Nutritional Requirements for Growth of *Vicia hajastana* Cells and Protoplasts at a Very Low Population Density in Liquid Media*

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**Summary.** When *Vicia hajastana* Grossh. cells or protoplasts were cultured at a high population density (ca. 5000 cells or protoplasts/ml), they were able to grow in a mineral-salt solution supplemented with sucrose (or glucose), a few vitamins, and 2,4-dichlorophenoxyacetic acid. They were not able to survive when cultured at a low population density unless the medium was supplemented with zeatin, naphthalene-1-acetic acid, nucleic-acid bases, amino acids, other sugars, sugar alcohols, and organic acids. *Vicia* cells were able to grow at an initial population density of 25–50 cells/ml in this defined medium. The population density could be lowered to 1–2 cells/ml with good growth when the mineral-salt medium was enriched with organic acids, sugars, sugar alcohols, coconut water, and casamino acids. The protoplasts also grew best in a medium enriched with these supplements. Three individual protoplasts were isolated and each one was cultured in a separate dish containing 4 ml of this medium. Within 30–40 days, each one had grown indefinitely and formed a mass of cells (ca. 10⁷).

**Introduction**

A very high initial population density (ca. 10000 cells/ml) was required for growth of plant cells in a mineral-salt liquid-culture medium. The initial population density could be lowered to 1000–2000 cells/ml if conditioned medium, casein hydrolysate, or free amino acids were added to the culture medium (Stuart and Street, 1969, 1971). In order to isolate desirable types of cells such as mutants or heterokaryocytes, further reduction of the initial cell population density is desirable. Various organic compounds such as glucose, amino acids etc. can be absorbed and/or released by plant cells because of the permeability of the cell membrane (Stuart and Street, 1971; Sargent and King, 1974; Ruesink, 1973; Maretzki *et al.*, 1974). The inability of the plant cells to grow at a very low population density may be caused by excessive diffusion of metabolic intermediates into the medium, resulting in their dilution in the cell to a level below that required for survival (Ham, 1973). If this is the case, the cells should be able to grow at a very low initial population density in a medium enriched with the appropriate metabolic intermediates. The object of this study was to develop such a medium for *Vicia hajastana* Grossh. cells and protoplasts.

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Material and Methods

Vicia hajastana Grossh. cell suspension cultures, 1–2 days old and grown in Medium 1 (Kao et al., 1974) were used as the source of cells. The suspension culture consisted of single cells and clusters of up to 200 cells. When the population density was below 50 cells/ml, only cells and cell clusters which were able to pass through a 125-μm mesh-size stainless-steel filter were used. The average number of cells per cluster in the filtrate was 10 cells, ranging from 2 to 20 cells. The cell numbers were determined in two ways: (1) by calculation from packed cell volume divided by average cell size; (2) by direct counting using an inverted microscope.

Vicia protoplasts were obtained in the following way: 1 ml of 1-day-old suspension culture and 1 ml of an enzyme solution consisting of 7 mM CaCl₂, 0.7 mM NaH₂PO₄, 0.35 M sorbitol, 0.35 M mannitol and 3 mM MES [2-(N-morpholino)-ethanesulfonic acid] buffer, 2% Onozuka cellulase P 1500 (Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan), 2% Rhozyme HP 150 (Rehm and Haas Co. Canada, Ltd., West Hill, Ontario and 1% Sigma pectinase (Sigma Chemical Comp., St. Louis, Mo. USA) (pH 5.6; KOH) were mixed in a 60 × 15 mm Petri dish (Falcon) and incubated for 6 h at 24°. At the end of the incubation period, the protoplasts were passed through an 80 μm filter. The filtrate was centrifuged at 50 × g for 6 min. The supernatant was removed with a Pasteur pipette, and the protoplasts were resuspended in 10 ml of protoplast culture medium (Tables 1 and 3B) and again centrifuged. After the fourth washing, the protoplasts were resuspended in the same protoplast culture medium at various cell densities (Kao et al., 1973).

The mineral salts and various additives used for the media are listed in Table 1. All the media were filter-sterilized. The cells were cultured in plastic Petri dishes (Falcon or Kimble) in a thin layer of liquid (4 ml per 60 × 15 mm dish), incubated (24°) in diffuse cool-white fluorescent lamps (45 lux × 10 h) (40 W., Canadian General Electric, Winnipeg, Manitoba) inside a plastic box which served as a humidity chamber. The protoplasts were cultured either in a thin layer of liquid as were the cells, or in 100 μl drops as described previously (Kao et al., 1973). Unless otherwise stated, the experiments were repeated at least 3 times.

Results

I. Cells (Table 2)

Although Vicia cells cultured at a high population density were able to grow in a mineral-salt medium supplemented with sucrose, glucose, vitamins and 2,4-dichlorophenoxyacetic acid (2,4-D), or 2,4-D plus zeatin and 1-naphthaleneacetic acid = NAA (Media 1 and 2, Table 2B), they were not able to survive when they were cultured at a low population density unless the medium (Medium 2) was supplemented with amino acids, nucleic acid bases, other sugars, sugar alcohols, and organic acids of the tricarboxylic-acid cycle (Medium 6). The minimum cell density required for growth in this medium was 25–50 cells/ml. The population density could also be lowered considerably when the mineral-salt medium (Medium 3) was supplemented with Bacto vitamin-free casamino acids (Difco Lab., Detroit, Mich., USA) and coconut water (Medium 7). The number of cells per ml required for growth in this medium was 50–250. The population density could be further lowered when Medium 7 was enriched with sugars, sugar alcohols and organic acids (Medium 8). We were able to grow 1–2 cells/ml in this medium. The time required to grow from a few cells to ca. 10⁷ cells in 4 ml of this medium was between 20 and 30 days.

Only slight gain was observed when only free amino acids and nucleic-acid bases were added to Medium 2 (Medium 5). This was also true when only organic acids or organic acids in combination with the sugars were used to enrich Medium 2 (Media 3 and 4).