained under photoautotrophic conditions. The characterization of oxygen exchange and of some enzymes and metabolites of carbon assimilation indicated that chlorophyllous callus, grown on carbohydrate free medium, developed the photosynthetic pathway typical of C3 plants. By comparing the fresh weight of callus cultivated in the light or in non-photosynthetic conditions (in darkness or in the light +3-(3,4-Dichlorophenyl)-1,1-dimethyleurea) we concluded that growth depended to about 70 to 88 % on photosynthesis.

Materials and methods

Plant material. Ten tetraploid clones of *Solanum tuberosum* L. have been tested : cvs BF15, Denali, Désirée, DTO-33, Panette, Haig, Institut de Beauce, Kennebec, Serrana, Superior. They were cultured in test tubes on medium containing MS salts (Murashige and Skoog 1962), 1.5% sucrose, vitamins from Morel and Wetmore (1951) with 0.8% agar (Gum Agar, Sigma). Tubes were incubated in a growth chamber (25°C) with a daylength of 18 hours (125 µmol m⁻² s⁻¹; Grolux Sylvania fluorescent / cool white tubes, 1/1).

Protoplast isolation and culture. Protoplasts were isolated from the upper leaves of 4 week old plantlets. About one gram of plant material was sliced into 1 mm strips and placed for one hour in a Petri dish containing 20 ml of hypotonic medium (MS salts, Morel and Wetmore vitamin mixture, 1 mg l⁻¹ 2,4-D, 0.4 mg l⁻¹ BAP, 2 g l⁻¹ glucose, 72 g l⁻¹ mannitol, pH 5.8.). After 16 hours of dark incubation at 28°C, the protoplasts were filtered through a 100 µm steel mesh and washed twice with the culture medium by centrifugation at 800 rpm for 5 min. The pellet was then resuspended in the same medium. The protoplasts were counted in a haemocytometer and diluted to 5 x 10⁴ protoplasts ml⁻¹. The plating efficiency was defined as the number of callus as a percentage of the initial number of cultured protoplasts. Protoplasts and callus cultures were carried out in modified VKM medium (Bokelmann and Roest 1985), regeneration and rooting on modified carbohydrate free MS medium.

*O₂ exchange measurements. Oxygen exchange rates of chlorophyllous callus were determined using the mass-spectrometric ¹⁶ O₂:¹⁸ O₂ isotope technique (Dixon et al. 1984). The combination of these two techniques was successfully used in the isolation of photosynthetic variants of *Nicotiana plumbaginifolia* (Rey et al. 1990). The circuit was first sparged with air. After completion of the sparging, 2 ml of ¹⁸ O₂ (98.6 ¹⁸ O₂ atom % from Eurisotop, France) were injected into the closed circuit. The gas circuit was connected to a mass spectrometer (MM 14.80, V.O. Instrumenta). The variations in gas concentration were followed by measuring the mass-peaks m/z=32 (¹⁶ O₂), m/z=36 (¹⁸ O₂) and m/z=40 (argon used as internal reference gas) in the ion source of the mass spectrometer. Gas exchange was measured under three different experimental conditions (25 min each) :
(i) in the light (150 or 800 μmol m⁻² s⁻¹) under air; (ii) at the same light intensities at saturating CO₂ concentration (1%); (iii) in darkness. Light was supplied with a 120 W metal halide lamp (Osram).

**Biochemical characterization.** The initial (non activated) and total (HCO₃⁻/Mg²⁺ activated) RubPCase activities were assayed as previously described (Rey et al. 1990) by determining the amount of ^14CΟ₂ incorporated into acid stable products. PePCase activity was measured in a similar manner, 2.1 mM phosphoenolpyruvate, 0.35 mM malate dehydrogenase and NAD replacing the ribulose 1,5-bisphosphate in the reaction mixture.

Chlorophyll and protein content were determined on aliquots of the crude extract used for RubPCase and PePCase assays. Chlorophyll a and b were extracted in an 80/20 acetone water mixture and determined according to the method of Lichtenthaler and Wellburn (1983). Soluble protein concentrations were measured spectrophotometrically by the Bradford method (1976). Bovine serum albumin was used as a standard.

For malate content, about one g of callus was homogenized in 5 ml of 5% (v/v) perchloric acid and incubated one night at 4°C. The mixture was neutralized using 5 M potassium bicarbonate and centrifuged for 10 min at 2000 rpm. The malate content of the supernatant was measured spectrophotometrically according to Möllering (1985).

Determination of starch and sucrose content was carried out following the protocol described by Rey et al. (1990). Aliquots were incubated with amyloglucosidase (EC 3.2.1.1., Sigma) for starch and with β-fructosidase (EC 3.2.2.2, Sigma) for sucrose. Glucose and fructose resulting from hydrolysis were measured enzymatically (Bergmeyer et al. 1974; Bernt and Bergmeyer 1974).

### Results and discussion

#### Culture protocol

We established a protocol for photoautotrophic growth and plantlets regeneration of protoplast derived callus of *Solanum tuberosum* using some modified procedures from Bokelmann and Roest (1983) and Rey et al. (1989).

The composition of the different culture media are presented in Table 1.

The main steps of the protocol are:

**Day 1-7:** The freshly isolated protoplasts (20-50 μm in diameter; 3-6 x 10⁶ per g of fresh weight) were cultured in 3 cm diameter plastic dishes containing 3 ml of modified VKM medium (Bokelmann and Roest 1983) adjusted at an osmolality of 540 mOsm kg⁻¹ with mannitol and a glucose concentration varying from 0.25 to 2 g l⁻¹. The Petri dishes were sealed and incubated at 25°C in the dark or in low light (50 gmol m⁻² s⁻¹). Around the fifth day, the first divisions were observed and chloroplasts turned brown.

**Day 8-14:** The suspension was diluted twice with the same liquid medium as above but without sugar and adjusted at an osmolality of 450 mOsm kg⁻¹ with mannitol and a glucose concentration varying from 0 to 2 g l⁻¹. The cells were then transferred to unsealed 9 cm diameter Petri dishes and placed in plexiglass cabinets flushed with moistened 2% CO₂ enriched air and illuminated 18 hours per day with a photon flux density of 125 μmol m⁻² s⁻¹. Around the fifteenth day, the first divisions were observed and chloroplasts turned brown.

**Day 15-29:** The developing cell aggregates were cultured another fortnight after dilution with an equal volume of a semi-solid (0.4% Gum Agar) modified VKM medium adjusted at an osmolality of 340 mOsm kg⁻¹ with 45 g l⁻¹ mannitol and without glucose, VKM sugar and 2,4-D.

**Month 2-3:** Small green callus (1-2 mm diameter) were transferred, about 20-30 per dish, on growing medium (previous medium at 0.4% Gum Agar). After 2-3 months, they reached a size of 0.5-1 cm diameter.

### Table 1: Composition of the different culture media.

<table>
<thead>
<tr>
<th>Day</th>
<th>MINERALS:</th>
<th>CARBOHYDRATES:</th>
<th>VITAMINS:</th>
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<tbody>
<tr>
<td></td>
<td>VKM salts</td>
<td>VKM sugars and sugar alcohols</td>
<td>VKM vitamins</td>
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<tr>
<td>1-7</td>
<td>+</td>
<td>+</td>
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<tr>
<td>8-14</td>
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<td>+</td>
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<tr>
<td>15-29</td>
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<td>+</td>
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</table>

**Day 1-7:**

- **GROWTH REGULATORS:**
  - 2,4-D (mg l⁻¹): 0.2
  - NAA (mg l⁻¹): 1
  - ZEA (mg l⁻¹): 0.5
  - GA3 (mg l⁻¹): 0.5
  - IAA (mg l⁻¹): 0.5

**Day 8-14:**

- **GROWTH REGULATORS:**
  - 2,4-D (mg l⁻¹): 1
  - NAA (mg l⁻¹): 0.01
  - ZEA (mg l⁻¹): 0.5
  - GA3 (mg l⁻¹): 0.5
  - IAA (mg l⁻¹): 0.1

**Month 3-4:** Regeneration was carried out by subculturing callus (10 callus per dish) on solidified (0.8% Gum Agar) medium containing MS salts, 1 mg l⁻¹ ZEA, 0.01 mg l⁻¹ NAA and GA₃ and adjusted at an osmolality of 250 mOsm kg⁻¹ with 14 g l⁻¹ mannitol.

**Month 6-7:** Adventitious shoots approximatively 1 cm long were excised from the callus and rooted in test tubes flushed with moistened 2% CO₂ enriched air on semi-solid (0.6% Gum Agar) medium containing only MS salts and 0.1 mg l⁻¹ IAA. Root formation occurred within two weeks.

#### Varietal effect

The successful initiation of the protoplast culture depended on the cultivars tested.

The protoplasts of the cvs Denali, Institut de Beaubais, Désirée lyed after two or three days of culture, those of cv BF15 did not divide and those of cvs Fannette, Serrana and Superior started a few divisions that stopped after the first dilution. Taking into account these observations, some modifications of the culture protocol (osmotic pressure adjustment, different medium composition or sequences of dilution) should be defined for successful photoautotrophic culture of each cultivar, in the same manner as for heterotrophic cultures (for example on cv BF15, Laine and Ducrœux 1987).

Three cultivars (cvs Haig, Kennebec, DTO-33) succeeded in developing cell aggregates, green callus and...