NUTRITIONAL AND KARYOTYPIC CHARACTERIZATION OF A HAPLOID CELL CULTURE OF DAUCUS CAROTA

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SUMMARY

The purpose of this study was to optimize growth conditions for a strain of haploid carrot callus and to follow its karyotypic changes in a long span of time. The strain has been maintained in liquid suspension since September 1977. It has remained predominantly haploid in its karyotype since that time. The original explant was initiated and subsequently subcultured in Gamborg's B5 medium. The components of the B5 medium were omitted one at a time and sequentially added back to determine their minimum, optimum, and maximum nontoxic concentrations. These changes were made in the original formula: the addition of an organic buffering agent and an increase in the iron and other micronutrient concentrations. Using this slightly modified B5 medium, we assessed the effect on growth by single additions of amino acids, different carbon sources, growth regulators, and vitamins. No improvement in plating efficiency resulted from addition of any of these compounds. We conclude that there are factors limiting the plating efficiency of the haploid cells other than these tested, or that single additions will not make a discernible difference, or that growth promoting factors cannot be exogenously supplemented to cultured cells.

Key words: haploid; carrot culture; low density plating; karyotype stability.

INTRODUCTION

Since 1970, when Carlson reported the isolation of leaky autotrophs from tobacco cell cultures, much effort has been devoted to mutant isolation from a variety of cultured plant cells (1-3). Generally, mutant production from plant tissue cultures has not fulfilled the optimistic expectation projected 10 yr ago. The incidence of genetic markers recovered, such as antimetabolite resistance, occurs spontaneously at a frequency of $10^{-4}$ to $10^{-7}$ (2), a rate which is 10- to 100-fold less frequent than observed in whole plants (4).

One possible explanation for this lack of success is the population density dependency that is particularly characteristic of plant cell cultures. In most cases, the population density must be $10^5$ to $10^6$ cells/ml in order for normal growth to occur. All growth stops when the population is diluted below these critical levels. Mutant selection is dependent upon the isolation of the rare, unique individual in a population and its ability to grow back to a usable amount of material. This bottleneck can sometimes be circumvented if the dying wild type cells, killed by a selective process, can furnish the necessary nutrients for the few surviving cells. In most cases, however, the selection must rely on laborious and time consuming testing because of this limitation.

There are many advantages in the use of the carrot tissue culture system, and they have been applied to somatic genetic studies, i.e., mutant isolation (5) and protoplast fusion (6). Primarily its greatest advantage lies in the lasting embryogenic potential and ease of cell dissociation. In

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1977, a callus was initiated from the hypocotyl of a haploid carrot in Dr. J. Straub's lab (personal communication). This callus has maintained a steady state of haploidy (between 70 to 90%) since its initiation and is capable of regenerating haploid plants. This cell line could offer great promise for the selection of recessive mutations, but the density dependency of the suspension cultures of this cell line can be limiting to growth.

In these studies, we attempted to lessen the density dependency by testing a variety of single additions to see if anything could stimulate the growth promotion supplied by the densely growing cultures. We did this by suspending single cells and very small clumps in a thin layer of agar at a density that would be on the borderline of survival. These isolated cells should be very responsive to changes in the exogenous medium and could furnish a sensitive and quantitative assay for the promotion of growth.

**MATERIALS AND METHODS**

The culture was initiated from a haploid carrot, *Daucus carota* L. variety "Juwarot," isolated as a twin seedling by Ms. Truckenbrodt in 1973 and was kindly supplied by Dr. J. Straub. The suspension culture was initiated in September, 1977, in our laboratory by transferring the callus to liquid B5 medium (7) supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-DD) and placed on a 60 c/min rotary shaker under continuous light at 27 °C. A friable cell line designated HA with a mass doubling time of 3.5 d, as measured turbidimetrically (8), was established. It was subcultured biweekly by dilutions of 1:10 with fresh medium. Upon transfer to auxin-free medium, HA underwent somatic embryogenesis. Flowering plants of normal morphology have been regenerated.

To test the effects of single additions of the compounds on growth, a modified Bergmann thin-layer plating technique was used (9). The B5 formulae were made by deleting one component at a time. Cells to be used in the experiment were grown for 1 wk prior to plating in deficient medium to deplete storage pools in the cells. The "starved" cells were collected by serially filtering through nylon sheets with pore sizes of 500, 200, and 53 μm. The population collected in this manner was comprised of 90% single cells and 10% small clumps consisting of 2 to 10 cells. The cells and clumps were collectively referred to as colony-forming units (cfu). Cell counts were done by serial dilution and direct microscopic observations. These HA cells, when diluted to $4 \times 10^5$ cfu/ml, resulted in a 2% plating efficiency. If diluted to $1.5 \times 10^5$ cfu/ml, the plating efficiency dropped to 0.22%, and at $10^5$ cfu/ml or less the plating efficiency was zero. These same cells, when supplied with a feeder layer, will yield 40 to 50% plating efficiencies at cell densities less than 100 cfu/ml (unpublished data). For our assay, cells were suspended at $2.5 \times 10^5$ cfu/ml in half the intended volume of B5 medium, and an equal volume of B5 containing 1% agar was gradually added to achieve a final concentration of $1.25 \times 10^5$ cfu/ml. Cells prepared in this manner routinely gave between 150 to 175 colonies in 5 ml of standard B5 medium. Aliquots of a test substance were delivered into the plates in volumes less than 0.5 ml prior to the addition of the cell agar mixture. All organic substances were filter sterilized for use in the assay. A gentle swirl completed the mixing of the test compound with the basal medium and the cells. Plates were sealed with parafilm strips and incubated in the dark at 27 °C for 3 wk. The number of colonies were counted by an automatic colony-counting machine (Biotran II, New Brunswick, NJ) with the lower threshold of discrimination set at 200 μm. In order to make a colony visible the isolated cells would have to complete six to eight divisions in the thin layer of agar.

Cells from suspension cultures were used as material for chromosome counting. In this way, we hoped to give a realistic portrayal of the status of the ploidy of the cultured cells. Four days after subculture cells were suspended in B5 medium plus 0.25% colchicine and incubated for 16 to 18 h. Cells were then collected by centrifugation and fixed in Carnoy's fixative; prior to staining, a hydrolysis in 1N HCl at 60 °C for 5 min was done to soften the rigid cell wall. Following washes with 45% acetic acid, these hydrolyzed cells were stained with carbofuchsin according to the method of Kao (10).

**RESULTS**

**Karyotype.** The first chromosome counts of the culture were done in December of 1977 (Fig. 1). In the first 14 months, the relative proportions of haploid, diploid, and aneuploid cells did not change. Of 358 mitotic figures scanned, 322 (90%) showed 9 chromosomes, 30 (8.5%) had 18 chromosomes, and 6 (1.6%) disclosed an intermediate or aneuploid number. During the next 12