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The Influence of Culture Conditions on Mitotic Activity in Protoplasts Derived from *Pisum* Root Cortical Explants

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With 12 Figures

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Summary

Protoplast cultures were prepared from explants of the roots of seedling peas. In defined, synthetic media these cultures were mitotically active. A variety of culture conditions were investigated and the influences of these conditions on the mitotic activity of the protoplasts were observed. Marked inhibition of mitoses were observed after exposure to high light intensities, and in the absence of a proper exogenous supply of hormones. The protoplasts showed extreme sensitivity to the nature and concentration of the exogenous hormone supply. The protoplasts were mitotically active at low population densities (6,000–8,000 protoplasts/ml of medium). They did not divide in cultures in which glucose was supplied as a carbohydrate source, but divided actively in cultures in which sucrose (2%) was supplied. The responses to temperature and pH were similar to most protoplast systems which have been reported. The definition of a wide variety of optimal culture conditions resulted in high mitotic activity in protoplast cultures with low population densities.

1. Introduction

A wide variety of tissues and media are currently used for the production and culture of naked protoplasts. Much of this early work has been reviewed by COCKING (1972). Mitotically-active protoplasts derived from sugar cane cell suspensions (MARETZKI and NICKELL 1973), tobacco leaves and petals (TAKEBE and NAGATA 1973, VASIL and VASIL 1973), carrot callus, embryos and leaves (REINERT and HELLMANN 1973, WALLIN and ERIKSSON 1973), *Asparagus officinalis* cladodes (MACKENZIE *et al.* 1973), *Petunia* leaf mesophyll, petals and callus (HESS *et al.* 1973) and pea root cortical explants (LANDGREN and TORREY 1973) were reported at Colloques Internationaux du C.N.R.S. and these reports were published in a volume entitled *Protoplastes et Fusion de Cellules Somatiques Vegetales.*

Since the International Colloquium, reports of mitotic activity in protoplasts of pea mesophyll (CONSTABEL *et al.* 1973), carrot root (KAMEYA and UCHIMIYA 1973), and the mesophyll of *Ipomoea* and *Calystergia* (HARADA 1973) and *Phaseolus* (PELCHER *et al.* 1974) have been made. There have been additional reports of mitotic activity in cultures of *Petunia* protoplasts (DURAND *et al.* 1973), *Asparagus* protoplasts (BUI-DANG-HA and MACKENZIE 1973) and in protoplasts derived from haploid tobacco (OHYAMA and NITSCH 1972). The culture media used in the stimulation of mitotic activity in protoplast cultures have included modifications of B 5 (GRAMBOW *et al.* 1972), LINSMAIER and SKOOG (BAWA and TORREY 1971), Takebe's media I and II (TAKEBE and NAGATA 1973), S 2 (BOGERS 1973) and S 2 M (LANDGREN and TORREY 1973).

Even so, reports on the influences of culture conditions on mitotic activity of protoplasts in culture are fragmentary. Such information would help to identify the specific nutritional, hormonal and cultural requirements of naked protoplasts, leading to improved cell division and plating efficiency of protoplast experiments.

In this report are presented studies that have been made of the cultural conditions necessary for the preparation and propagation of isolated pea root protoplasts. Details on the methods of production and the handling of the protoplasts in culture are presented.

2. Materials and Methods

2.1. Explant Production

One hundred-fifty seeds of pea (*Pisum sativum* cv. Little Marvel) were surface sterilized with 200 ml of 5% Pittchlor for 30 minutes. All further manipulations were carried out using sterile procedures. The sterilized seeds were drained and then washed with three 250 ml aliquots of sterile, distilled water. They were imbibed in the dark for 8 hours at 25 °C, in three 10 cm petri plates each containing 15 ml of sterile distilled water. Following imbibition, the peas were germinated in 10 cm petri plates each containing 15 ml of 0.6% agar (10 peas per plate) for 48 hours. Roots 1.5–2.5 cm long were selected and the 10–11 mm region of these roots (measured from the tip) was excised. Root segments 1.4–1.7 mm in diameter were selected from those excised. The central cylinder and part of the inner cortex were removed using a mechanical punch. The tissues remaining, the epidermis and most of the cortex, constituted the explants which were studied. These explants are hereafter referred to as "pea root cortical explants". These methods of explant preparation were devised by LIBBENGA and used as reported by LIBBENGA and TORREY (1973). The protoplasts were isolated from these explants.

The media used were designated PS 2 M (0.6 M mannitol) and PS 2 MG (0.6 M mannitol). They were modified for protoplasts from S 2 M medium (TORREY and FOSKET 1970), a defined, synthetic high-salt medium developed for cultivation of pea root segments *in vitro*. Modifications of the medium included decreasing the concentration of sucrose from 4% to 2%, increasing the osmotic concentration by the addition of 0.6 M mannitol, adding 2 ppm of kinetin and in PS 2 MG, replacing the complex mixture of reduced nitrogen sources with 2×10^{-3} M glutamine and modifying the pH from 5.5 to 7.0 ± 0.1 with KOH.

2.2. Protoplast Production

Immediately after production, cortical explants were placed in 5 ml of PS 2 M (0.6 M mannitol) or PS 2 MG (0.6 M mannitol) media in a 6 cm plastic petri plate (Nuncware plates, supplied by Vanguard International Inc., Red Bank, New Jersey). This medium was used to precondition the cortical cells to an environment of high osmotic pressure. In experiments in which a component of the medium was under investigation, that component was deleted from this preconditioning medium, with the exception of the sugars used as osmotic agents. In the case of these sugars, a preconditioning medium for each of the sugars tested was

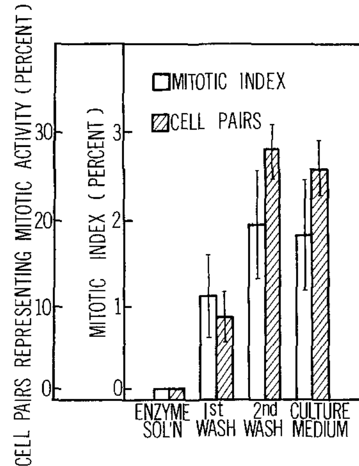


Fig. 1. The influence of washing on the mitotic index and the accumulation of mitotically produced cell pairs in ten-day-old cultures of pea root protoplasts. Protoplasts were isolated in the media indicated, and were cultured at 25 °C in the dark at population densities of 6,000–8,000 cells/ml. Each point represents four replicates from two experiments with a total sample size greater than 1,200 cells. The vertical bars represent ± one standard deviation from the mean of the four replicates

prepared. After 30 minutes in the preconditioning medium, the cortical explants were transferred to an identical medium with 6% (w/v) Cellulase "onozuka" 1500 (Lot 223011) and 2% (w/v) Macerozyme (commercial pectinase, Lot 40064, both from the All Japan Biochemical Co.) added. This enzyme incubation medium had been filter-sterilized through a Swinnex-25 0.45 µm filter, into a 6 cm plastic petri plate. Incubation in the enzyme medium was for 17 hours, in the dark, at 25 °C.

After enzyme incubation, the cortical explants were removed from the incubation medium using stainless steel forceps. The explants were then placed for 30–40 minutes in each of two 5 ml changes of fresh culture medium PS 2 M (0.6 M mannitol) or PS 2 MG (0.6 M mannitol) in 6 cm plastic petri plates. Two washings in this enzyme free medium were sufficient to release the protoplasts from the inhibitory effects of the enzyme incubation medium (Fig. 1). Protoplasts isolated in the second washing medium showed patterns of mitotic activity identical to those observed in protoplasts isolated according to the standard procedures. Experimental culture media modifications were made as indicated in the observations.

During the washing process, which decreased the concentration of the commercial wall-degrading enzymes in the cortical explants, isolation plates and culture plates were prepared.

Plastic 6 cm petri plates were used for both preparations. In the isolation plates, six 50 µl droplets of the appropriate fresh culture medium (free of enzymes) were arranged in a circle. These drops served as the medium in which the protoplasts were isolated. The culture plates each contained twenty 10 µl droplets of fresh medium arranged around the periphery of the dish. These droplets served to saturate the atmosphere of the plates. Following washing, one cortical explant was placed in each of the droplets in the isolation plate, using forceps. Each explant was gently teased under a dissecting microscope, using stainless steel dissecting needles. This procedure was carried out working from the inner cortex toward the epidermis. Teasing released the protoplasts from the constraint of the weakened cell walls. The needles were used to remove large pieces of cell wall debris, and the drops were left undisturbed in the dark for 10–20 minutes. During this time, the protoplasts settled to the bottom of the drop.

The protoplasts from one isolation plate were collected using mouth suction with a Pasteur pipette with an orifice of 150–200 µm, 4–5 times the diameter of a protoplast. The pooled protoplasts were distributed in the culture plates. Two to six separate 50 µl droplets were placed in the center of each plate. Each culture droplet contained 300–400 protoplasts.

2.3. *Protoplast Culture*

The culture plates were sealed with strips of Parafilm. They were stored in 2 cm deep 10 cm petri plates, each of which contained a 9 cm filter paper wetted with distilled water. The large plates were wrapped in aluminum foil and incubated in a precision scientific (General Electric) model 850 dark incubator at 27 °C unless otherwise noted. After production, the protoplasts were not exposed to light, except when they were sacrificed to sample for mitotic activity. Variations in the culture conditions were made as indicated in the section on Results.

2.4. *Staining Procedures*

The stain used to determine the frequency of mitotic activity was 0.5% synthetic orcein (Gurr's) in 65% acetic acid. A drop of stain was placed on a slide. The protoplasts from a single culture drop were collected using mouth suction with a fine pipette. The protoplasts were added to the stain and the mixture was allowed to sit for 1–2 minutes. The drop was covered with #0 cover slip without added pressure and the preparation was sealed with finger nail lacquer. The cells were observed immediately, or the slide was refrigerated and observed during the following 24–48 hours.

The slide was scanned using brightfield illumination with a 20× objective. The mitotic index was calculated as the sum of the metaphase, anaphase and telophase figures divided by the total number of cells in the culture. Prophase figures were not included in the mitotic index because of the difficulties in distinguishing between prophase and interphase figures when stained as described. Thus mitotic values are substantially lower estimates than the actual numbers.

"Cell pairs representing mitotic activity" was a statistical figure determined by dividing the sum of mitotically produced two-cell, three-cell, four-cell and greater colonies in stained preparations by the initial number of cells in the culture. Care was exercised in determining the origin of cell pairs as wall formation tends to bond cells in clusters (POJNAR and COCKING 1968). In cell pairs which were derived from single protoplasts, the characteristic spherical shape of the original mother cell was generally maintained to a large extent (Fig. 11). The divisions tended to be unequal, resulting in daughter cells of different sizes, while nuclear size in daughter cells tended to be uniform. Cross walls could be visualized using phase optics, staining with Calcofluor (NAGATA and TAKEBE 1970), or by lowering the condensor during normal brightfield microscopic observation.

Cell clusters produced by the adhesion of cells with newly formed cell walls tended to be non-spherical. The original size and shape of the cells involved in clumping were maintained to a large extent. There also tended to be significant nuclear size variations between adhering cells.
Unless otherwise noted, two samples were taken on each sample day for each treatment. Duplicate experiments for each treatment were run. Mean and standard deviations of the four samples for each point were determined using those functions so designated on the Monroe model 1766 calculator.

3. Results

The protoplasts isolated from these explants were spherical and varied in diameter from 25–55 μm. Smaller sub-protoplasts, derived primarily from the epidermis, were often present in the cultures. The sub-protoplasts were either nucleate or enucleate, and occasionally showed a reduced vacuole. Preliminary studies indicated that light, population density, temperature, sugars, pH, and the auxin and cytokinin concentrations influenced mitotic activity in cultures of these protoplasts.
The cyclic nature of mitotic events in pea root protoplast cultures and the susceptibility of these cultures to perturbations in the timing of mitotic events, led to the use of the accumulation of cell pairs as a measure of mitotic activity. This accumulation was a direct reflection of past mitotic events. It was not influenced by the sampling time, as was the mitotic index, and was relatively free from influences other than those of experimental modifications. By the tenth day in culture, active populations of protoplasts had accumulated large numbers of cell pairs. Although not all cell pairs would continue to develop and form cell colonies, the sampling of cell pairs allowed a rapid analysis of a large number of media and culture conditions, without the need for large cell populations or the lengthy interval necessary for plating efficiency counts. The small population size allowed observation of the entire population, and a record of actual mitotic events provided a verification of the observations of accumulated cell pairs. The mitotic index also provided an indication of the mitotic course (high or low activity) which a culture was following.

3.1. Light

Pea root protoplasts were first observed to be sensitive to light as a result of the failure of early experiments in which cultures were observed under a compound, inverted microscope daily or biweekly. Experiments on the influence of light on pea root protoplasts were complicated by the use of dim white, incandescent light (at the level of the isolation *ca.* 700 lux of mirror-reflected light from a Wotan 380179 bulb) in the isolation of protoplasts, during the teasing of the cortical explants after enzyme incubation. This 15 minutes exposure may have contributed to the lag period which preceded

the first mitosis in pea root protoplast cultures (Fig. 12). It did not, however, totally inhibit subsequent mitoses. Attempts to use dim green, fluorescent light during the isolation procedures were not successful, because the low differences in optical density between the medium and the naked protoplasts rendered the protoplasts almost invisible.

When pea root protoplasts were exposed to 5 minutes of high intensity, incandescent light (*ca.* 11,000 lux at the level of the cultures, as measured with a Gossen Luna-Pro exposure meter) in ranges normally found during brightfield microscopy using a Unitron inverted microscope with EL-1B bulb, mitotic activity was effectively inhibited (Fig. 2), whether the light was filtered with a green gel filter (reducing intensity to *ca.* 3,000 lux) or not. Culture under cool, white fluorescent lights (Sylvania White F 15T8W, *ca.* 1,400 lux at the level of the cultures) with a 16 hours day and 8 hours night did not totally inhibit division, but did reduce the accumulation of mitotically produced cell pairs to *ca.* 40% of that seen in cultures which were only exposed to light during the protoplast isolation procedures. Under the day-night culture conditions, the first mitosis appeared on the seventh or eighth day in culture. The high mitotic index seen for these conditions in Fig. 2 represented a second peak of mitotic activity on the tenth day in culture.

Attempts were made to improve the mitotic activity of pea root protoplasts isolated under incandescent light by an exposure to far-red light (6 minutes, 100 cm from a General Electric 300 W Reflector Floodlight, filtered through a 5 cm waterbath and plastic red and far-red filters). Far-red treated cultures had mitotic indices and accumulations of cell pairs after ten days in culture within one standard deviation of the controls which were only illuminated during protoplast isolation. These results are summarized below.

Light regime	Mitotic index (%)	Accumulated cell pairs (%)
Isolation, dark (10 days)	1.2 ± 0.4	32.1 ± 3.8
Isolation, far-red (6 min.), dark (10 days)	1.6 ± 0.6	30.6 ± 3.0

3.2. Population Density

The limitations imposed on this culture system by population density of cells in culture are indicated by Fig. 3. Each point in this figure represents a single culture sacrificed after 10 days incubation in the dark. Since each culture had a volume of 50 µl, populations under 2,000 cells/ml were in fact represented by fewer than 100 cells per culture. This fact should be weighed in the interpretation of the mitotic index of populations in this range. An accumulation of cell pairs, supported by the observation of mitotic figures,

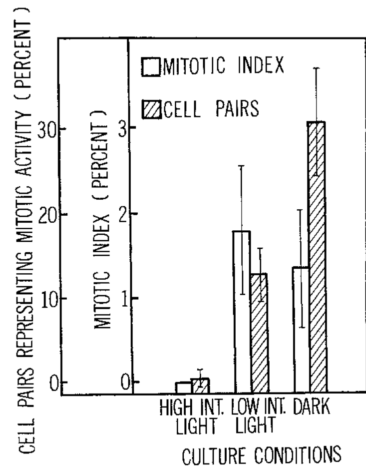


Fig. 2. The influence of light on mitotic index and cell pair accumulation in pea root protoplasts after 10 days of culture in PS 2 MG (0.6 M mannitol). All treatments were initially exposed to ca. 700 lux of dim, white incandescent light during the teasing of the cortical explants. High intensity light represented a subsequent five-minute exposure to 11,000 lux of white incandescent light. Low intensity light represented culture under 1,400 lux of cool, white fluorescent light, with a 16 hour day. Each point represents four replicates from two experiments and a total cell population greater than 1,200 cells. The vertical bars are ± one standard deviation from the mean of the four replicates

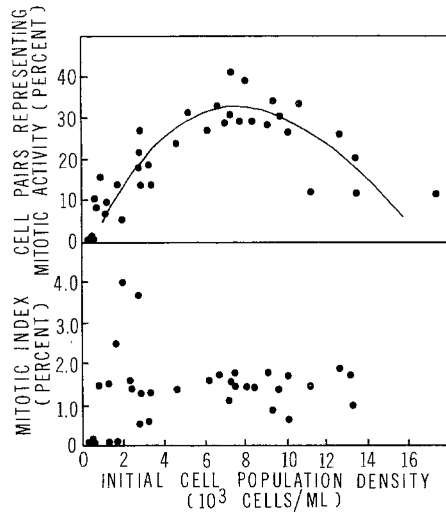


Fig. 3. The influence of cell population density on the mitotic index and cell pair accumulation in pea root protoplasts after 10 days of culture in the dark in PS 2 MG (0.6 M mannitol). Each point represents a single culture

occurred in populations with as few as 500 cells/ml. Below this population density, no cell divisions or cell-pair accumulations were observed. The decrease in the accumulation of cell pairs observed in cultures with more than 8,000 cells/ml was probably the result of an increase in the differentiation of a slowly-dividing or non-dividing population of protoplast derivatives. This differentiated population is currently under study.

3.3. *Temperature*

Attempts to culture protoplasts at temperatures ranging from 19–29 °C were carried out (Fig. 4). The highest accumulation of cell pairs on the tenth day of culture occurred at 27 °C. No mitotic activity as indicated by either mitotic figures or an accumulation of cell pairs was observed at 19 °C. From 21–27 °C, there was an increase in mitotic activity corresponding to increased temperature. Mitotically-produced cell pairs accumulated more slowly at 29° than at 27 °C.

3.4. *Sugars*

The response of pea root cortical explant protoplasts to sugars involved the role of these sugars both as osmotic agents and as carbohydrate sources. Originally, attempts are made to combine these two roles. No mitotic activity was observed when sucrose, glucose or ficoll were used as osmotic agents in concentrations ranging from 0.5–0.8 M or when combination of these sugars with $Ca(NO_3)_2$, glutamine, the S 2 M reduced nitrogen mixture, mannitol or sorbitol were used (mean concentration of individual osmotic agents: 0.2 M, total osmoticum concentration: 0.8 M). The protoplasts produced in plasmolyzing concentrations of sucrose were not spherical, but were rather collapsed, as though the density of the medium were excessive. Mitotic figures were only observed when mannitol (0.6–0.8 M), sorbitol (0.6 M) or mannitol (0.3 M) and sorbitol (0.3) together, were used as osmotic agents in combination with 2% sucrose (0.058 M) as a carbohydrate source. Fig. 5 summarizes the mitotic activity of pea root protoplasts grown in these conditions. Mannitol as an osmoticum was slightly superior to sorbitol or combinations of sorbitol and mannitol. This effect was especially evident in the pea root protoplasts when low levels of mitotic activity were caused by non-osmotic factors (*e.g.*, exposure to fluorescent light during culture, kinetin concentrations of 1/2 to 1 ppm). Under these conditions, mitotic activity was not evident in cultures in which sorbitol was used as an osmotic agent, either separately, or in combination with mannitol. When mannitol was used as the osmotic agent, mitotically-produced cell pairs were limited to 8–10% during the first 10 days of culture under these inhibitory conditions.

Sugars and sugar alcohols used as osmotica in producing protoplasts were not readily metabolized by the cells. As in most tissue culture systems, more

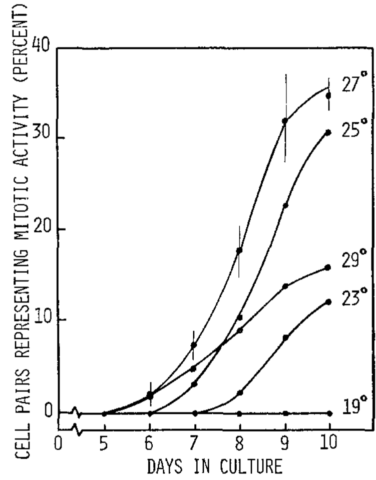


Fig. 4. The influence of temperature on the time course of cell pair accumulation in pea root cortical explant protoplasts after 0–10 days in culture in PS 2 MG (0.6 M mannitol). Day 9 at 29 °C represents only three samples as does day 10 at 19 °C; the other points represent four samples from two experiments (approximately 1,200 cells). The vertical bars are ± one standard deviation

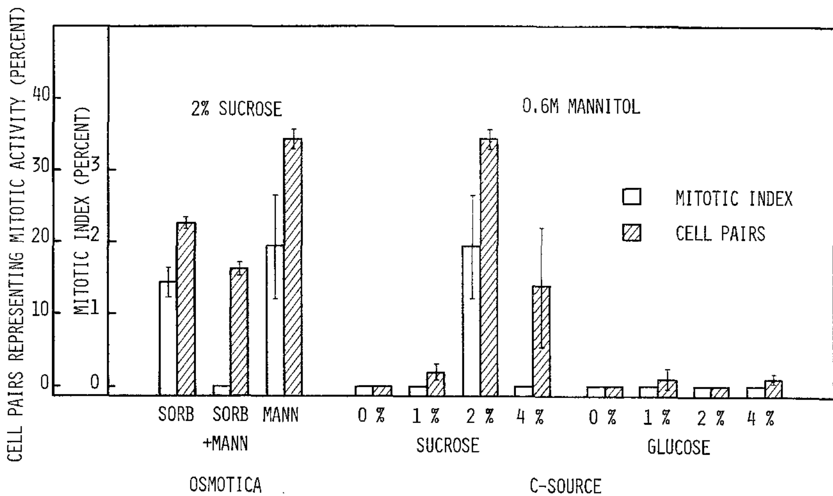


Fig. 5. The influence of sugars as osmotica and carbohydrate sources on the mitotic index and cell pair accumulation in pea root protoplasts after 10 days in culture in PS 2 MG (osmoticum concentration 0.6 M, divided equally in the case of the mixture). Each point represents four replicates from two experiments and a total cell number greater than 1,200 cells. Vertical bars represent ± one standard deviation from the mean of the four replicates

commonly metabolizable sugars were necessary as carbon and energy sources for the protoplasts in culture. Sorbitol and mannitol in the absence of sucrose failed to stimulate mitoses. Glucose and sucrose, in particular, were tested as carbon-energy sources. In this system, sucrose was effective, while glucose was not (Fig. 5). The optimum sucrose concentration tested was 2% (0.058 M).

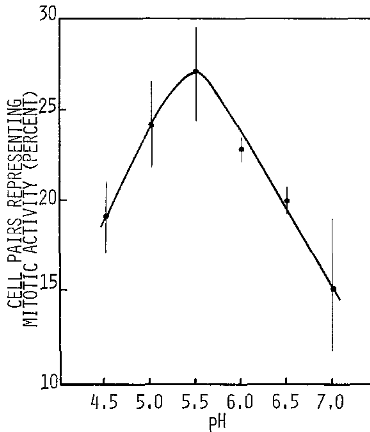


Fig. 6. The influence of pH on mitotically produced cell pair accumulation in pea root protoplasts after 10 days of culture in PS 2 M (0.6 M mannitol). Three samples are represented by the points at pH 6.0 and 6.5, while the other points represent four samples from two experiments (approximately 1,200 cells). The vertical bars are ± one standard deviation from the mean

3.5. Hydronium Ion Concentrations

The study of the influences of pH and reduced nitrogen source on the growth of pea root cortical explant protoplasts was complicated by an apparent change in the stock solutions which were used to prepare the media. In early experiments, using medium PS 2 MG with 0.6 M mannitol, glutamine alone as a reduced nitrogen source proved effective for inducing mitotic activity and an optimum response resulted when the initial pH was 7.0. When fresh stock solutions were prepared, glutamine was no longer effective by itself as a reduced nitrogen source. Attempts to determine the nature of the change responsible for these results were not successful. It was observed, however, that the mixture of reduced nitrogen compounds normally used for S 2 M was a suitable source for protoplast culture. Experiments in culturing pea root protoplasts with the S 2 M nitrogen mixture have been repeated with several different sets of stock solutions, and single experiments have been made with this medium to verify the observations on light, population densities, and temperature, which were originally made using PS 2 MG (0.6 M mannitol). Experiments to determine which components of the S 2 M reduced

nitrogen mixture were required for good growth proved to be inconclusive. Results from several replicates of deletion experiments (omitting one component of the mixture) and of experiments in which single sources of reduced nitrogen were used, proved to be too variable to allow final conclusions to be made.

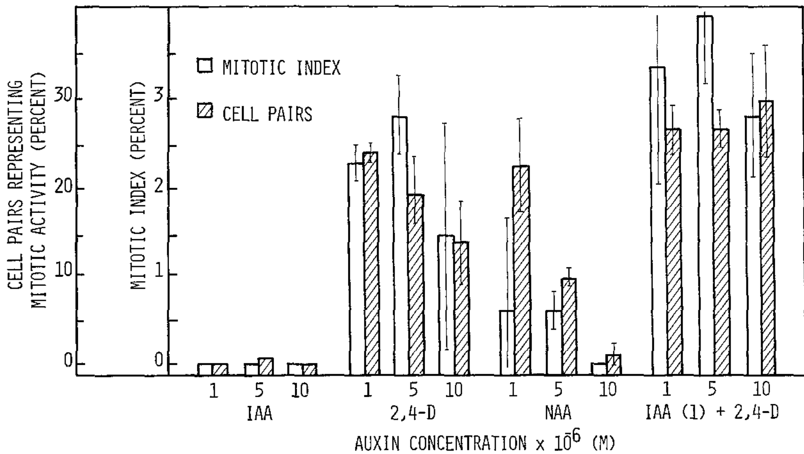


Fig. 7. The influence of various auxin concentrations on the mitotic index and cell pair accumulation in pea root protoplasts after 10 days of culture in PS 2 M (0.6 M mannitol). Each point represents four replicates from two experiments with a total cell number greater than 1,200 cells. The vertical bars are ± one standard deviation from the mean of the four samples

When the S 2 M reduced nitrogen mixture was used, the influence of initial pH on the accumulation of mitotically-produced cell pairs in cultures of pea root cortical explants (Fig. 6) indicated a pH optimum at 5.5 ± 0.1. The buffering capacity of the medium was contributed to by the high salt concentrations and by the reduced nitrogen sources themselves. After five days in culture, a medium with an initial pH of 5.5 ± 0.1 was found to have a pH of approximately 5.0. After ten days incubation, the same medium was found to have a pH of approximately 4.5 (as determined with Brom Cresol Green).

3.6. *Auxins*

The sensitivity of the protoplasts to the hormones in the medium was probably amplified by the direct contact of the cell surface with the medium. The responses of pea root cortical explant protoplasts to varying concentrations of IAA, 2,4-D and NAA and combinations of IAA and 2,4-D are indicated in Fig. 7. The absence of mitotic activity in media containing only IAA (as an auxin) in the presence of 2 ppm kinetin was observed. NAA and 2,4-D sufficed to promote cell division in the presence of kinetin. The frequency

of crosswall formation between daughter cells resulting from cell division in cells derived from protoplasts was greatly reduced when NAA alone was used as the auxin, or when low (1×10^{-6} M) 2,4-D concentrations were used in conjunction with IAA at 10^{-6} M. Under these conditions, crosswalls were not evident using phase optics or lowering the condensor during brightfield observations of orcein-stained preparations, procedures which generally made the crosswalls visible. Staining with Calcofluor indicated that the percentage

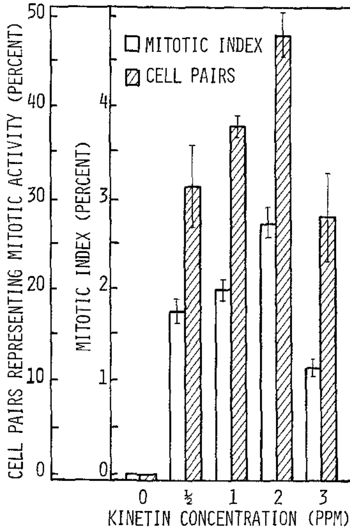


Fig. 8. The influence of kinetin concentration on the mitotic index and cell pair accumulation in pea root protoplasts after 10 days of culture in PS 2 M (0.6 M mannitol). Each point represents four replicates from two experiments with a total cell number greater than 1,200 cells. The vertical bars are ± one standard deviation from the mean of the four samples

of cells with crosswalls (3–5%) was much lower than the percentage which had apparently undergone mitosis (*ca.* 30%). When auxins were used in media lacking exogenous cytokinins, no cell divisions were observed.

3.7. *Kinetin*

Kinetin was the sole cytokinin studied. The effects of kinetin were initially tested over a range of concentrations from 0.01–10 ppm. In this range, mitotic activity was limited to media with 1 ppm kinetin. This observation led to the testing of kinetin concentrations over a much narrower range (0.5–3 ppm). The sensitivity of pea root protoplasts to small changes in kinetin concentration (Fig. 8) was much greater than that observed in the intact cortical explants when they were cultured directly on S 2 M.

3.8. The Development of Cultured Protoplasts with Time

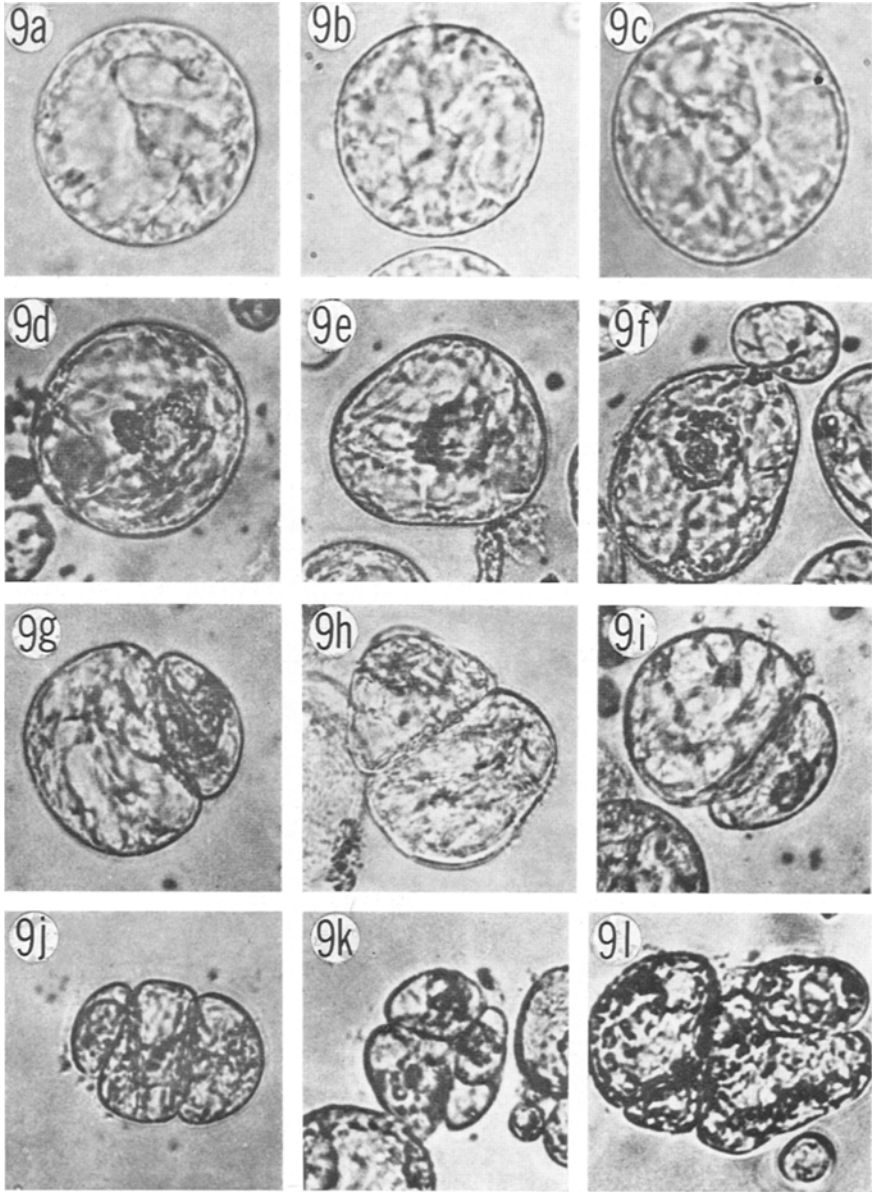
The appearance of cells taken from cultures under optimal growth conditions after 0 to 11 days of incubation is shown in Fig. 9. The formation of a new outer cell wall was observed after 3–5 days in culture. Presence of a wall was indicated by a change from the spherical shape and was also made evident by the occurrence of "budding" on the fifth day in culture. The presence of "buds", which result when the cytoplasm of enlarging cells ruptures at a weakening in the newly formed cell wall, was a condition frequently observed, but not involved in the normal developmental process. Excessive "budding" was not conducive to cell division, while small "buds" neither stimulated nor inhibited that process. This phenomenon should in no way be confused with the reproductive processes observed in certain fungi. The cell walls of cells after three days in culture stained lightly with Calcofluor. After six days in culture, the cell walls stained heavily and cell pairs were evident. After 9–11 days in culture, subsequent mitoses led to the formation of cell colonies with 3, 4, and 8 cells respectively.

Figs. 10 and 11 illustrate the appearance of mitotically active cells derived from pea root cortical explant protoplasts stained with modified aceto-orcein. Fig. 10 shows the stages of mitosis which were readily identified using this stain, while Fig. 11 illustrates the appearance of mitotically-produced 2-, 3-, 4-, 5-, and 10-celled colonies.

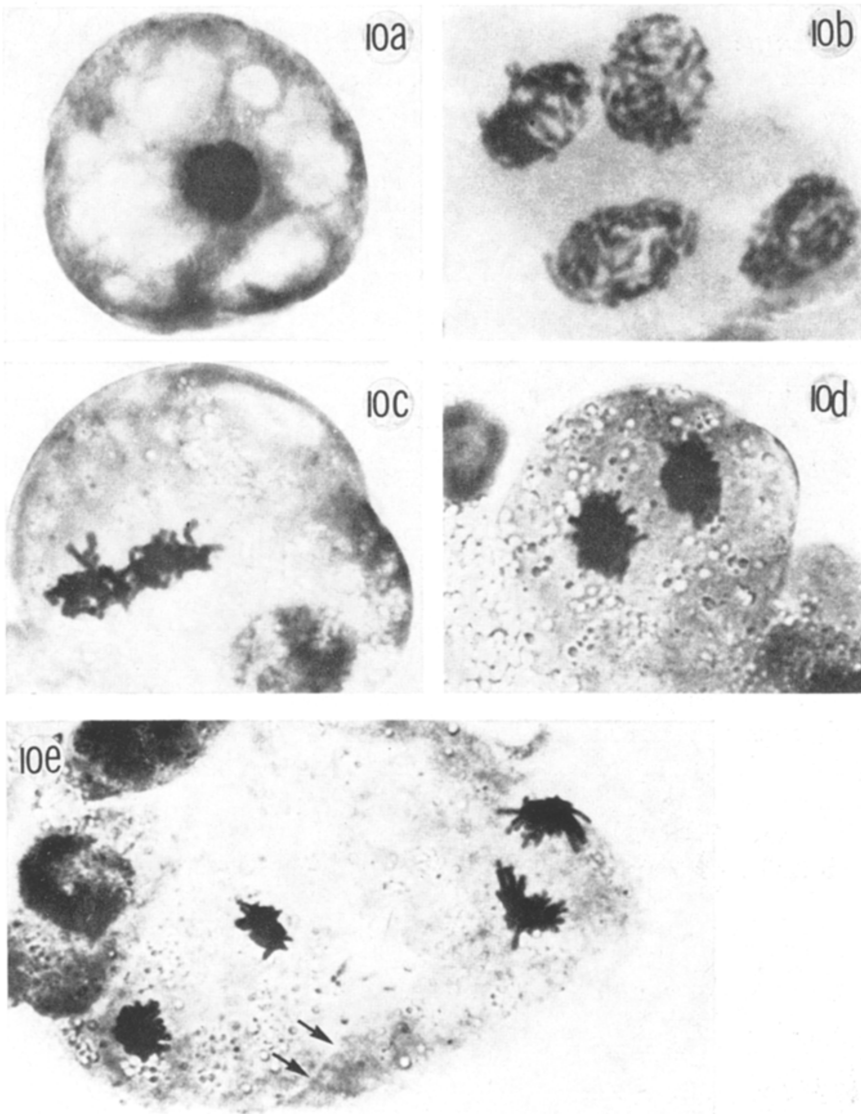
Observations of the time course of mitotic activity in stained preparations indicated an initial mitotic peak after 6–7 days in culture (Fig. 12). Further mitotic activity occurred with an apparently damped two-day cycle time. The accumulation of mitotically produced cell pairs coincided with the observation of mitotic figures and continued until cultures were 10–12 days old. After this time, mitotic activity was limited to divisions in small colonies which had already formed. There was a marked decrease in mitotic activity after 15 days of culture.

4. Discussion and Conclusions

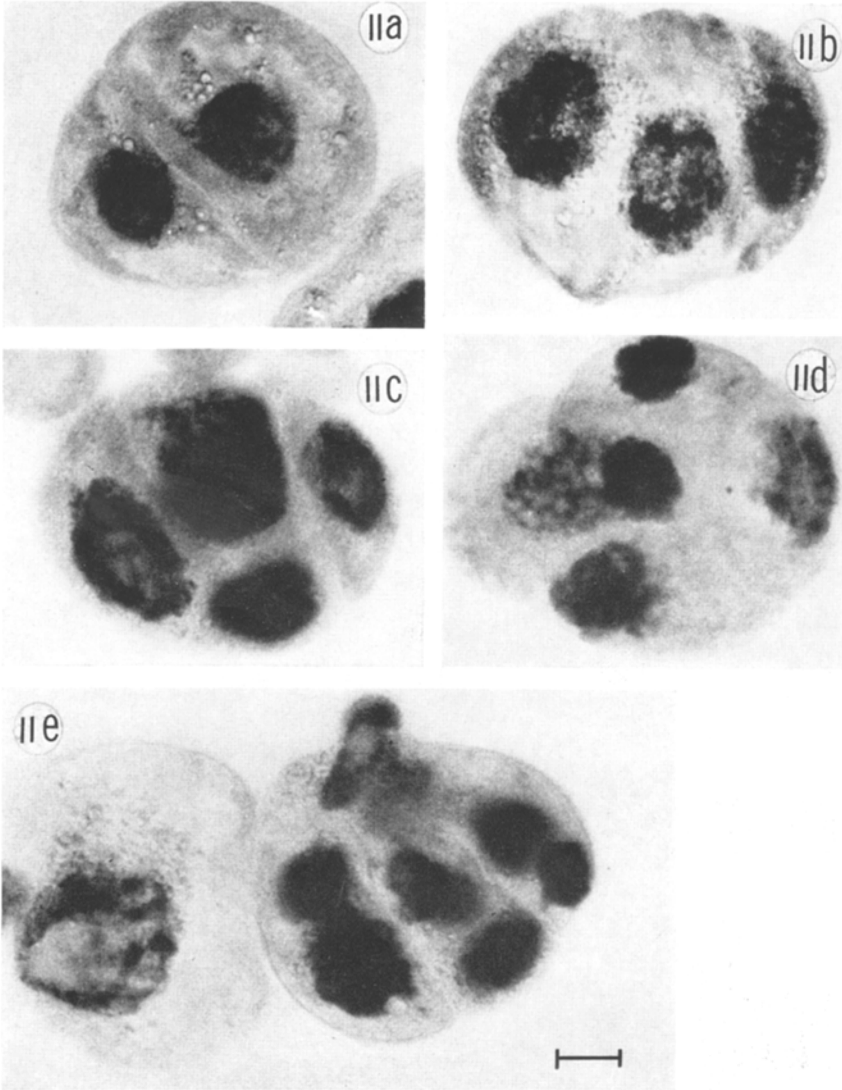
The successful culture of protoplasts derived from cortical explants of the roots of pea seedlings was achieved on a defined, synthetic medium. The medium was derived from S 2 M, which, with the addition of 1 ppm kinetin, had been used in the culture of intact pea root cortical explants. Many of the conditions used for the culture of intact cortical explants were apparently optimal for the protoplasts as well. These included the maintenance of dark culture conditions, the initial pH, the reduced nitrogen sources, and the auxin concentrations (both IAA and 2,4-D were used). The optimal population density and temperature for culture of pea root protoplasts were determined.



Figs. 9 *a–l*. The appearance of the most advanced cells in cultures of pea root cortical explant protoplasts in PS 2 M (0.6 M mannitol) observed after 0–11 days, respectively. The "bud" formed on the cell after five days in culture is not a normal step in this developmental sequence. All ×600



Figs. 10 *a–e*. Fixed and stained preparations of cells derived from cultures of pea root protoplasts. *a* Interphase cell from culture day 0. *b–e* Cells from culture days 8–15. *b* Four late prophase nuclei in a cell colony apparently derived from a single protoplast. *c* Metaphase plate of undetermined ploidy. *d* Early telophase figure. *e* Late anaphase and early telophase figure in two adjacent cells. The two dividing cells were apparently derived from a single protoplast. Arrows indicate the crosswall between these two cells. All $\times 800$



Figs. 11 *a–e*. Fixed and stained preparations of cells derived from cultures of pea root protoplasts. *a–e* Represent mitotically produced cell colonies containing 2, 3, 4, 5, and 10 cells, respectively. Taken from culture days 6–12. The horizontal bar represents 10 µm. All ×800

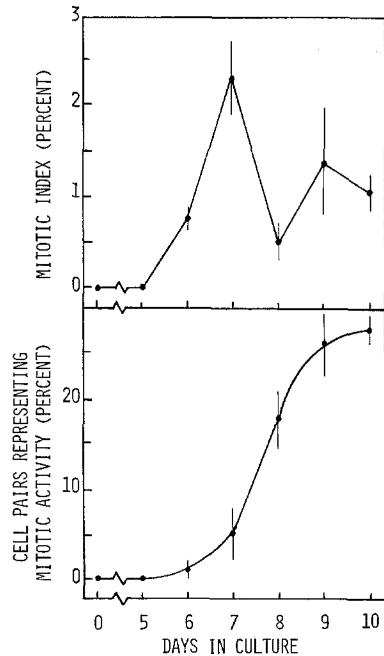


Fig. 12. The time course of the mitotic index and accumulation of mitotically produced cell pairs in cultures of pea root protoplasts after 0–10 days incubation in PS 2 M (0.6 M mannitol) in the dark at 27 °C, with 300–400 cells/50 μl culture drop. Each point represents four replicates from two experiments

Ferre-Simpson (1975) found that exposure to white light inhibited the increases in DNA and fresh weight of cortical explants in culture. This effect was evident only after the 10–11 mm root segments were excised from the seedling. It was observed that protoplasts from this explant showed a similar sensitivity to light. The light inhibition in cortical explants was alleviated to some extent by the use of dim, green fluorescent light during the isolation and transfer procedures. Protoplast production procedures precluded the use of dim, green fluorescent light during the teasing of enzyme-treated cortical explants. This procedure required the use of dim, white incandescent light, in exposures which may have contributed to the six-day lag preceeding mitotic activity.

Enzmann-Becker (1973) observed that protoplasts from the mesophyll of tobacco leaves were sensitive to high-intensity light (3,000 lux) after isolation and until about the third day in culture. These protoplasts were chlorophyllous and showed greater mitotic activity under high light intensity than under low (400 lux) after 48 hours in culture. This period corresponded approximately with the time necessary for the deposition of a Calcofluor positive cell wall (Nagata and Takebe 1970). Also during this period many

cytological changes were occurring, including repairs (WILLISON 1973) of damage to organelles resulting from osmotic stress and enzyme treatment (GIGOT *et al.* 1973, MACKENZIE *et al.* 1973).

When light was used in the culture of protoplasts, the exposure was generally between 700 (DURAND *et al.* 1973) and 3,000 lux (TAKEBE *et al.* 1971). Light was used primarily with protoplasts derived from leaf mesophyll tissue. Protoplasts from *Asparagus* cladodes (BUI-DANG-HA and MACKENZIE 1973), carrot suspension cultures (WALLIN and ERIKSSON 1973) and barley mesophyll (SCHASKOLSKAYA *et al.* 1973), as well as the pea root protoplasts reported in this paper, grew best when protected from exposure to light during culture. The inhibition of protoplasts by light does not seem to be mediated by phytochrome.

Protoplast cultures have been difficult to maintain at low population densities on defined, synthetic media. Culture levels are maintained in the range of 1.5×10^4 (GRAMBOW *et al.* 1972, NAGATA and TAKEBE 1971) to 5×10^5 cells/ml (BINDING *et al.* 1973, DURAND *et al.* 1973, HARADA 1973) . These populations are maintained in cultures of up to 10 ml, resulting in a total culture population of over 10^6 cells (TAKEBE and NAGATA 1973). The cortical explant protoplast system lent itself to the study of small numbers of cells. The staining and counting procedures described allowed single cultures of 50 µl to be analysed in some detail. Studies were made on cultures containing 300–400 cells and time course studies could be made on total populations as small as 8,000–9,000 cells. In this system, mitotic figures were observed in cultures containing as few as 50 cells (1,000 cells/ml), however, such cultures failed to show an accumulation of cell pairs. The pea root cortical explant protoplast had an absolute requirement for exogenous auxin and cytokinin regardless of the population density.

The response of cultured pea root protoplasts to temperature was essentially biphasic. At temperatures up to 27 °C, increased mitotic activity was accompanied by an acceleration of the first observed mitosis. Above 27°, mitotic activity began early, but was inhibited after only 8 days in culture. Most protoplast cultures are maintained between 25° (BINDING *et al.* 1973, GRAMBOW *et al.* 1972, HARADA 1973, WALLIN and ERIKSSON 1973) and 28 °C (REINERT and HELLMAN 1973, TAKEBE *et al.* 1971).

A wide variety of sugars have been used as osmotic agents and as carbohydrate sources in protoplast cultures. Mannitol from 0.3 M (DURAND *et al.* 1973) to 0.7 M (NAGATA and TAKEBE 1971) has been used as an osmotic agent in mitotically active systems, as has sorbitol from 0.15 M (KAO *et al.* 1970) to 0.6 M (REINERT and HELLMANN 1973). Sucrose at concentrations as high as 0.6 M was used in the culture of *Asparagus officinalis* protoplasts (BUI-DANG-HA and MACKENZIE 1973). In mitotically active pea leaf mesophyll protoplasts from the cultivar Century, glucose and sucrose were effective as carbohydrate sources and a mixture of sorbitol (0.3 M) and mannitol (0.2 M)

was used to maintain the medium at a high osmotic pressure (CONSTABEL *et al.* 1973). These observations are in sharp contrast to mitotic patterns observed in pea root protoplasts. These contrasting results may be due to varietal differences (unpublished results) or may be related to differences between the roots and leaves of peas as sources of protoplasts. Whatever the cause of these differences, they emphasize the necessity of a pragmatic analysis of sugars both as osmotic agents and as carbohydrate sources.

Most protoplast media used for the study of mitotically active populations are initially adjusted to a pH of 5 (REINERT and HELLMANN 1973) to 6.4 (BUI-DANG-HA and MACKENZIE 1973). The optimum pH for pea root cortical explant protoplasts was *ca.* 5.5. If cell division could again be stimulated in PS 2 MG medium at pH 7.0, further study of this unusual pH optimum would be justified.

The inability to visualize crosswalls in cultures of pea root protoplasts incubated in media containing NAA or IAA plus low concentrations of 2,4-D is similar to observations made of *Haplopappus* protoplasts (ERIKSSON and JONASSON 1969) and of carrot protoplasts (HELLMANN and REINERT 1971). In the pea protoplasts the nature of the crosswall seemed to be directly related to the auxin used in culture. ERIKSSON and JONASSON used 1 mg/l (*ca.* 5×10^{-6} M) NAA, while HELLMANN and REINERT used 5×10^{-8} M 2,4-D in the culture of their protoplasts which showed modified crosswall synthesis. The failure of pea root protoplasts to show cell enlargement after cell division (Fig. 11) when cultured in media containing both IAA and 2,4-D was also noted.

Cytokinins are not regularly investigated over such narrow concentration ranges as those used in this study. The 20% increase in mitotic activity between 1 ppm and 2 ppm kinetin and the 35% decrease in activity between 2 ppm and 3 ppm kinetin would indicate the significance of such studies for protoplast systems.

The production of protoplasts directly from root explants allowed a well defined genetic and developmental population to be studied. These protoplasts divided on a defined, synthetic medium. Optimal conditions were determined for culture, and 8–10 celled colonies of mitotic origin were obtained after 2 weeks in culture. The sensitivity of pea root cortical explant protoplasts to sucrose in the medium was more marked than that of the tissues from which they were derived. These protoplasts were also influenced in their mitotic activity by a two-fold increase in kinetin concentration and in their cytokinesis by a five-fold increase in 2,4-D concentration. As a mitotically active population, they should be suitable for fusion studies and for studies of infection with *Rhizobium.*

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