Effect of Chilling Temperatures upon Cell Cultures of Tomato

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ABSTRACT

The effect of chilling temperatures upon cell cultures of tomato (Lycopersicon esculentum Mill cv ‘VF36,’ and cv ‘VFNT Cherry,’ and L. hirsutum Humb. & Bonpl.) was tested. Doubling times for L. esculentum were 2 to 3 days at 28°C, and 3 to 8 days at 12°C. No growth was observed at 8°C, indicating an abrupt limit to growth between 8 and 12°C. Fluorescein diacetate staining indicated that 80% to 90% of the cells were alive when cells were maintained at 8°C for up to 2 weeks. When cultures kept at 8°C for up to 30 days were transferred to 28°C, growth resumed quickly, and at a rate virtually identical to that for unchilled cells. Similar results were found for cells maintained at 0°C. Cells of ‘VFNT Cherry’ and of L. hirsutum. Under certain conditions, cultures slowly doubled in fresh weight and cell volume at 8°C but additional growth at 8°C did not occur, nor could growth be maintained by subculture at 8 or 9°C. The results are contrary to reports that cell cultures of tomato die when exposed to temperatures below 10°C for 1 or 2 weeks. Our observations indicate that chilling temperatures quickly inhibit growth of tomato cells, but do not kill them.

MATERIALS AND METHODS

Plant Material. Seeds of Lycopersicon esculentum Mill cv VF36 and cv ‘VFNT Cherry,’ and Lycopersicon hirsutum Humb. & Bonpl. collected from a population near Rio Casma, Perú (LA 1777) at an altitude of 3000 meters, were provided by Dr. C. M. Rick, University of California, Davis. Seeds were sterilized with 25% chlorine bleach and germinated on agar.

Cell Suspension Culture. Callus was initiated from root or hypocotyl of sterile seedlings germinated on agar. Each culture originated from a single seedling. The medium for callus and suspension cultures consisted of M & S major salts (9) with either 21 mM NH₄NO₃ (ammonium nitrate medium) or with 20 mM NH₄Cl plus 20 mM sodium succinate (ammonium medium), plus M & S minor salts (9), Nitsch’s vitamins (11), 100 µM iron EDTA, 3% sucrose, 2 mg/l 2,4-D, and 0.1 mg/l BA or isopentenyl adenine. The medium is modified from the procedures of S. E. Barsel (personal communication). Cultures were maintained on orbital shakers at 180 to 200 rpm or on a reciprocating shaker at 64 cycles-min⁻¹. All stock cultures were maintained at 28°C. ‘VF36’ and L. hirsutum stocks were maintained in 200 ml of medium in 500 ml flasks and transferred every 7 to 10 days by transferring 25 ml of cells to 175 ml of fresh medium. Stock cultures of ‘VFNT Cherry’ were maintained in 125 ml flasks.

Callus from all tissues was initiated on NH₄NO₃ medium. Suspension cultures were initiated from 2 to 6 months after initiation of callus, and used for experiments from 2 months to 1 year afterwards. Suspension cultures of L. esculentum cv ‘VFNT Cherry’ were maintained in NH₄NO₃ medium, while cultures of cv VF36 and L. hirsutum were maintained in ammonium medium. The cultures grew well in either medium, results were similar using either medium, and choice of NH₄NO₃ or ammonium media was not significant to the behavior of the cultures.

Growth Measurements. Several methods were used to assess the effect of temperature on growth.

A. Weight Determinations. Stock cultures were maintained in 200 ml of medium in 500 ml flasks at 28°C. Inoculum from a culture in the early stationary phase of growth was diluted 1 to 5 into 30 ml of medium in a series of 125 ml flasks. The flasks were immediately transferred to the indicated temperatures. At 2 days intervals, flasks were harvested by vacuum filtration onto Whatman No. 4 filter paper on a Büchner funnel, and fresh weight and dry weight were determined.

B. SCM Measurements Using Stationary Phase Cells. A nondestructive means of measuring the growth of the cell cultures was needed. The cultures were thick; also the cells settled rapidly when not agitated, so measurements of the optical density of the cells in a side arm did not seem appropriate. Instead, a method was developed to measure the settled cell volume. Inoculum from a culture in the early stationary phase of growth was diluted 1 to 9 into 45 ml of medium in a series of 300 ml side arm flasks.

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3 Abbreviation: SCM, settled-cell-to-medium ratio.
which were immediately transferred to the indicated temperatures. To determine the SCM, the flasks were tipped to fill the side arm with a uniform sample of the total culture, and left with the side arm vertical for 20 min, then the ratio of the heights of the settled cells and the cells plus the medium in the side arm was determined. A ratio of 1.0 indicates that the settled cells fill the side arm. Fresh weight, dry weight, and SCM increased in proportion to each other over the range of SCMs from 0.1 to 0.9. There was a similar increase in cell number up to an SCM of 0.5; above 0.5 there was no further increase in cell number. SCM could be measured as often as once a day without affecting the rate of growth; more frequent determinations were avoided.

C. SCM Determinations Using Logarithmic Phase Cells. Cells in middle-logarithmic phase were used to inoculate 300 ml side arm flasks. Volumes were adjusted to give an initial SCM of approximately 0.05, in 45 ml of medium. Flasks were kept at 28°C until reaching an SCM of approximately 0.15, then transferred to the indicated temperatures. All temperatures given are accurate to 1.0°C.

Fluorescein diacetate staining was used to determine cell viability. The procedure was carried out as described by Widholm (21). Stained cells were examined using a Zeiss Photomicroscope III and a minimum of 200 cells were counted for each determination.

RESULTS

The initial research plan was (a) to determine the lowest temperature at which cells of L. esculentum survived and grew, (b) to measure the rate at which chilled cells of L. esculentum die off, (c) to compare the minimum survival temperature of L. esculentum with that for the other cell lines, and (d) to compare the die-off rate at temperatures below the minima for the different cell lines.

Stationary phase cells grown at 28°C were diluted into fresh medium and were incubated at 28, 12, or 8°C (Fig. 1). At 2 d intervals, samples of cells were examined for viability using the fluorescein diacetate procedure. Fresh weight, dry weight, and medium pH were also determined; two flasks were used for each data point. Flasks of cells placed at 28 and 12°C increased in fresh weight and dry weight, and the pH of the medium dropped from 5.8 to 5.0. Doubling times are shown in Table I. Flasks of cells placed at 8°C or 5°C showed no change in fresh weight, dry weight, or medium pH. However, there was no significant difference in viability of cells maintained for 2 weeks at 28, 12, or 8°C, as measured by fluorescein diacetate staining (Fig. 1). Fluorescein diacetate staining correlated with ability to plasmolyze in a hypertonic medium and with lack of staining by phenoisafiranin. The only instance of significant cell death was in late-stationary phase cell grown at 28°C (not shown).

Many observations were made of the microscopic appearance of the cells. Cells grown at 28°C had large vacuoles, many cytoplasmic strands crossed the vacuoles, and the nucleus was suspended in the center of the vacuole by cytoplasmic strands. There was vigorous cytoplasmic streaming around the periphery of the cell, across the vacuole, and around the central nucleus. Cells maintained at 8°C for 4 d or more also had a large central vacuole, but few cytoplasmic strands crossed the vacuole. Instead, most of the cytoplasm was restricted to the periphery of the cell, and the nucleus was in the peripheral cytoplasm instead of the center of the cell.

Attempts were made to determine a minimal temperature for survival, below which cells died. However, even if the cells were maintained at 0°C in an ice bath for 1 week, they still showed rapid regrowth when transferred back to 28°C (Fig. 2). There was no significant change in double time whether cells were exposed

<table>
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<th>Species</th>
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<th>Doubling Time</th>
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<tr>
<td>L. hirsutum</td>
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* See "Materials and Methods."

Fig. 1. Cell viability as a function of time. Cells from stationary phase cultures of L. esculentum cv VF36 were inoculated into fresh medium, and incubated at 8°C (C), 12°C (△), or 28°C (●) for the indicated times. Samples were stained with fluorescein diacetate and the percentage of cells that were fluorescent is indicated as the percentage of live cells. Each measurement is the average for two flasks. Doubling times are shown in Table I.
and drained were grey appearance of the cold-treated that of to both 28°C, went one conditions, tial) and rotary inoculated in grown at 28°C (VF36) or maintained at 8°C and then transferred to 28°C (arrows) after 8 (C) or 12 d (Φ).

to 0°C for 0 to 7 d.

Details of survival and limitations to growth around 10°C were then examined. Cells of L. esculentum and L. hirsutum showed a lag of up to 3 d before entering the exponential stage of growth at 28°C when transferred to fresh medium from a culture in the early stationary phase of growth (Fig. 3). There was no growth if the newly inoculated cultures were kept at 8°C, but if cells were maintained at 8°C for up to 12 d, then transferred back to 28°C, the cells grew with a doubling time and a lag time similar to those of cells not exposed to the cold (Fig. 3).

If the cells were transferred from 28°C to cooler temperatures during the early exponential phase of growth, the results were more variable (Fig. 4; Table I). Cells of L. esculentum cv VF36 stopped growth for approximately 50 h when transferred to 12°C, then resumed growth, with a doubling time 1.5 to 4 times longer than at 28°C (Table I). In the experiment shown in Figure 4, cells of L. esculentum and L. hirsutum did not grow at 6°C, and increased slightly in volume at 8°C. Both sets of cells resumed growth at 28°C with little change in doubling time after a month at 6 or 8°C (not shown). In other experiments (not shown), both L. esculentum and L. hirsutum doubled in weight at 9°C. In no case could growth be maintained by transferring cells that had grown at 8 or 9°C to new medium at 8 or 9°C, and cells grown at 8 or 9°C never achieved more than one doubling in fresh weight. Similar results were found whether cells were grown on ammonium medium or on NH₄NO₃ medium.

Additional observations were made on cells grown in large flasks to produce sufficient material for membrane preparations. A rotary shaker at 9°C was used for this purpose. Cells were inoculated from late exponential stage into 180 ml of fresh medium in 500 ml flasks, grown at 28°C for 4 d (early exponential) and transferred to 9°C for up to 2 weeks. Under these conditions, cells of both L. esculentum and L. hirsutum underwent one doubling in 2 weeks, and were then harvested. There was a noticeable difference in appearance between cells of L. esculentum and cells of L. hirsutum after 2 weeks at 9°C. At 28°C, both cultures were yellow-white in color and were fluffy and drained easily when collected on a Buchner funnel. The appearance of the cold-treated L. hirsutum cells remained similar to that of the controls, but the cold-treated L. esculentum cells were grey and formed a compact grey mat that drained poorly.

Fig. 3. Settled-cell-to-medium ratio as a function of time for cells kept at 8°C, then transferred to 28°C. Cells from stationary phase cultures of L. esculentum cv VF36 (A) or L. hirsutum (B) were used to inoculate fresh medium in side-arm flasks. Cells were grown at 28°C (■) or maintained at 8°C and then transferred to 28°C (arrows) after 8 (C) or 12 d (Φ).

Fig. 4. Settled-cell-to-medium ratio as a function of time for cells kept at different temperatures. Side-arm flasks were inoculated with cells from cultures in the mid-logarithmic phase of growth and incubated at 28°C until the cells reached an SCM of approximately 0.2. One flask was left at 28°C (Φ) and the others were transferred (arrow) to 12°C (C), 8°C (D), and 6°C (■). A, Cells of L. esculentum cv VF36 in ammonium medium. B, Cells of L. esculentum cv VF36 in ammonium nitrate medium. C, Cells of L. hirsutum in ammonium medium.
LOW TEMPERATURE LIMIT FOR GROWTH OF TOMATO CELLS

when collected on a Büchner funnel. The difference in appearance was not reflected in fresh weight, yield of protein, yield of membrane, or yield of proton transport activity of microsomal membranes collected from the cells. Cell cultures of both species gave a similar yield in terms of fresh weight, total protein, and individual membrane fractions (6). Because the L. hirsutum cells were white and the L. esculentum cells grey when maintained for 2 weeks at 9°C, we tested whether the L. esculentum cells might have increased levels of phenolics or other low mol wt solutes. Extracts of the cells were examined for the presence of methanol extractable fluorescent or ninhydrin positive solutes (such as amino acids and polyamines). However, both species showed a large increase in such solutes when cells were chilled.

Experiments using whole plants of L. esculentum and two accessions of L. hirsutum were carried out to examine the extent of the differences that can be observed in whole plants of a lineage similar to that of the cell cultures. One to 2-month-old plants, from 30 to 60 cm high were transferred from the greenhouse to growth chambers and kept for 3 weeks with day temperatures of 25°C and night temperatures of either 20°C or 8°C. The day length was 13 h, and temperatures were ramped from the day temperature to the night temperature for a period of 4 h, beginning 2 h before and ending 2 h after the lights were turned off or on. The first experiment was carried out in May and used 30 cm plants as starting material. The second experiment was in October and used 60 cm plants as starting material. At the end of each experiment the foliage in the chamber with 20°C nights was visibly much denser than in the chamber with 8°C nights. In the first experiment the plants of L. esculentum and the middle-altitude accession of L. hirsutum showed significant inhibition of fresh weight, dry weight, and leaf area by cold nights, and the high altitude accession of L. hirsutum did not. In the second experiment, the plants of all three accessions had reduced fresh weight, leaf area, and Chi/g fresh weight ratios compared to the plants grown with warm nights. In both experiments, the plants of the high altitude accession appeared green and healthy under both cold and warm nights. In neither experiment were plants of L. esculentum killed by the cold nights; symptoms of chilling injury were minor ones such as an increase in purple coloration of the veins, and some leaf curling and epinasty; there were no sizable areas of necrotic lesions on leaves. Exposure to 8°C for 4 h a night for 3 weeks inhibited growth of the plants but apparently caused little cell death. Response of the middle-altitude accession plants of L. hirsutum was variable with some plants showing bleaching of leaves, and development of purple coloration in the lamina (unlike L. esculentum, which developed purple in the veins), and other plants showing no symptoms of injury under the cold nights regime.

DISCUSSION

Although high altitude accessions of L. hirsutum are potential sources of genes for chilling resistance, we found little difference in the effect of temperature upon growth and survival of cells of L. esculentum and L. hirsutum. This is consistent with reports that the differences in response to chilling of the intact plants are also subtle. For example, correlations have been found between altitude of accession and effect of temperature on cytoplasmic streaming in trichomes of L. hirsutum (12), rate of amino acid uptake by leaf slices (15), minimum temperature for seed germination (7, 14), and growth (20). Smilie and Norst (18) found that the photosynthetic activity of detached leaves of L. hirsutum declined more slowly than that of L. esculentum at 0°C. However, no difference was found in composition of leaf polar lipids (14), or lipids of root mitochondria (3). Herner and Kamp (7) found only a slight difference in survival of L. esculentum and a high altitude accession of L. hirsutum, after exposure to temperatures between 0 and 5°C, and Dalziel and Breidenbach (3) reported that although rooted cuttings of L. hirsutum survived longer than those of L. esculentum at constant 5°C, they eventually also died. Our own experiments, comparing growth of plants of L. esculentum and L. hirsutum when exposed to 8°C nights for 3 weeks, found no visible symptoms of chilling injury on leaves of a high altitude accession of L. hirsutum, but in one experiment growth of the L. esculentum plants was inhibited as much as for L. esculentum. Vallejos et al. (20) report a reduction in growth for both species when exposed to a chilling regime of 12°C days, 5°C nights, though less reduction for a high altitude accession of L. hirsutum than for a low altitude accession of L. hirsutum. None of the findings cited above are inconsistent with our claim that cell lines of L. esculentum and L. hirsutum both had a similar "low temperature limit to growth."

It has been suggested that an important phenomenon to be examined in chilling injury is the low temperature limit to growth (17). We observed such a limit for the suspension cultures of tomato cells, as none of the cell lines we tested maintained growth below 12°C. Our results suggest that the primary lesion of chilling injury of tomatoes may be one that prevents a critical step in cell division or cell expansion. The cells are not killed; however, instead, chilling the cell suspensions preserves them in a non-growing state. The cell lines may be protected by the sterile nutrient medium from the secondary effects of chilling that kill the cells of the intact plant, such as eventual nutrient imbalance, desiccation, disease, or malfunctioning of phloem transport. It is not possible to use the cell cultures to explore all aspects of chilling injury of intact plants. For instance, chilling injury of tomato plants is influenced by the time of day at which the chilling stress is applied (8) and by RH (7). However, the suspension cultures may be a useful system to separate the primary causes of chilling injury from the secondary effects that make it difficult to study chilling injury of whole plants.

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