MULTIFACTOR ANALYSIS OF ENVIRONMENTAL PRE-CONDITIONING OF TOMATO SEEDLINGS ON PROTOPLAST CULTURE AND DEVELOPMENT

By

RANDALL PAUL NIEDZ

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ABSTRACT

MULTIFACTOR ANALYSIS OF ENVIRONMENTAL PRE-CONDITIONING OF TOMATO SEEDLINGS ON PROTOPLAST CULTURE AND DEVELOPMENT

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Leaf mesophyll protoplasts were enzymatically isolated from 24-30 day old seedlings of Lycopersicon esculentum P.I. 367942 grown either in a controlled environment chamber (CEC) or in vitro (IV). Prior to protoplast isolation, donor seedlings were pre-conditioned in a 3x3 factorial arrangement of 0, 12 or 24 hours at 10°C and 0, 24 or 48 hours in the dark. The variables measured included protoplast yield, 24-hour viability, 30-day microcalli diameter and shoot morphogenesis. Multifactor fixed effect linear models were constructed to determine the influence of seedling source and cold/dark pre-conditioning on the observed variables.

Protoplasts were cultured in the dark (27°C) in a liquid modified Kao and Micahyluk (KM) medium over a semi-solid modified Murashige and Skoog (MS) medium. Both media contained the growth regulators (mg/L): 2,4-dichlorophenoxyacetic acid (1.0), alpha-naphthaleneacetic acid (1.0) and 6-benzylaminopurine (0.5). To stimulate shoot morphogenesis protoplast-derived calli were cultured in the light (cool white fluorescent lamps; 30 $uEm^{-2}s^{-1}$) on modified MS medium with 2 mg/L zeatin.

Protoplasts from CEC donor seedlings pre-conditioned with

0 hours dark/12 and 24 hours cold died in 24-48 hours.

Protoplast yield from CEC seedlings preconditioned with 12 and 24 hours of cold was reduced an average of 27.4% (compared to 0 hours cold over all levels of dark). However, the subclass 12 hours cold/48 hours dark was not significantly different from 0 hours cold/all levels of dark. In contrast, yield from IV seedlings was not significantly reduced by cold preconditioning, but decreased an average 43.9% by a 48 hour dark treatment over all levels of cold. Protoplast viability was improved an average of 14.1% of CEC seedlings receiving a 48 hour dark treatment, and was 0% for the 2 treatments 12 and 24 hours cold/0 hours dark. For IV seedlings preconditioned with 12 and 24 hours cold/0 hours dark protoplast viability was 6.9% greater than compared to 0 hours cold.

CEC microcalli diameter of 12 and 24 hour cold were 12.5% less than 0 hour cold. In contrast, IV microcalli from 12 hour cold/0 hour dark were 11.8% larger than microcalli from all other IV preconditioning treatments.

Shoot morphogenesis from CEC seedlings was 10.3% less from 0 hours dark compared to 24 and 48 hours dark, and 25.4% less from 24 hour cold compared to 0 and 12 hour cold preconditioning of IV seedlings.

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ABBREVIATIONS

BAP - 6-benzylaminopurine
NAA - alpha-naphthalenacetic acid
2,4-D - 2,4-dichlorophenoxyacetic acid

CEC - controlled environment chamber
IV - *in vitro*

C - factor cold
D - factor dark
S - factor source
CD - cold x dark interaction
CS - cold x source interaction
DS - dark x source interaction
CDS - cold x dark x source interaction

$uEm^{-2}s^{-1}$ - Micro Einstein per square meter and second

INTRODUCTION

Most plant breeding programs follow a process of recombination and selection within an elite germplasm pool to produce superior cultivars. Techniques such as induced polyploidy, wide hybridization, mutant induction and *in vitro* methods supplement primary breeding operations by providing genetic variability difficult and/or impossible to obtain otherwise. *In vitro* methods can be classified into three general catagories; 1) the micropropagation of shoot tips to produce virus-free stock, multiply hard to propagate species and expensive F1 hybrids, 2) the culture and manipulation of protoplasts, cells and tissue to produce genetically useful individuals and, 3) vector introduced genes.

Plant regeneration from protoplasts has been demonstrated in over 70 species, half of which are represented by the Solanaceae. Successful application of *in vitro* techniques such as exogenous DNA and chromosome uptake by protoplasts, somatic cell fusion and cell selection to improve a species economically, requires a base technology of efficient plant regeneration from protoplasts. Factors important in successfully regenerating plants from cultured protoplasts include genotype, growth conditions of the donor plant, pre-conditioning of donor tissue, types and

concentrations of isolation enzymes, composition of the culture medium and the environmental conditions during isolation and culture. In tomato and potato the physiological condition of the donor plant is of critical importance in achieving successful isolations of viable protoplasts capable of sustained divisions (160,164,168). Tabaeizadeh et al. (175) found that a cold/dark treatment of whole plants and detached leaves significantly increased the number of protoplast-derived calli though the effect was not as great with detached leaves. Shahin (160) reported the necessity of dark and low temperature preconditioning of donor tomato tissue in isolating viable protoplasts. Likewise, Shepard and Totten (165) used a short photoperiod and low temperature preconditioning of donor plants. Without such preconditioning, protoplasts failed to undergo division regardless of the culture medium used. How these factors influence the various stages of growth from initial cell viability to shoot formation from protoplast-derived calli is little understood as much of the research to date is empirical and/or anecdotal.

This study attempts to identify those stages of protoplast development most influenced by dark and cold preconditioning of the donor plant by preconditioning donor tissue and measuring protoplast yield, viability, rate of cell colony growth and shoot morphogenesis. The extent that such treatments are necessary in achieving shoot regeneration can then be examined by determining the contribution of cold

and dark on each measured variable. The tomato, Lycopersicon esculentum, is used in this study as it is the world's leading vegetable crop and is also a prominent species for somatic cell genetic manipulations.

LITERATURE REVIEW

I. History of the Tomato

The cultivated tomato, Lycopersicon esculentum, originated in western South America and is a member of the family Solanaceae. All ten species in the genus Lycopersicon are $2n=2x=24$ (146). Domestication is thought to have first occurred in Mexico. When the tomato was first taken to Europe in the 16th century it was thought to be poisonous due to deadly glycoalkaloids present in the other members of the nightshade family such as belladonna and henbane.

The tomato currently ranks second to potato in economic importance among vegetables, with a combined value for processing and fresh market fruit exceeding 800 million dollars per year in the United States (111). In addition to its culinary appeal the tomato is one of the best known crop plants in terms of its established genetics and cytogenetics (147). A favorable biology such as a short life cycle, ecological versatility, high seed yields (up to 20,000 seeds per plant), diploid genome, self-pollinating and amenability to hybridization are responsible for this species appeal to researchers. Plant breeders have made significant improvements during the past 50 years as follows:

1. Increased yields - larger and greater numbers of fruit per plant, improved flower set and a more concentrated fruit set
2. Plant habit changed to accomodate cultural and harvesting operations - the sp gene for determinate growth is mainly responsible for such improvements

3. Improved handling and storage

4. Pest resistance.

II. In Vitro Culture

A. Tissue culture

The first successful in vitro culture of tomato was reported by White in 1934 (199). He succeeded in culturing isolated root tips in liquid suspension culture. The roots grew indefinitely for over a year. This indicated to White that root growth was not dependent on the top of the plant. Twenty years later root cultures were again utilized, but from the wild species Lycopersicon peruvianum (127). Some of the cultured roots formed shoots that were transferred to soil.

It was not until 1973 that routine shoot morphogenesis was first reported using cultured internodes of L. esculentum (42). Numerous reports followed detailing rapid shoot morphogeneous from a variety of tissues: leaves (8,35,74,80,87,106,129,133,137,178), stem internodes (41,103), apical meristems (86), cotyledons (66,92,134,195) and hypocotyls (66,71,103,131,210). The general methodology consisted of placing explants from in vitro or greenhouse seedling explants onto a semisolid medium containing inorganic salts, macronutrients and micronutrients, usually those of Murashige and Skoog (120) , vitamins of the B group (thiamine, pyridoxine and nicotinic acid), glycine, myo-inositol, sucrose and an auxin and/or cytokinin.

Using 64 treatment combinations of indole-3-acetic acid (IAA) and 6-furfurylaminopurine (kinetin), Padmanabhan (133) classified tomato as one of the plant species with morphogenetic expression regulated by the auxin/cytokinin ratio as first discovered by Skoog and Miller (169). When an auxin is used, IAA is reported to be optimum for achieving shoot morphogenesis (80,87), with alpha-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) almost always inhibitory to shoot morphogenesis (7,131,195). Moreover, 2,4-D is generally most useful when initiating or maintaining callus and suspension cultures. The cytokinins zeatin, 6-benzlyaminopurine (6-BAP), kinetin and 6-(gamma,gamma-dimethylallylamino)-purine (2iP), in increasing efficiency, all seem to initiate shoots of tomato with or without an accompanying auxin.

Although there are numerous reports on shoot morphogenesis in the cultivated tomato, these experiments achieved shoot formation from primary leaf, stem or hypocotyl explants (8,80,87,131,133,178) and not from secondary or older callus. DeLanghe and DeBruijne (41) studied shoot regeneration in L. esculentum and L. peruvianum. They hypothesized that the lower shoot-forming capacity of L. esculentum was due to a higher endogenous level of gibberellic acid (GA), as addition of GA to the culture medium resulted in a high production of callus and fewer shoots. Adding 1000 ppm chlormequat (CCC), a GA inhibitor, to the culture medium had no effect on the shoot-forming

ability of the explants. However, if the donor seedlings were sprayed daily with 2000 ppm CCC callus derived from these seedlings maintained a undiminished shoot-forming capacity after 2 years.

Meredith (109) studied shoot regeneration from 17-28 month old callus cultures of L. esculentum and L. pimpinellifolium. L. pimpinellifolium callus did not regenerate shoots, but callus from L. esculentum 'VFNT Cherry' formed shoots, however the shoots were morphologically abnormal and could not be rooted. Plantlets regenerated from younger, 4-month-old callus, were normal and rooted easily.

Behki and Lesley (7) studied the response of secondary leaf callus to the nitrogen source used in the culture medium and the growth regulators used in the induction and differentiation media. They found that decreasing the amount of nitrogen (MS salts used) inhibited shoot regeneration. A similar inhibition of shoot regeneration occurred when the NH_4^+ concentration equalled or exceeded the NO_3^- concentration (NH_4^+:NO_3^- is 1:2 in MS). NO_3^-, however, could be substituted for NH_4^+ without any deleterious effect on shoot formation. They also found in both the callus induction and the differentiation media the duration of the induction period and the presence of light during the induction period was important.

B. Genetic control of shoot morphogenesis

In addition to media components, a strong genetic effect on shoot morphogenesis is found among L. esculentum genotypes (56,64,71,96,97,131,134,186,210). The wild Lycopersicon species L. peruvianum is in general superior to L. esculentum in shoot forming-ability (103,118,178,209).

Ohki et al. (131) was the first to study the genetic transmission of shoot-forming capacity in tomato. The F_1 hybrid whose seed parent had a higher shoot-forming capacity was superior to the reciprocal hybrid in shoot formation indicating a maternal effect. Heterosis was also observed in this particular hybrid. The other hybrid whose seed parent was the poorer of the two parents possessed an intermediate regeneration capacity.

In contrast to Ohki et al. (131), Frankenberger et al. (57) found no reciprocal cross differences or heterosis. Regressing shoot formation of the hybrids onto the mid-parent values gave a heritability estimate of 0.98 indicating additive gene action. A highly significant general combining ability indicated that shoot-forming ability could be predicted from the general combining ability estimates of the parents. A possible explanation for some of the discrepancies between these two experiments (i.e. reciprocal cross differences and heterosis) might be the culture media used. Frankenberger et al. (57) used one culture medium whereas Ohki et al. (131) used three media chosen from a screening test of 35 IAA and 2iP combinations. In only 2 of

the 3 media were reciprocal cross differences and heterosis observed suggesting that the capacity of the hybrids in comparison to their parents depends also upon the growth regulators in the medium. With only one medium, different from any that Ohki et al. (131) used, Frankenberger et al. (57) could have missed observing these effects. Behki and Lesley (8) evaluated different tomato genotypes on media with a range of growth regulator concentrations and demonstrated that the type and concentration of auxins and cytokinins which stimulated shoot formation from leaf tissue differed among genotypes.

Because of the high totipotency of L. peruvianum, some workers have hypothesized the presence of regeneration controlling genes in L. peruvianum and have proposed interspecific hybridization as a means to transfer those genes into the cultivated tomato (32,183). The ability to form shoots appears to be a dominant trait in reports to date (95,97,128,183). Thomas and Pratt (183) developed a tomato genotype L2, with an ancestry of 75% L. peruvianum and 25% L. esculentum. L2 was selected for self-fertility, high rate of callus growth, and high efficiency of regeneration of shoots from callus. Kut and Evans (97) found the F_1 hybrids L. esculentum x Solanum lycopersicoides and L. esculentum x L. hirsutum to regenerate as well as the more responsive wild parent. Koornneef et al. (95) developed a tomato genotype MsK93 for superior shoot regeneration from cell cultures. Like L2, MsK93 contains the favorable shoot regeneration

genes from L. peruvianum but its genetic backround is greater than 50% L. esculentum.

One strategy not yet employed in tomato is the selection for regeneration potential as successfully pursued by Bingham et al. (16) in alfalfa. Bingham and his co-workers identified five fertile regenerable genotypes. Regenerants from these were intercrossed and the progeny screened for regeneration. Shoot morphogenesis was increased from 12 to 67% after two cycles of recurrent selection.

Why L. esculentum is more recalcitrant than L. peruvianum and L. chilense is not known. There are some observations that may contribute to its morphogenetic response. First, chromosome changes are negatively correlated with the capacity of cultured tissues to regenerate shoots (203). Plant cells in culture from a wide range of species exhibit a high incidence of polyploidy, aneuploidy and chromosomal rearrangements (6,55,38,98,173,204). Bayliss (6) reported that of 55 species studied only 11 retained the original chromosome number while in culture. Murata and Orton (121) showed that even when the chromosome number remains unchanged there may be a high incidence of chromosomal rearrangement. The extent of chromosomal variation is influenced by several factors. First, differences of cultivars within a species, for example, celery (19), corn (4) and oats (107), indicate a genetic effect. Ogura (130) reported a dominant Mendelian trait inducing chromosome instability in tobacco. Second,

culture conditions such as the concentration and type of growth regulators used in the culture medium can also influence chromosomal stability (89,132,203). Third, Cassells (25) suggested that high endogenous auxin levels in tomato may suppress shoot formation from callus cultures. Comparative assessments of auxin levels in non-regenerating genotypes, regenerating genotypes and wild Lycopersicon species have yet to be done.

C. Protoplast culture

Protoplasts were first isolated from tomato roots by Cocking (33) followed by cotyledon tips (32) and placental tissue from immature fruits (63). These were also the first successful protoplast isolations using enzymatic digestion rather than mechanical methods (31). One recent exception to the prevalent use of cell wall degrading enzymes is the mechanical isolation of protoplasts from auxin conditioned callus of Saintpaulia ionantha (African violet) (13). Though these early attempts demonstrated the feasibility of recovering large numbers of protoplasts using fungal derived enzymes, the protoplasts rapidly degenerated. Pojnar et al. (139) reported the first observation of cell wall synthesis using protoplasts isolated from tomato fruits. The first report of sustained cell division was by Nagata and Takebe (123) for tobacco leaf mesophyll protoplasts. The following year they reported the regeneration of plants from protoplast-derived calli (122).

L. peruvianum with its high morphogenetic capacity was

the first Lycopersicon species regenerated from protoplasts (118,149,207,209). Plants of L. peruvianum var. dentatum and L. chilense also are easily regenerated from callus and cell suspension-derived protoplasts (73,138). Numerous early attempts to regenerate plants from protoplast-derived calli of L. esculentum failed (27,45,117,118,166,177,209). Morgan and Cocking (115) screened fourteen cultivars of L. esculentum for their morphogenetic potential using a petiole-derived callus assay. The cultivar 'Lukullus' produced many green meristematic regions which readily developed into shoots. When protoplasts were isolated from leaf tissue of Lukullus they divided and formed calli that regenerated shoots when placed onto MS salts and vitamins (120), 2% sucrose, 0.8% agar and 1 mg/L zeatin. However, of the nine cultivars from which protoplasts were isolated, plants were only recovered from 'Lukullus'. Protoplasts were isolated from cell suspension cultures derived from two-year old callus cultures of 'Lukullus' (94). Shoots were regenerated from callus derived from protoplasts but they were abnormal and failed to root. However, using cotyledon protoplasts from the cultivar 'Nadja' morphologically normal plants were recovered (93). Shahin (160) used pre-conditioned in vitro grown seedlings and was able to regenerate plants from fourteen tomato cultivars. However, he could not isolate protoplasts from greenhouse or growth chamber grown seedlings. In our laboratory we have succeeded in regenerating fertile plants from both in vitro and growth

chamber grown seedlings of seven diverse genotypes (124,125) without pre-conditioning.

D. Anther Culture

Most attempts to regenerate haploid tomato plantlets via anther or microspore culture have failed (21,30,39,44,65,101,161,162,205,206) except for L. peruvianum (144). In tomato, haploid plantlets have only been obtained from cultured anthers (23,64,212,213) but not in large numbers. Although, haploid embryoids were obtained culturing uninucleate microspores in liquid medium (192). Gresshoff and Doy (64) reported the recovery of abnormal haploid plantlets from one of forty-three tomato lines studied. The factors identified as essential for haploid plantlet recovery were an amenable genotype, anthers cultured when the pollen mother cells were still in metaphase I and the use of light during plantlet development.

Given a responsive genotype, one of the principle factors affecting haploid induction is the stage of microsporogenesis at which the anthers are cultured (76,172). The correct stage must be determined experimentally for each species. Several workers (3,44,150) reported on the beneficial effect of a cold pretreatment on anthers which disrupts microsporogenesis. This finding led Zamir et al. (206) to use fifteen male-sterile mutants representing four stages when microsporogenesis was blocked - pre-meiotic, meiotic, tetrad and microspore. Ms 10^{35}, a male-sterile mutant characterized by an arrest in the development of

meiocytes at the tetrad stage, formed haploid callus; however, no plants were regenerated. Ziv et al. (212,213) using ms 10^{35} and the culture system of Zamir et al. (206) succeeded in recovering haploid plants. Anthers of heterozygous plants (tf/+) carrying the allele for trifoliate were placed into culture (212). All plants regenerated were trifoliate; thus, they were believed to be doubled haploids of sporogenic origin. It was unknown why normal plants were not recovered.

E. Uses

In vitro culture systems have been used to study a wide variety of physiological, genetic, developmental and breeding problems.

Warren and Routley (197) used callus cultures of resistant and susceptible cultivars to Phytophthora infestans to determine if the disease response in culture correlated with the reaction of the whole plant. Some differences were noted but they were not as delineated as at the whole plant level. A somaclonal variant resistant to race 2 of Fusarium oxysporum f. sp. lycopersici was selected from the progeny of plants regenerated from callus cultures of UC82B (50,112). No selective agents were used and resistance was determined to be governed by a single, dominant gene. Scala et al. (69,156) were able to select cell lines from cotyledon-derived cultures showing higher partial resistance to F. oxysporum f. sp. lycopersici race 1 filtrate than unselected controls. No plants were regenerated. Of 370 plants

regenerated from leaf discs of a fully TMV susceptible line six were selected as putatively resistant (5). Progeny testing revealed varying degrees of resistance. Screening for tobacco mosaic virus (TMV) resistance at the callus and protoplast level, Toyoda et al. (184) found significant differences between callus from resistant and susceptible genotypes. These differences were not observed at the protoplast level. Zhuk et al. (211) used cell suspension cultures to show that TMV infection increases the number of metaphases with concomitant disturbances of the spindle and in the frequency of chromosome aberrations at anaphase and telophase. No ploidy changes were observed.

Somatic hybrids have been recovered between potato + tomato (108,148,163), Solanum lycopersicoides + tomato (70), Solanum nigrum + tomato (68,79), tomato + S. rickii (128) and L. peruvianum + Petunia hybrida (174). Binding (14) achieved cell division between petunia + tomato heterokaryons but no plants were recovered.

Meredith (110) selected variant cell lines tolerant of aluminum. Variant cell lines were stable but no plants were regenerated making it difficult to rule out epigenetic effects. Suspension cultures of L. peruvianum were exposed to a step-wise increase of the concentration of cadmium sulphate (9). Exposure of tolerant and sensitive genotypes to Cd, Cu or Zn led to the intracellular accumulation of a low molecular weight cystein-rich cadmium-binding protein. This protein was increased 5-fold in selected cell lines.

Epigenetic effects could not be ruled out as plants were not regenerated for genetic analysis. Tolerance to NaCl was observed between cultivars using cultured cotyledon explants (54). Differences were noted on the basis of explant growth and callus proliferation. Ellis (48) was unable to isolate cell lines resistant to the herbicide metribuzin using cell suspension cultures. However, Harrison et al. (72) detected differences in response of tomato cultivars to metribuzin using callus cultures initiated from the hypocotyl. The sensitivity of this assay was decreased due to a high level of variabilility. Paraquat-tolerant tomato mutants isolated from cell cultures had a 30-fold increase in whole plant tolerance (181). A detailed genetic analysis was not performed though some of the progeny of tolerant individuals retained the trait. When cell suspension cultures of a L. esculentum x L. peruvianum hybrid were irradiated with 294 rads/min for a total of 11,760 rads glyphosate tolerant cell lines were selected (170).

Tomato protoplasts were able to take up laboratory made positively charged lipid vesicles without any fusion treatment (28) indicating a potential vehicle for delivering compounds directly into the cell.

Root cultures were compared to stem-derived callus for sugar uptake (29). Roots grew better with sucrose than glucose and fructose while callus grew well in sucrose and glucose and slightly less well on fructose. Coleman and Greyson (34) studied root formation using leaf discs and

found that GA inhibited rooting when IAA was present but stimulated rooting in the dark without IAA.

The *in vitro* response of ovaries from newly opened flowers can be used in screening genotypes for parthenocarpic progeny to be used in breeding the same season (69). Rastogi and Sawhney (145) studied the nutritional and hormonal factors controlling flower morphogenesis by culturing young floral buds. Embryo culture has been used to obtain interspecific hybrids between *L. esculentum* and *L. peruvianum* (18,95) and *L. esculentum* and *L. minutum* (187).

Utilizing tissue culture induced variation in tomato, Evans and Sharp (51) recovered thirteen single gene mutations including male-sterile, jointless, tangerine fruit, lethal albino, virescent, indeterminate, mottled and green base. Buiatti et al. (20) found 17.04% of the progeny of tissue culture-derived plants had chlorophyll mutations or other morphological abnormalities. These mutants segregated 3:1. Polyploids and chimeras were also found. Plants regenerated from hypocotyl segments from a homozygous (*LaLa*) leafless lanceolate mutant exhibited both leafy and leafless phenotypes (157). Progeny of leafy regenerates segregated into homozygous mutants (*LaLa*), heterozygotes (*La/+*) and normal plants (*+/+*) indicating genetic reversion. These studies suggest that tissue culture induced variation may be of value to tomato breeders.

Tissue culture provides a convenient way to transform plants with *Agrobacterium tumefaciens* (106). Kanamycin

resistance was incorporated into 4 cultivars using stem explants (11). Tomato protoplasts were used to study the hormone relationships of a number of Agrobacterium transformed cell lines (194).

III. Preconditioning of donor tissue

Protoplasts have now been isolated from every plant tissue and organ including tissue derived callus and cell suspension cultures. The physiological condition of the donor plant markedly effects the yield, viability and subsequent differentiation of the protoplasts.

A. Genotype

Given suitable culture medium and environmental conditions, genotype is probably the single most important factor determining the physiological response of plant protoplasts (125,160). As mentioned earlier, profound differences are observed even between cultivars within the same species. Niedz et al. (125) regenerated plantlets from six diverse tomato genotypes. The frequency of calli forming shoots ranged from 2-22% with two genotypes not responding. Likewise, Morgan and Cocking (115) found only one tomato cultivar, 'Lukullus', of 14 tested, that was capable of shoot morphogenesis from protoplast-derived calli.

Arguably it might be stated that all genotypes are capable of shoot morphogenesis from protoplasts if only the proper cultural conditions are found. However, such conditions have not always been found even with a considerable search. For instance, many investigators have

attempted to regenerate plantlets from cereal protoplasts but only in a few cases, sugar cane (193), pearl millet (193) and rice (37,202), has success been reported. In Zea mays, Potrykus (140) tested tens of thousands of culture media to no avail. To date, only 3 lines of maize have been reported (113) which are capable of cell wall formation and cell division from protoplasts - inbred line A188, Black Mexican Sweet and B73.

B. Greenhouse, CEC, In vitro

Greenhouse grown plants have been used to isolate and regenerate plants from protoplasts (26,114,115,175). However, because of varying light and temperature regimes year-round repeatability is difficult (198). Controlled environmental chambers (CEC) reduce some of the variability associated with greenhouse grown plants (1,2,12,24,67,126,152,155,165,168). For instance, leaves taken from pea plants in a CEC yielded protoplasts that divided and formed callus, while leaf-derived protoplasts from greenhouse grown plants died and only rarely divided (2).

Aseptically grown plant material provides a number of advantages over greenhouse and CEC grown plants. First, light, temperature and nutrient supply are uniformly controlled and, second, tissue sterilization with toxic substances is avoided (81,171). In one case it was the only successful method to isolate and culture tomato leaf mesophyll protoplasts (160).

C. Light

Little work has been done on the effects of duration, intensity and quality of light on protoplast source plants. Bhatt and Fassuliotis (12) found a long photoperiod (16 hr) and high light intensity (7500 lux) beneficial for consistently high yields of eggplant protoplasts capable of division. When plants were grown under a 10 hour photoperiod and high light or lower light (4300 lux) and 12 or 16 hour photoperiod, protoplasts failed to divide.

In potato, protoplast division was only observed when source plants were previously conditioned for 4-10 days under a 6 hour photoperiod of 7000 lux with fluorescent light (164). Binding (15) found protoplast yield of _in vitro_ grown haploid and diploid _Petunia hybrida_ dependent on several factors including an optimal light intensity of 7000 lux, similar to both previous studies.

Watts et al. (198) discovered that illumination in excess of 25,000 lux of greenhouse grown tobacco plants decreased protoplast yield. Tal and Watts (177) reported that greater numbers of viable tomato protoplasts, as measured by their plating efficiency, were obtained from plants preconditioned under low temperature (15^{o}C) and high relative humidity (82%). Modification of membrane characteristics was suggested for the increased stability of protoplasts under these conditions.

The production of viable protoplasts from greenhouse-grown plants depended on a dark pre-conditioning treatment of

shoot cuttings 30-162 hours prior to isolation (36). Shahin (160) recommended transferring donor plants 48 hours before isolation to the dark to reduce starch accumulation and to favor the osmotic adjustment of the cells after isolation.

Cell divisions and colony formation were high when Solanum pennellii plants were transferred 6 days before isolation to short-day conditions (8/16 hour photoperiod)(49).

Protoplasts isolated from freshly removed leaves of greenhouse or CEC grown Vicia narbonensis plants did not divide (46). A 2-4 day dark treatment was not beneficial. However, when the leaves were first pre-cultured on basal medium plus BAP and p-chlorophenoxyacetic acid (CPA) for 7-8 days, the isolated protoplasts divided.

D. Nutrition

Cassells and Barlass (27) reported that protoplast viability from leaves of greenhouse grown tomatos was increased by feeding with calcium nitrate (0.1 M twice weekly) and low light intensities (2.52-10.8 MJ $m^{-2}day^{-1}$, 16 hr photoperiod). Protoplast stability seemed dependent on a relatively high calcium content (4-4.7% of dry matter) of the leaves. Supplemental feeding of donor plants with calcium also helped increase protoplast stability of tobacco (197). The authors believe feeding is important as it increases membrane calcium and may have other beneficial effects connected with increased nitrate and chloride uptake and with anion/cation interactions. One difference between tomato and

tobacco in these two previous studies is the influence of the flowering state. Poor quality protoplasts were obtained once a tobacco plant entered the flowering state but not in tomato. It is suggested that endogenous changes are occurring that are incompatible with the isolation of stable protoplasts.

E. Tissue age and source

The age of the tissue affects protoplast isolation and viability (102,119,191). David et al. (40) report that yield and plating efficiency are increased in expanding cotyledons compared to fully expanded cotyledons of Pinus pinaster. Likewise, isolation of protoplasts from cotyledons of cotton led to an increase in the number of damaged protoplasts partly due to the increase in incubation time (91). Kao and Michayluk (84) found protoplasts from the youngest, not fully expanded leaves divided sooner then those from older leaves. Wallin et al. (196) found the use of very young leaves (3-5 mm) in buds from in vitro cultured shoots of apple increased protoplast yield.

Berry et al. (10) obtained the highest yields and plating efficiencies from protoplasts derived from cotyledons of in vitro seedlings of Lactuca sativa. Using in vitro seedlings of sunflower, Lenee and Chupeau (100) found that only protoplasts isolated from hypocotyls divided with repeatibly high plating efficiencies (60%).

F. Senescence retardents

Sawhney et al. (153,154) found that pretreating detached oat leaves for 18 hours in a solution containing a senescence retardant (eg. cycloheximide, kinetin, L-lysine, L-arginine) increased protoplast stability. An associated decrease in nuclease activity and an improvement in the incorporation of protein and nucleic acid precursors was also observed. Conversely, the addition of L-serine, a senescence promoter, to the conditioning solution decreased protoplast release and did not improve stability. High RNase activity, low rates of amino acid and nucleoside incorporation are phenomena associated with senescent leaves.

G. Temperature and humidity

Mesophyll protoplasts of rape plants grown at 19-21^{o}C and 40-45% relative humidity were capable of sustained division and subsequent plant regeneration. Protoplasts derived from plants grown at a higher temperature and humidity, 26^{o}C and 70% respectively, failed to survive (88).

MATERIALS AND METHODS

I. Protoplast isolation and culture

A. Source Material

Seeds of P.I. 367942 (L157) were obtained from the North Central Regional Plant Introduction Station, Ames, Iowa. A single plant selection was made and selfed seed collected from this individual. The process was repeated for a total of 3 generations to reduce any heterogeneity that may have existed in the original seed sample.

B. Seedling Preparation

1. CEC grown seedlings

Seeds of L157 were sown on soil-less planting medium (VSP, Bay Towing Co., Houston, TX) in plastic containers and germinated in a controlled environment chamber (CEC). Total light intensity of 150 $uEm^{-2}s^{-1}$ was provided by cool-white fluorescent lamps and 6 incandescent bulbs providing 20 $uEm^{-2}s^{-1}$, both on a 14 hour photoperiod. The temperature was $27^{o}C/25^{o}C$ day and night, respectively. Seedlings were transplanted 10-12 days following sowing into VSP in plastic 4-cell (2"x2") pack containers and grown under the same environment. Seedlings were watered daily with a 20-20-20 fertilizer at 150 ppm N, with the pH adjusted to 6.2 with 50% H_3PO_4.

2. <u>In vitro</u> grown seedlings

Seeds of L157 were surface sterilized with 70% ethanol for 3 minutes followed by 30 minutes in a solution containing

30% (v/v) Big Chief Bleach (Patterson Laboratories, Detroit, MI) and 6 drops/l Tween-20 (Sigma Chemical Co., St. Louis, MO), and subsequently washed 4 times with sterile distilled water. Thirty to forty seeds were sown per container (Magenta Corp., Chicago, IL) containing 50 mls of MS medium plus 30 g/l sucrose. Ten to twelve days after sowing seedlings were cut at the hypocotyl, and the apical portion reinserted into fresh MS medium. Seed germination and seedling growth were under a light intensity of 35 $uEm^{-2}s^{-1}$, 16 hr photoperiod provided by cool-white fluorescent lamps and a $27^{o}C/25^{o}C$ day/night temperature regime.

3. Cold and dark pretreatment of donor tissue

In vitro and CEC grown donor plants were preconditioned immediately prior to protoplast isolation, cold treatments included 0, 12 and 24 hours at $10^{o}C$. Dark treatments included 0, 24 and 48 hours dark at $26^{o}C$. All possible combinations (9 cold/dark treatments) of cold and dark were tested. The 9 cold/dark treatments were given to 2 donor tissue sources, CEC and in vitro, for a total of 18 treatment combinations (3x3x2 factorial). All preconditioning treatments were carried out in CEC.

C. Protoplast Isolation

1. CEC grown seedlings

The first fully expanded leaves of 3-week old plants, following any preconditioning, were excised and surface sterilized for 20 minutes in a solution containing 15% (v/v) Big Chief Bleach (Patterson Laboratories, Detroit, MI) and 6

drops/l Tween-20 (Sigma Chemical Co., St. Louis, MO) and subsequently washed 4 times with sterile distilled water. Leaflets were sliced transversely into 1 mm strips and plasmolyzed for 1 hour in CPW salt solution (58) containing 13% (w/v) sorbitol at pH 5.7. This plasmoticum was removed and replaced by a filter-sterilized enzyme solution containing 3.0% (w/v) Meicelase-P (Meiji Seiki Kaisha Ltd., Tokyo, Japan), 0.1% (w/v) Macerase (Calbiochem, San Diego, CA), 9.0% (w/v) sorbitol and CPW salts at pH 5.7. Leaf strips plus enzyme solution were incubated at 27^{o}C in the dark on a gyratory shaker (45 rev/min) for 4-6 hours. Digested tissue was filtered through a 60 um sieve, the filtrate was collected and placed into 16-ml screw-capped tubes and centrifuged at 35 x g for 10 min. The supernatent was discarded and the protoplast pellet resuspended in protoplast washing medium (KM-T w/o growth regulators and 0.4 molar sorbitol as osmotic stabilizer). The protoplasts were washed 2 times in protoplast washing medium and finally resuspended in flotation medium and centrifuged at 100 x g for 10 min. Flotation medium was prepared by mixing 1 part Lymphoprep (Accurate Chemical and Scientific Corp., Westbury, NY), 2 parts CPW salts plus 0.5M sucrose, adjusting the pH to 5.8, and filter-sterilizing the solution. After centrifugation, protoplasts at the surface were removed and resuspended in culture medium, KM-T (Table A18). Protoplasts were counted and plated in 2 mls of KM-T over 2 mls of A4 (Table A18) in 60 x 15 polystyrene culture dishes (Falcon,

Becton, Dickinson and Co.) at a density of 3 x 10^4 protoplasts/ml. A4 was sterilized by autoclaving for 15 minutes at 15 p.s.i.

2. In vitro grown seedlings

All leaf tissue of 3-week old transferred shoot tips, following any preconditioning, were excised and sliced into 1-2 mm sections. Leaf sections were placed into a filter-sterilized enzyme solution containing 1% (w/v) Cellulysin (Calbiochem, San Diego, CA), 0.1% (w/v) Macerase, 0.4 molar sorbitol and CPW salts at pH 5.7. Incubation of in vitro tissue was the same as described for CEC tissues except the time was 4-5 hours. Purification was as previously described for CEC tissues. Plating density was 6 x 10^4 protoplasts/ml in 2 mls KM-T over 2 mls A4.

D. Protoplast Culture and Shoot Morphogenesis

The protoplasts were initially cultured at 27°C in the dark for 7-10 days and then transferred to low light (3-4 $uEm^{-2}s^{-1}$ from cool-white fluorescent lamps). Two to three weeks after isolation, depending upon the cell division rate, the liquid medium containing the cells and cell colonies was removed and placed onto fresh medium, KM-T with 0.3 molar sucrose, the 2,4-D deleted and 0.6% purified agar added (Table A19). Thirty day-old calli were removed by pipette and placed onto Whatman No. 1 filter paper overlaid on MS + 2 mg/L zeatin (MS2Z) with 0.15 M sucrose. Filter paper with attached calli was transferred to MS2Z with 0.087 molar

sucrose in another 2 weeks. As individual calli reached 3-4 mm in diameter they were removed and placed directly onto MS2Z medium. Calli showing shoot organogenesis were transferred to regeneration medium containing 1 mg/L zeatin (trans isomer) for further shoot development. Shoots were excised and rooted on MS medium without growth regulators. Rooted shoots were transplanted into VSP soilless mix and kept under clear plastic for 2 weeks before transfer to the greenhouse.

II. Experimental design and data analysis

The four variables measured were protoplast yield, viability, cell colony diameter and shoot morphogenesis. Yield was calculated as protoplasts released/two gram of fresh weight of tissue in 25 ml enzyme solution. Percent viable protoplasts twenty-four hours after isolation was determined by fluorescein diacetate staining (200) and visual observation. Microcalli diameter was measured thirty days after plating with an ocular micrometer. Shoot morphogenesis was the number of calli that underwent shoot morphogenesis during 3 months. The method of analysis used in these experiments was the Least Squares Approach (158,159).

RESULTS

There were two empty subclasses for the variables microcalli diameter and shoot morphogenesis from CEC grown seedlings - 12 hr cold/0 hr dark ($CD_{12,0}$) and 24 hr cold/0 hr dark cold ($CD_{24,0}$). Protoplasts isolated from CEC grown seedlings pre-conditioned in either manner died within 24-48 hours.

I. Yield

A. Test of interaction effects

Using the sums of squares for the models R(u,CDS) and R(u,C,D,S,CD,CS,DS)(Table A1) the cold x dark x source (3-way) interaction is significant at the 1% level (Table A5). Testing seedling source separately, the cold x dark (2-way) interaction is significant at the 5% level for both CEC grown seedlings and *in vitro* (IV) grown seedlings (Table A5).

B. Goodness of fit

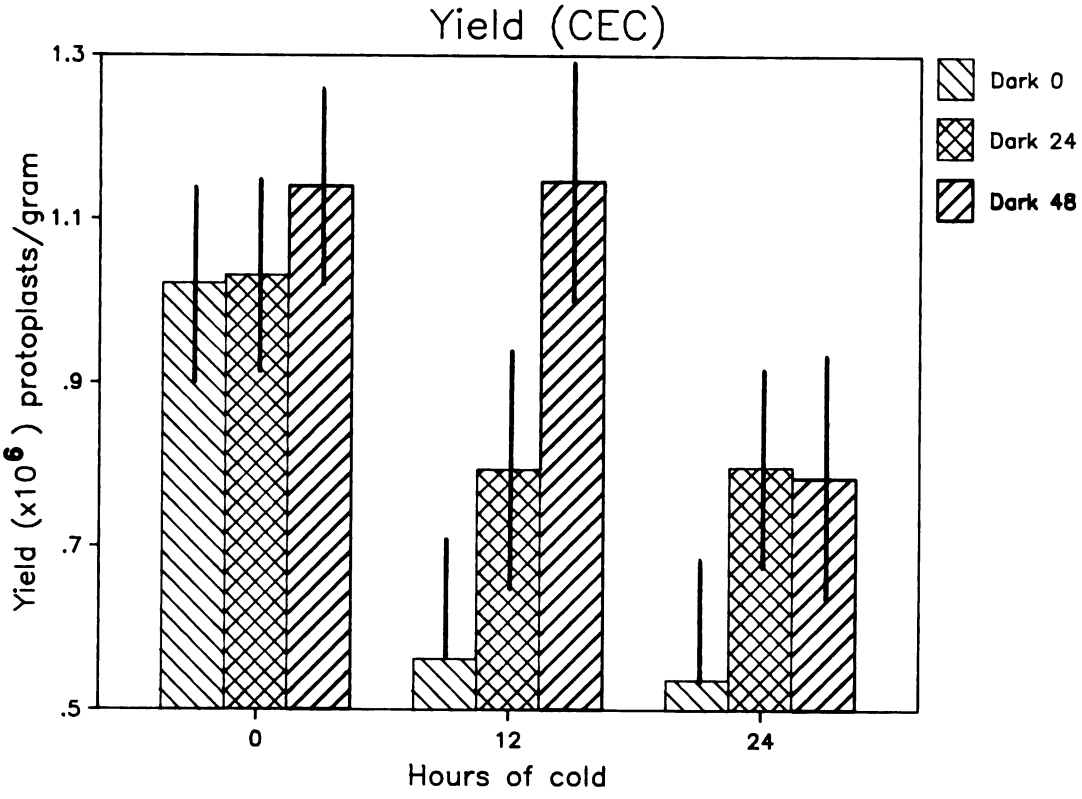
The 3-way interaction model accounts for 90.9% of the observed variation in yield and the model is significant at the 1% level (Table A7). The 2-way interaction model for CEC and *in vitro* grown seedlings accounts for 88.7% and 92.3% (Table A1) of the variation in protoplast yield, respectively. Both 2-way interaction models are significant at the 1% level (Table A6).

C. Contrasts

The smallest subclass means for the variable yield are simply subclass averages $u + CD_{ij}$ (Fig. 2 & Table A8). Because of a significant interaction, comparisons between classes within a factor (the most interesting kind) cannot be made but are limited to comparisons between subclasses or groups of subclasses. For CEC grown seedlings, the contrast to compare all treatments receiving 0 hours of cold to all treatments receiving 12 and 24 hours cold, $2(CD_{0,0} + CD_{0,24} + CD_{0,48}) - (CD_{12,0} + CD_{12,24} + CD_{12,48} + CD_{24,0} + CD_{24,24} + CD_{24,48})$(Table A9), is significant at the 1% level, and indicates that cold preconditioning decreases protoplast yield from CEC grown seedlings. However, the treatment 12 hours cold and 48 hours dark is not significantly different from the 3 treatments of 0 hours cold over the three dark levels (Table A9). For IV grown seedlings the contrast to compare all treatments with 48 hours dark to all treatments receiving less than 48 hours dark, $2(CD_{0,48} + CD_{12,48} CD_{24,48}) - (CD_{0,0} + CD_{0,24} + CD_{12,0} + CD_{12,24} + CD_{24,0} + CD_{24,24})$(Table A9), is significant at the 1% level indicating a 48 hour dark treatment decreases yield over all levels of cold. Some other contrasts are listed in Table A9. Four preconditioning treatments, $CD_{0,0}$, $CD_{0,24}$, $CD_{0,48}$ and $CD_{12,48}$, resulted in the highest yields from CEC seedlings (Table A17). Three preconditioning treatments, $CD_{0,24}$, $CD_{24,0}$ and $CD_{12,0}$ resulted in the highest protoplast yield from IV seedlings (Table A17).

Figure 1. **Effect of cold and dark pre-conditioning on protoplast yield. (a) CEC grown seedlings. (b) IV grown seedlings.**

a)


Yield (CEC)
1.3
1.1
.9
.7
.5
Yield (x10⁶) protoplasts/gram
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

b)

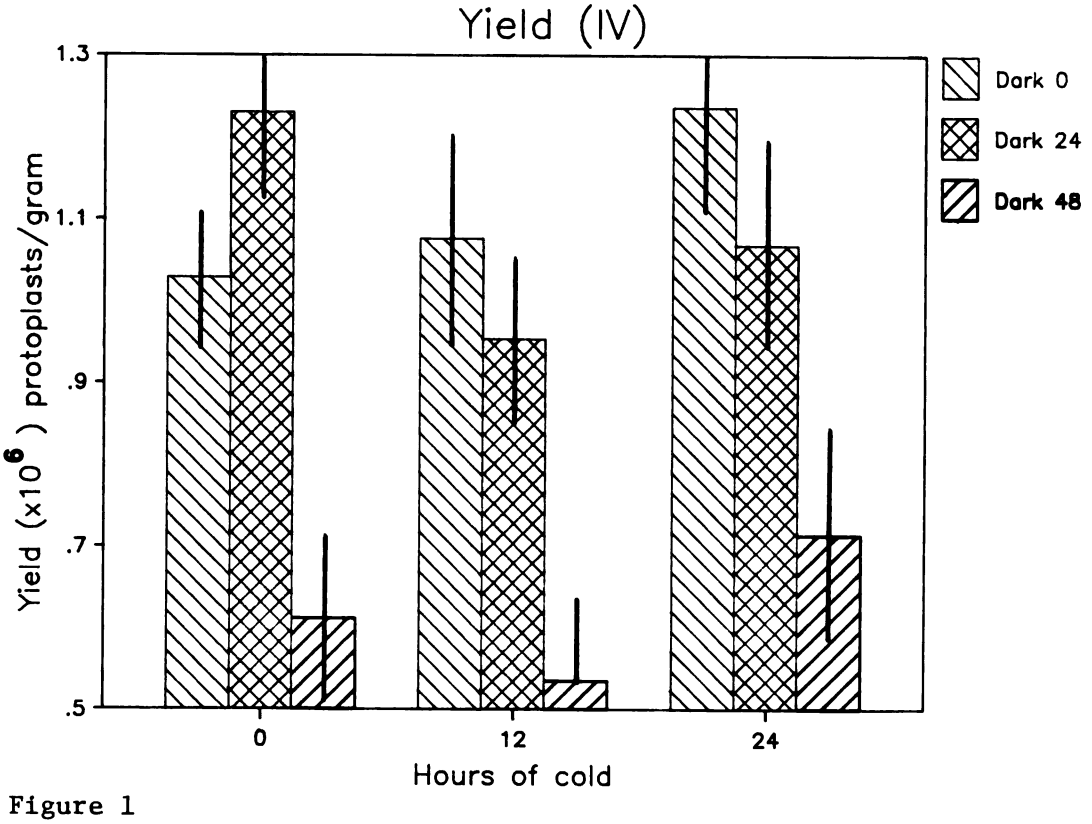

Yield (IV)
1.3
1.1
.9
.7
.5
Yield (x10⁶) protoplasts/gram
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

Figure 1

II. Viability

A. Test of interaction effect

Using the sums of squares for the models R(u,CDS) and R(u,C,D,S,CD,CS,DS)(Table A2) the cold x dark x source interaction is significant at the 1% level (Table A13). Testing seedling source separately, the cold x dark interaction is highly significant for CEC grown seedlings and significant for IV grown seedlings (Table A5).

B. Goodness of fit

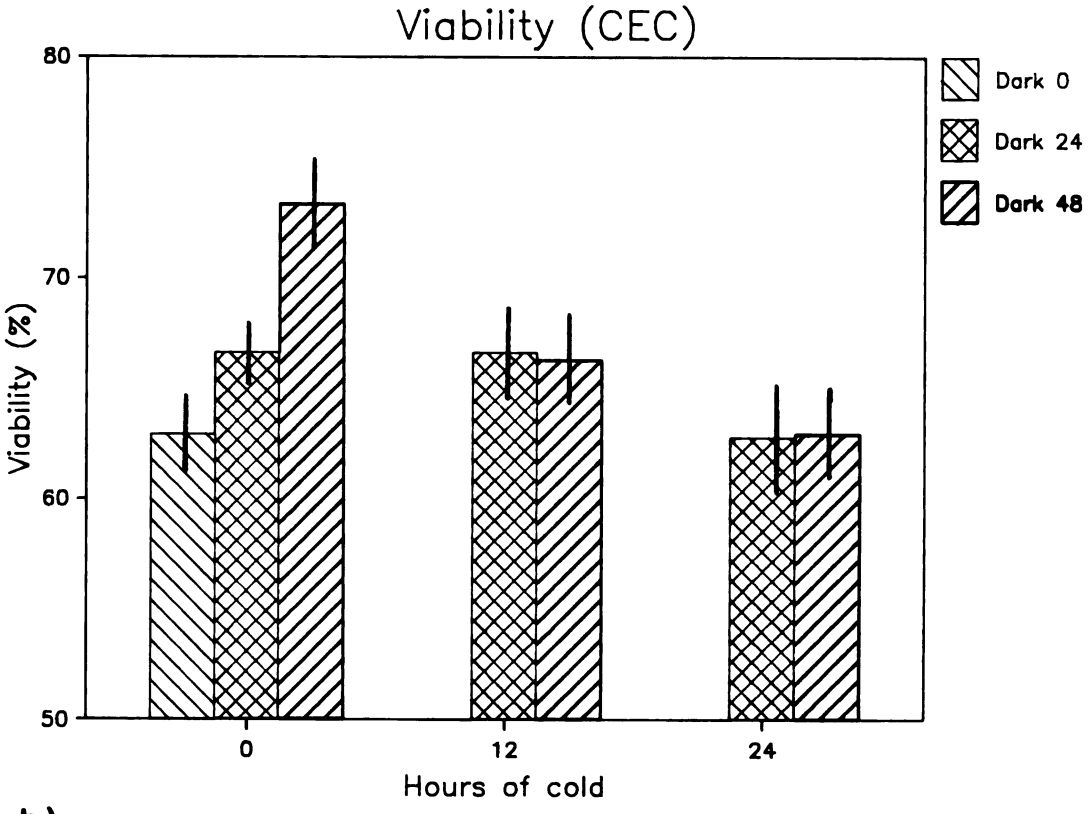
The 3-way interaction model is significant at the 1% level (Table A7) and accounts for 96.4% of the observed variation in protoplast viability. The 2-way interaction models are significant at the 1% level (Table A6) and account for 98.0% and 22.7% in the variation of protoplast viability for CEC and IV derived protoplasts, respectively.

C. Contrasts

The smallest subclass means for the variable viability are subclass averages (Fig. 4 & Table A10). As for protoplast yield comparisons between classes within a factor are not possible. The contrast $CD_{0,48} - CD_{0,24}$ (Table A11) indicates $CD_{0,48}$ gave the highest viability for CEC grown seedlings. However, the contrasts $CD_{12,0} - CD_{24,48}$ and $CD_{12,0} - CD_{12,24}$ (Table A11) indicate the highest viability for IV grown seedlings was obtained from the treatments $CD_{12,0}$, $CD_{12,24}$ and $CD_{24,48}$. For CEC seedlings the preconditioning treatment $CD_{0,48}$ had the highest viability while for IV seedlings $CD_{12,0}$ had the highest viability

Figure 2. Effect of cold and dark pre-conditioning on protoplast viabiltiy. (a) CEC grown seedlings. (b) IV grown seedlings.

a)


Viability (CEC)
80
70
60
50
Viability (%)
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

b)

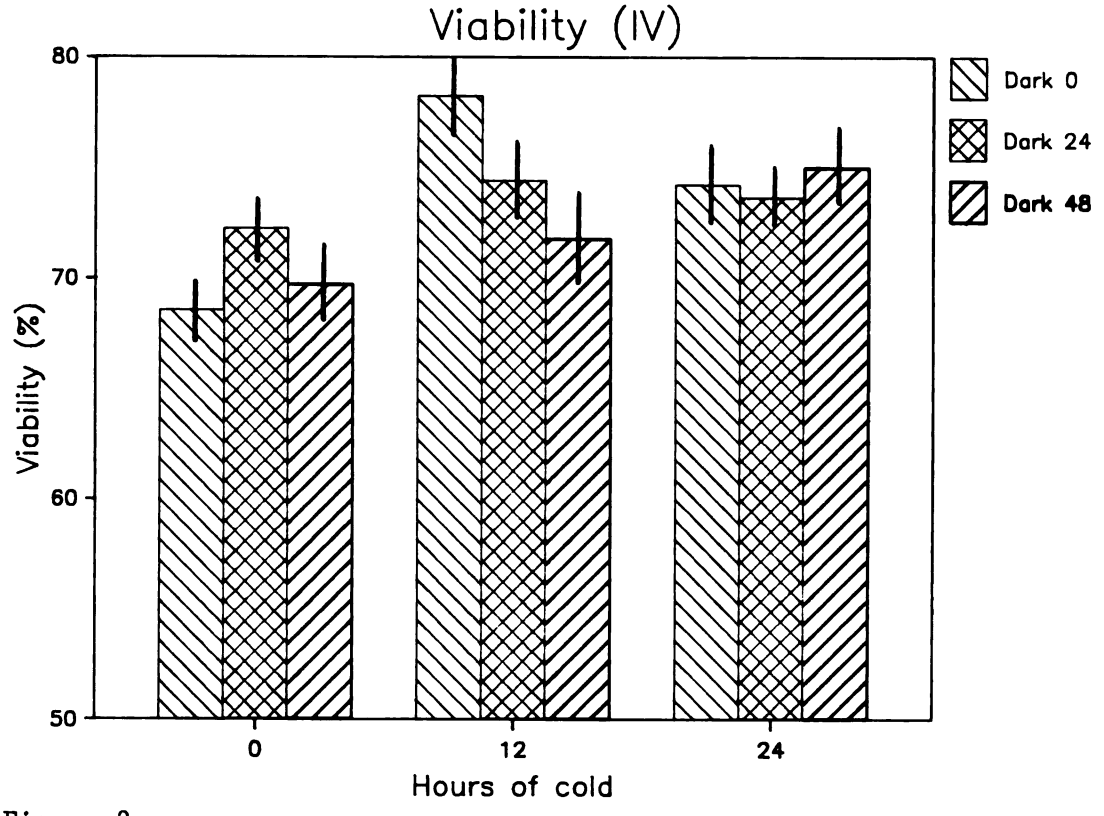

Viability (IV)
80
70
60
50
Viability (%)
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

Figure 2

(Table A17).

IV. Microcalli diameter

A. Test of interaction effect

Using the sums of squares for the models R(u,CDS) and R(u,C,D,S,CD,CS,DS)(Table A3) the cold x dark x source interaction is significant at the 1% level (Table A5). Testing seedling source separately, the cold x dark interaction is not significant for CEC grown seedlings and highly significant for IV grown seedlings (Table A5).

B. Goodness of fit

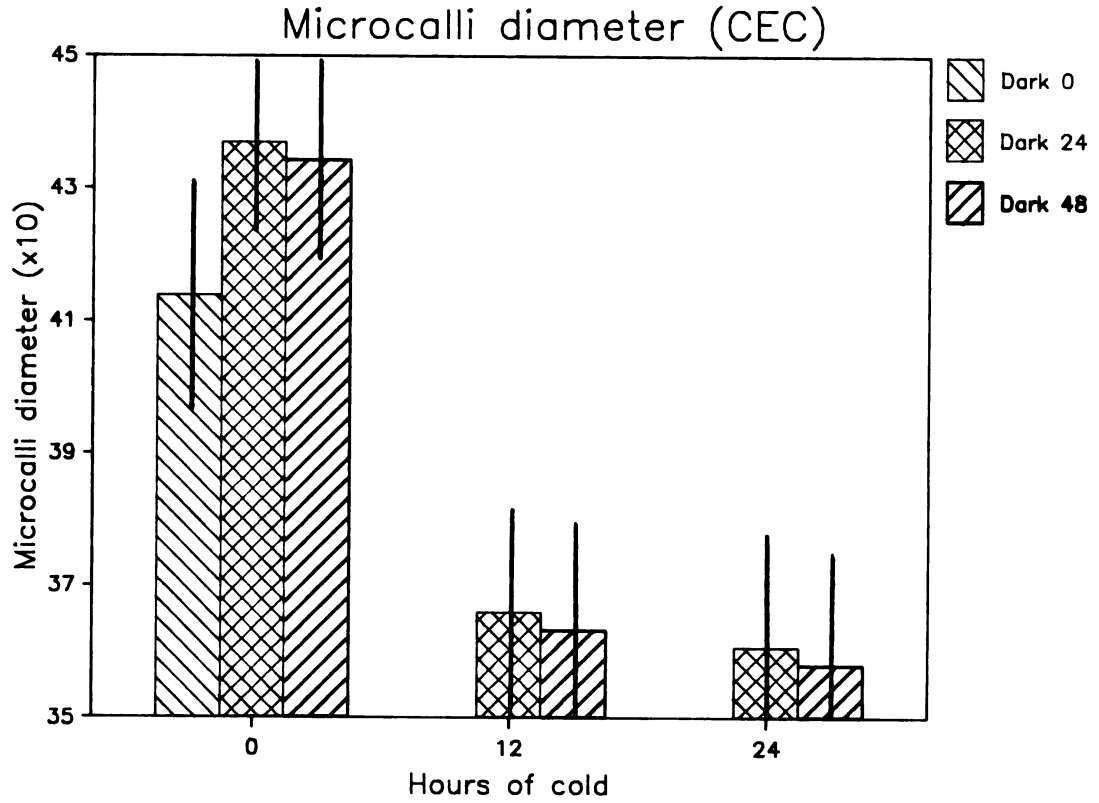
The 3-way interaction model accounts for 73.6% of the variation in microcalli diameter and is significant at the 1% level (Table A7). The 2-way interaction model accounts for 29.3% of the observed variation in microcalli diameter from IV grown seedlings and is significant at the 1% level (Table A6). Whereas, the 2-way interaction model is not significant for accounting for microcalli diameter from CEC grown seedlings (Table A5). The full model R(u,C,D) accounts for 37.6% (Table A3) of the observed variation and is significant at the 1% level.

C. Contrasts

The smallest subclass means for microcalli diameter of IV and CEC grown seedlings are listed in Table A12 and Fig. 6. Referring to Table A17, single factor evaluation of CEC source seedlings shows the factors cold and dark together accounting for 37.6% of the variation, and considered separately accounting for 35.2% and 2.7% of the variation,

Figure 3. Effect of cold and dark pre-conditioning on microcalli diameter. (a) CEC grown seedlings. (b) IV grown seedings.

a)


Microcalli diameter (CEC)
Microcalli diameter (x10)
45
43
41
39
37
35
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

b)

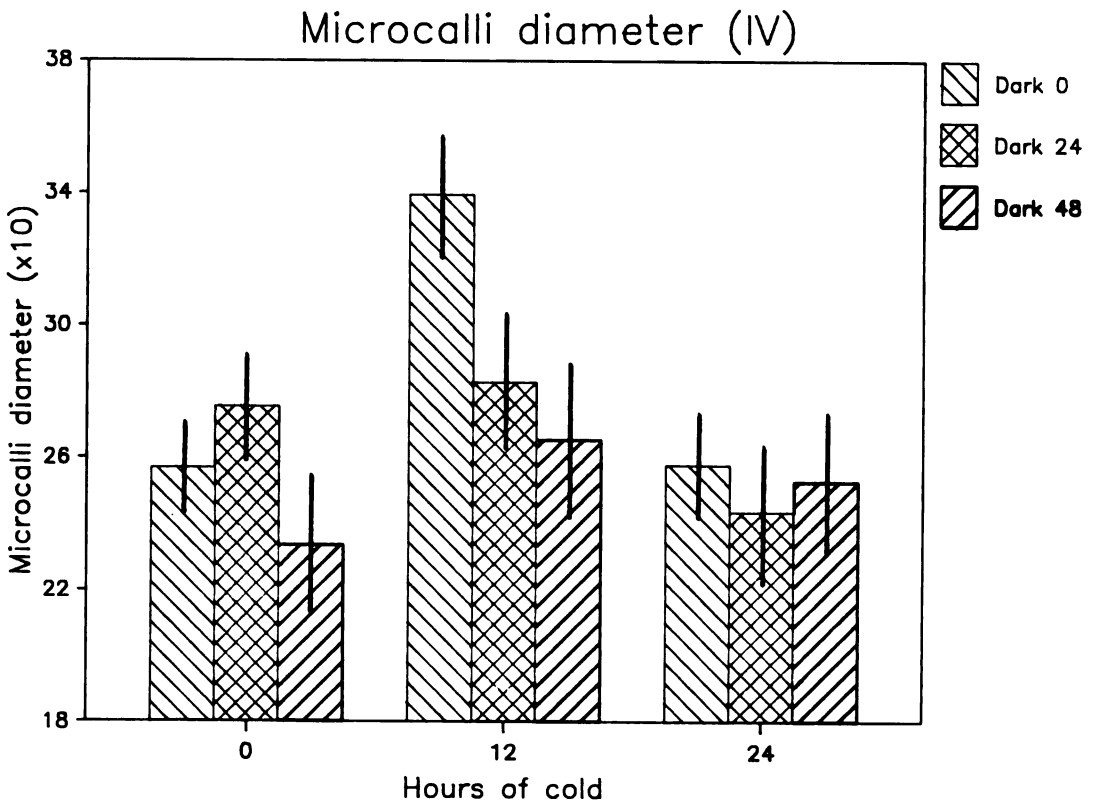

Microcalli diameter (IV)
Microcalli diameter (x10)
38
34
30
26
22
18
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

Figure 3

respectively. The factor dark is not significant.

Factors cannot be examined individually for IV source seedlings due to interaction. The contrast $2CD_{12,0} - (CD_{0,0} + CD_{0,24})$(Table A14) shows the treatment $CD_{12,0}$ having the highest microcalli diameter for IV source seedlings. The largest microcalli diameter from CEC seedlings were observed from the two treatments $C_0 + D_{24}$ and $C_0 + D_{48}$, while for IV seedlings the largest microcalli was observed from the treatment $CD_{12,0}$ (Table A17).

IV. Shoot morphogenesis

A. Test of interaction effect

Using the sums of squares for the models R(u,CDS) and R(u,C,D,S,CD,CS,DS)(Table A4) the cold x dark x source interaction is not significant (Table A5). Testing seedling source separately, the cold x dark interaction is not significant for both CEC grown seedlings and IV grown seedlings (Table A5).

B. Goodness of fit

The 3-way interaction model accounts for 41.4% of the variation in shoot morphogenesis and the model is significant at the 1% level (Table A7). The full model with no interaction accounts for 25.4% of the variation in shoot morphogenesis from CEC grown seedlings and 38.9% from IV grown seedlings.

C. Contrasts

The smallest subclass means for shoot morphogenesis of CEC and IV grown seedlings are $u + C_i + D_j$ (Table A14)

As CEC and IV grown seedlings had no significant interaction effect (Table A5) between cold and dark pre-conditioning single factor effects were assessed (Table A17). The two factors, cold and dark, together describe 39.61% and 25.38% of the variation in shoot morphogenesis from CEC and IV source seedlings, respectively. Cold accounts for <1% of the variation in shoot morphogenesis and dark accounts for 38.40% for CEC source seedlings. For IV source seedlings, cold and dark describe 13.77% and 23.34%, respectively. Cold and dark together or individually are significant at the 1% level from both sources (Tables A5).

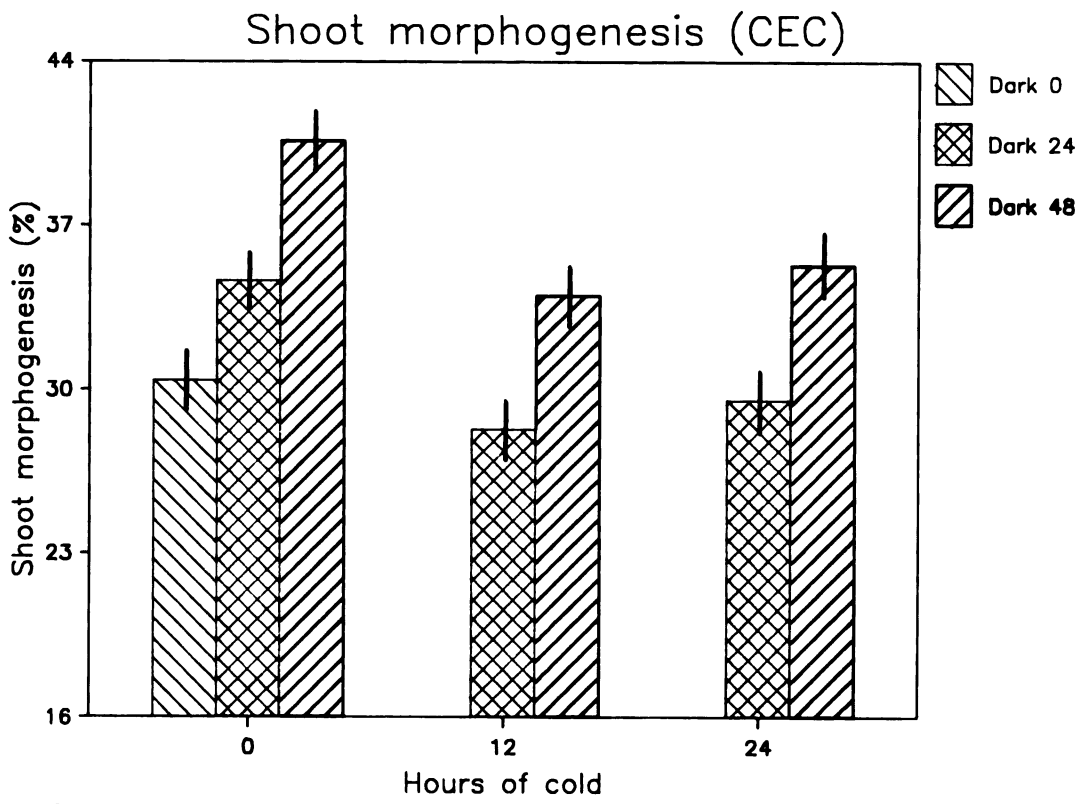
For CEC grown seedlings the contrasts $C_0 = C_{12} = C_{24}$ and $D_0 = D_{24} = D_{48}$ are both significant at the 1% level indicating differences exist among the classes within both the factors cold and dark. These two contrasts are equivalent to the models R(C|u,D) and R(D|u,C), respectively. Contrasting the highest class within each factor with the other two classes, $2C_0 - (C_{12} + C_{24})$ for the factor cold and $2D_{48} - (D_0 + D_{24})$ for the factor dark, shows both contrasts significant at the 1% level. Contrasting the two lower classes of each factor for CEC calli, $C_{12} - C_{24}$ and $D_0 - D_{24}$ for the factors cold and dark respectively, results in the cold contrast being not significant and the dark contrast being significant at the 1% level. It seems the cold

preconditioning treatments, 12 or 24 hours, decreases regeneration and that a dark preconditioning treatment progressively increases regeneration with a 48 hour treatment being the most effective. From CEC seedlings $C_0 + D_{48}$ resulted in the highest frequency of shoot morphogenesis, while for IV seedlings six preconditioning treatments were equally high - $C_0 + D_{0,24\ \&\ 48}$ and $C_{12} + D_{0,24\ \&\ 48}$ (Table A17).

For CEC seedlings $CD_{0,48}$ (and $C_0 + D_{48}$)(Table A17) ranks as the "best" (highest treatment mean) preconditioning treatment under all 4 measured variables, while for IV seedlings, $CD_{12,0}$ (and $C_{12} + D_0$) ranks as the "best".

Figure 4. Effect of cold and dark pre-conditioning on shoot morphogenesis. (a) CEC grown seedlings. (b) IV grown seedlings.

a)


Shoot morphogenesis (CEC)
Shoot morphogenesis (%)
44
37
30
23
16
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

b)

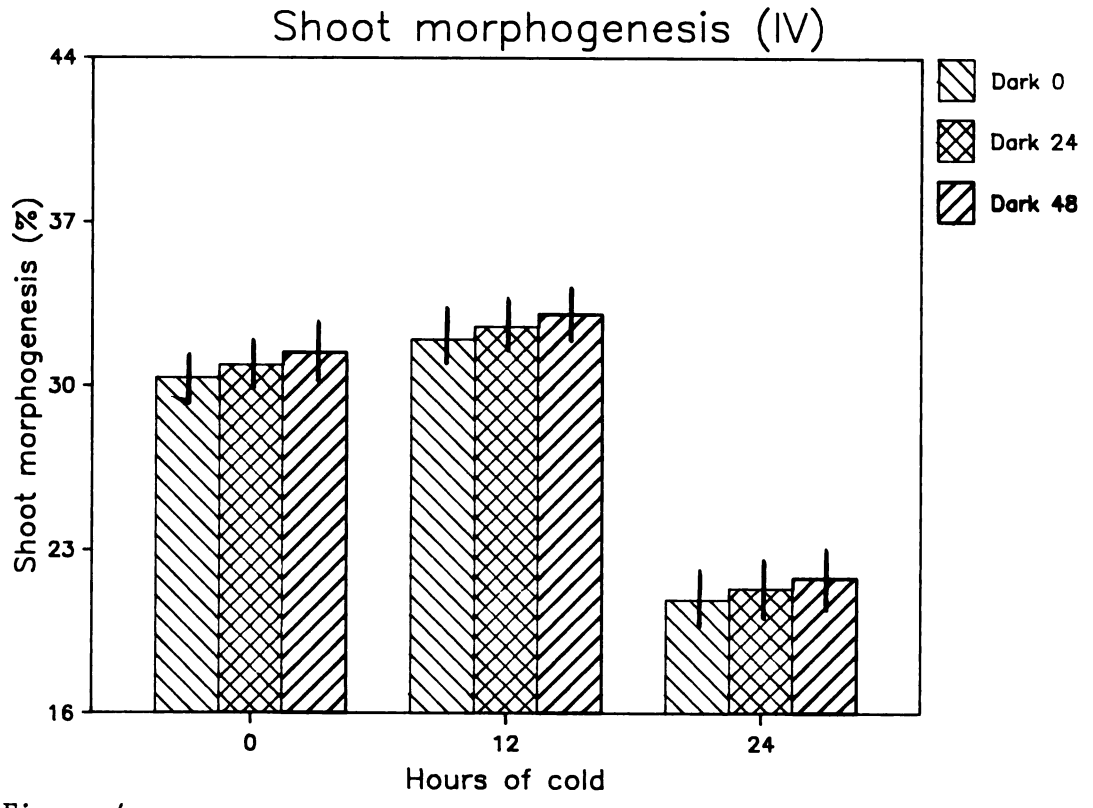

Shoot morphogenesis (IV)
Shoot morphogenesis (%)
44
37
30
23
16
0
12
24
Hours of cold
Dark 0
Dark 24
Dark 48

Figure 4

DISCUSSION

P.I. 367942 (L157) was chosen for this study as it had the lowest frequency (2%) of shoot morphogenesis of the seven cultivars previously regenerated in our laboratory. The morphogenetic response of the other cultivars using the protocols developed for this research remains to be investigated.

I. Protoplast Yield

Protoplast yield from CEC and IV grown seedlings was differentially affected by cold and dark pre-conditioning. The decrease in yield from CEC seedlings under all cold treatments except $CD_{12,48}$ might be due to stress as the treatment times were too short to significantly alter cell wall composition. After 12 or 24 hours of cold, CEC seedlings were wilted. Other experiments with wilted seedling tissue always resulted in a decrease in protoplast yield (unpublished data). Perhaps the low yields associated with water stress are due to mechanical rather than physiological reasons. A wilted leaf does not cut as cleanly as a turgid one thereby resulting in damaged cells along the cut edge. This would limit enzyme ability to penetrate the intercellular tissue resulting in decreased yields. Accordingly, the two treatments with the lowest yield, 12 cold/ 0 dark and 24 cold/ 0 dark were also the most severly wilted. IV grown seedlings were not as affected by the cold treatments as CEC grown seedlings. As IV seedlings were grown under 100% relative humidity they would be less prone

to wilting and water deficits. It is difficult to compare protoplast yields from CEC and IV seedlings as the isolation enzymes used for each source were different. Varying toxicity, specificity and activity of the enzymes plus the possibility of differences in cell wall composition between the two sources makes contrasts between sources almost impossible. Valid contrasts are therefore limited to within source comparisons.

It is clear that cold pre-conditioning is of no beneficial value for increasing protoplast yield from either CEC or IV grown seedling tissue. A 24 hour dark pre-treatment did increase protoplast yield from IV seedlings. The presence of high tissue starch levels has been shown to correlate with a decrease in protoplast yield due to a decrease in the protoplasts ability to withstand mechanical handling (198). By dark treating tissue prior to isolation starch levels are reduced and protoplast stability under handling increases, and more protoplasts are recovered. Perhaps differences in cellular starch levels exist between CEC and IV grown seedlings to explain the beneficial effect of dark on IV seedlings-derived protoplast yield.

II. Protoplast Viability

There are a number of short-term methods of assessing protoplast viability:

1. Intact membrane exclusion of Evans blue dye (60,62,83) or phenolsafranine (53,77,85,105,185,200).

2. Intracellular accumulation of fluorescein (99,167,200) or neutral red (43).

3. Intracellular reduction of methylene blue (75).

4. Cyclosis (136,143).

5. Respiratory metabolism measured by oxygen uptake (176).

6. Photosynthetic activity (83).

7. Ethylene release (52).

Visual assessment of spherical protoplasts with the chloroplasts evenly distributed around the cell proved the most conservative estimate of predicting protoplast viability. Cyclosis is difficult to observe in mesophyll cells. Oxygen, photosynthetic measurements and ethylene release are only applicable to whole cell populations and dyes are more liberal viability indicators. For instance, protoplasts that were clearly of unacceptable quality (e.g. chloroplasts aggregated on one side and the plasmalemma membrane bulging outwards) would stain using fluorescein diacetate (FDA). Also, protoplasts will vary quantitatively in the amount of stain excluded or taken up. Estimates using FDA were consistently 15-20% higher than estimates obtained by visual observation (unpublished data).

Viability is between 50-60% for both CEC and IV derived protoplasts and though statistical differences were noted, the variances were so small that dark and cold pre-conditioning are of little practical consequence except in two classes - when CEC seedlings were exposed to light and cold viability dropped to zero.

The two empty subclasses caused by protoplast death from CEC grown seedlings preconditioned with a combination of light and cold were most likely due to photooxidation, a light and oxygen-mediated chlorosis (141) in cold sensitive species (190). A number of reports have distingushed between the response of plants to chilling in the dark and to chilling in the light (90,142,179,180,188). Chilling in the dark has little effect on photosynthesis (particularly PSII), but in the light chlorosis, photosystem damage (90,142,141,180,188,190) altered chloroplast ultrastructure (179,201) and cellular lipid degradation (141) are observed. These results suggest the potential use of protoplasts in tomato to assay for cold tolerant genotypes.

Other factors might also contribute to the differences in response to cold and dark preconditioning between CEC and IV seedlings. For instance, CEC seedlings were grown under higher light (150 $uEm^{-2}s^{-1}$) than IV seedlings (35 $uEm^{-2}s^{-1}$). Perhaps a difference in the cellular milieu such as osmotic pressure and membrane composition would contribute to the observed response. Also, the available carbon was supplied to each source differently, atmospheric CO_2 for the CEC seedlings and sucrose in the culture medium for the IV seedlings. Carbon source has previously been shown to profoundly effect cold tolerance of tomato cells (117).

III. Microcalli Diameter

Microcalli diameter at thirty days was indicative of the rate of cell colony growth. Only colonies composed of small

cytoplasmically-dense cells were measured, as colonies with large filamentous cell with large vacuoles are poor morphogenetically and would have distorted the data. For example, a single large filamentous cell could easily be the size of a entire colony of smaller cytoplasmically-dense cells. Large vacuolated cells constituted a large proportion of the cell types in CEC-derived cell colonies pre-treated with cold. The fewer cytoplasmically-dense cell colonies grow slower in these cultures than in cultures where the vacuolated cells were less numerous.

In spite of differences between the pre-conditioning treatments all treatments, except the two empty subclasses, yield cell colonies of acceptable quality and quantity. Colony size differed considerably between CEC and IV protoplast-derived colonies. Comparing the subclass means from CEC and IV sources in even the poorest CEC treatment, 48 dark/0,12,48 cold had a larger mean colony diameter than the best IV treatment, 0 dark/12 cold. Most probably the difference is due to the protoplasts from IV source seedlings dividing later (2-3 days) than protoplasts derived from CEC source seedlings. Also, the initial average protoplast size of the two source tissues differs considerably. CEC and IV derived protoplasts average about 35 uM and 10 uM, respectively. Initial protoplast size probably does not contribute greatly to thirty day colony diameter as the cells are virtually indistingushable after ten days.

IV. Shoot morphogenesis

With the exception of the two empty subclasses shoot morphogenesis was acceptable from all treatment combinations.

Within 7-10 days after p-calli were placed onto regeneration media darker green areas appeared in the callus from which shoots would eventually arise. Chlorophyll synthesis was generally observed to precede shoot morphogenesis. Calli varied from seven days to four weeks (experiment was terminated at this time) before the first green meristematic area appeared.

Interaction effects disappeared in both CEC and IV protoplast derived calli assayed for shoot morphogenesis. Analysis of single factor effects showed that only dark preconditioning effects were still present in CEC-derived calli, but both cold and dark effects were present in IV-derived calli. Cold treatments in all cases exerted no beneficial effect on shoot morphogenesis. Cold treatments were not significant for CEC-derived calli and accounted for a lesser proportion of the variation observed among IV-derived calli.

The effect of cold and dark on shoot morphogenesis is in contrast to their effect on microcalli diameter. For microcalli diameter of dark preconditioned CEC-derived calli was not significant. As previously explained for microcalli diameter a large proportion of the CEC-derived cells from cold preconditioned tissue were vacuolated resulting in slower growth of the calli. Also, an increase in the degree of browning was observed in these cultures. In all

treatments only calli of a compact and spherical morphology were chosen for transfer to shoot regeneration medium (MS2Z). As calli from all treatments grew rapidly and underwent chlorophyll synthesis it appears the variation due to cold was removed when morphologically desirable calli were selected.

SUMMARY AND CONCLUSIONS

Leaf mesophyll protoplasts of tomato were enzymatically isolated from CEC and IV grown seedlings previously subjected to cold (0, 12 and 24 hours at 10^{o}C) and dark (0, 12 and 48 hours) in a 3x3 factorial experiment. Protoplasts were cultured in KM-T over A4 solidified with 0.5% purified agar. Media were supplemented with 6-benzylaminopurine, alpha-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. Shoot regeneration was achieved on MS2Z.

Four variables were measured; protoplast yield, viability, microcalli diameter and shoot regeneration. As data was unbalanced and included two empty subclasses for 12 and 24 hours cold/0 hours dark/CEC the Least Squares Approach was used for data analysis.

CEC and IV grown seedlings responded differentially to cold and dark preconditioning. Cold did not benefit CEC protoplasts at any stage, but did increase viability and microcalli diameter for IV protoplasts. CEC grown seedlings were severly affected when subjected to cold and light as protoplasts died within 48 hours. This may have been due to several factors including photooxidation, lipid degradation and chloroplast ultrastructural changes. The use of tomato protoplasts to one, assay for cold tolerance and, two, study chilling injury so as to separate the primary causes of chilling injury from the secondary whole plant effects may be beneficial.

Further work on the effects of preconditioning could

include environmental preconditioning of detached tissue. The composition of the incubation medium during preconditioning could markedly influence the subsequent response. Also, preconditioning of freshly isolated or day old protoplasts might provide a means, as with the study of chilling injury, of seperating the secondary effects of preconditioning on the whole plant from the primary effects. The two best preconditioning treatments for all 4 variables were $CD_{0,48}$ and $CD_{12,0}$ (or $C_0 + D_{48}$ and $C_{12} + D_0$) for CEC and IV seedlings, respectively. This suggests a fundamental difference between CEC and IV seedlings regarding their response to cold and dark. Where CEC seedlings benefit from dark IV seedlings benefit from cold. Determining the difference would require growing CEC seedlings under IV conditions and vice versa. For instance, growing CEC seedlings enclosed in plastic bags to raise the relative humidity to 100% as for IV seedlings, or decreasing the light intensity from 150 $uEm^{-2}s^{-1}$ to 35 $uEm^{-2}s^{-1}$ for CEC seedlings and conversely increasing the light for IV seedlings. Also, the nutrients used could be more closely matched.

In spite of the statistical differences noted for cold, dark and donor plant on shoot regeneration, whole plant preconditioning is clearly unnecessary for successful culture and shoot regeneration from protoplast-derived tomato calli.

APPENDIX A

Summary statistics and analysis

Table A1. Reductions in sum of squares for yield.

Model		Sum of Squares	Degrees of freedom	R^2
y'y		41.919	47	100.0 %
R(u,CDS)		41.676	18	90.9 %
R(u,C,D,S,CD,CS,DS)		41.386	12	80.1 %
R(u,C,D)	CEC	18.281	5	72.7 %
	IV	23.098	5	84.4 %
R(u,CD)	CEC	18.454	9	88.7 %
	IV	23.222	9	92.3 %
R(u)		39.244	1	0.0 %

Table A2. Reductions in sum of squares for viability.

Model		Sum of Squares	Degrees of freedom	R^2
y'y		82.495	283	100.0 %
R(u,CDS)		82.183	18	96.4 %
R(u,C,D,S,CD,CS,DS)		80.553	12	77.5 %
R(u,C,D)	CEC	31.339	5	71.2 %
	IV	49.007	5	16.4 %
R(u,CD)	CEC	33.162	9	98.0 %
	IV	49.021	9	22.8 %
R(u)		73.871	1	0.0 %

Table A3. Reductions in sum of squares for microcalli diameter.

Model		Sum of Squares	Degrees of freedom	R^2
y'y		286,196.04	255	100.0 %
R(u,CDS)		281,397.67	16	73.6 %
R(u,C,D,S,CD,CS,DS)		281,030.18	12	71.6 %
R(u,C,D)	CEC	178,843.62	5	37.6 %
	IV	102,161.33	5	20.0 %
R(u,CD)	CEC	178,888.8	7	38.9 %
	IV	102,508.92	9	29.3 %
R(u)		268,016.79	1	0.0 %

Table A4. Reductions in sum of squares for shoot morphogenesis.

Model		Sum of Squares	Degrees of freedom	R^2
y'y		319,587.3	276	100.0 %
R(u,CDS)		315,924.5	16	40.6 %
R(u,C,D,S,CD,CS,DS)		315,850.9	12	39.4 %
R(u,C,D,S)		314,949.9	6	24.8 %
R(u,C,D)	CEC	156,891.2	5	25.4 %
	IV	158,874.6	5	38.9 %
R(u,CD)	CEC	156,894.2	7	21.5 %
	IV	159,030.3	9	43.5 %
R(u)		313,420.1	1	0.0 %

Table A5. Single factor evaluation.

Variable	Source	Factor(s)	df	partial F	sign.	R^2_f
Microcalli diameter	CEC	C,D	4	16.123	1%	37.61%
		C	2	29.132	1%	35.26%
		D	2	1.531	n.s.	2.78%
Shoot morphogenesis	CEC	C,D	4	22.846	1%	39.61%
		C	2	<1	n.s.	<1%
		D	2	44.885	1%	38.40%
	IV	C,D	4	10.372	1%	25.38%
		C	2	9.743	1%	13.77%
		D	2	18.572	1%	23.34%

Table A6. Goodness of fit for 2-way interaction model.

Variable	Source	R^2	Significance
Yield	CEC	88.7	1%
	IV	92.3	1%
Viability	CEC	98.0	1%
	IV	22.7	1%
Microcalli diameter	CEC	-	-
	IV	29.3	1%
Shoot morphogenesis	CEC	-	-
	IV	-	-

Table A7. Goodness of fit for 3-way interaction model.

Variable	R^2	Significance
Yield	90.9%	1%
Viability	96.4%	1%
Microcalli diameter	73.6%	1%
Shoot morphogenesis	-	-

Table A8. Smallest subclass means for Yield

	Means	
Smallest Subclass	CEC	IV
$u + CD_{0,0}$	1.022 ± 0.121	1.029 ± 0.083
$u + CD_{0,24}$	1.031 ± 0.121	1.230 ± 0.107
$u + CD_{0,48}$	1.140 ± 0.121	0.612 ± 0.107
$u + CD_{12,0}$	0.563 ± 0.148	1.077 ± 0.131
$u + CD_{12,24}$	0.794 ± 0.148	0.953 ± 0.107
$u + CD_{12,48}$	1.146 ± 0.148	0.536 ± 0.107
$u + CD_{24,0}$	0.538 ± 0.148	1.237 ± 0.131
$u + CD_{24,24}$	0.797 ± 0.121	1.068 ± 0.131
$u + CD_{24,48}$	0.784 ± 0.148	0.714 ± 0.131

Table A9. Contrasts for Yield

Source	h_o	F value	Sign.
CEC	$CD_{12,48} - CD_{0,48} = 0$	0.0046	n.s.
	$CD0,24 - CD12,24 = 0$	7.171	5%
	$CD_{12,48} - CD_{0,24} = 0$	1.688	n.s.
	$2CD_{0,48} - (CD_{0,0} + CD_{0,24}) = 0$	2.741	n.s.
	$CD_{12,48} - CD_{0,0} = 0$	1.963	n.s.
	$CD_{12,48} - CD_{12,24} = 0$	13.181	1%
	$CD_{12,48} - CD_{24,24} = 0$	15.550	1%
	$CD_{24,24} - CD_{24,0} = 0$	8.564	5%
	$2CD_{0,0} - (CD_{12,0} + CD_{24,0}) = 0$	40.543	1%
	$2CD_{0,24} - (CD_{12,24} + CD_{24,24}) = 0$	10.894	1%
	$(CD_{0,48} + CD_{12,48}) - 2CD_{24,48} = 0$	19.356	1%
	$CD_{12,48} - CD_{12,0} = 0$	36.158	1%
	$(CD_{24,48} + CD_{24,24}) - 2CD_{24,0} = 0$	9.575	1%
IV	$CD_{24,0} - CD_{0,24} = 0$	<1	n.s.
	$CD_{24,0} - CD_{12,0} = 0$	3.368	n.s.
	$CD_{24,0} - CD_{24,24} = 0$	3.758	n.s.
	$CD_{24,0} - CD_{0,0} = 0$	8.132	5%
	$CD_{0,24} - CD_{24,24} = 0$	4.145	n.s.
	$CD_{0,24} - CD_{12,24} = 0$	15.144	1%
	$CD_{24,48} - CD_{0,48} = 0$	1.643	n.s.
	$CD_{0,24} - CD_{0,0} = 0$	9.967	1%
	$2CD0,24 - (CD12,24 + CD24,24) = 0$	11.704	1%
	$CD_{0,0} - CD_{0,48} = 0$	42.90	1%
	$CDS_{24,0,IV} - CDS_{12,48,CEC} = 0$	<1	n.s.

Table A10. Smallest subclass means for viability

Smallest Subclass	Means	
	CEC	IV
$u + CD_{0,0}$	0.525 ± 0.018	0.559 ± 0.013
$u + CD_{0,24}$	0.547 ± 0.015	0.582 ± 0.014
$u + CD_{0,48}$	0.589 ± 0.021	0.566 ± 0.018
$u + CD_{12,0}$	0.000	0.622 ± 0.018
$u + CD_{12,24}$	0.547 ± 0.021	0.596 ± 0.019
$u + CD_{12,48}$	0.545 ± 0.021	0.579 ± 0.021
$u + CD_{24,0}$	0.000	0.595 ± 0.018
$u + CD_{24,24}$	0.524 ± 0.024	0.591 ± 0.015
$u + CD_{24,48}$	0.525 ± 0.021	0.600 ± 0.018

Table A11. Contrasts for viability

Source	h_o	F value	Sign.
CEC	$CD_{0,48} - CD_{0,24} = 0$	11.786	1%
	$CD_{0,24} - CD_{12,48} = 0$	<1	n.s.
	$CD_{0,24} - CD_{0,0} = 0$	6.702	1%
	$CD_{0,24} - CD_{24,24} = 0$	2.599	n.s.
	$(CD_{12,24} + CD_{12,48}) - (CD_{24,24} + CD_{24,48}) = 0$	4.202	5%
IV	$CD_{12,0} - CD_{24,48} = 0$	2.966	n.s.
	$CD_{12,0} - CD_{12,24} = 0$	3.143	n.s.
	$CD_{12,0} - CD_{24,24} = 0$	7.042	1%
	$CD_{12,0} - CD_{24,0} = 0$	4.636	5%
	$CD_{0,24} - CD_{0,48} = 0$	1.468	n.s.
	$(CD_{12,24} + CD_{24,24}) - 2CD_{0,24} = 0$	1.455	n.s.
	$CD_{24,24} - CD_{0,24} = 0$	<1	n.s.
	$CD_{24,48} - CD_{0,48} = 0$	6.818	1%
	$CD_{24,48} - CD_{24,24} = 0$	<1	n.s.
	$2CD_{0,48} - (CD_{12,48} + CD_{24,48}) = 0$	16.067	1%
	$CDS_{12,0,IV} - CDS_{0,48,CEC} = 0$	5.846	5%

Table A12. Smallest subclass means for microcalli diameter.

	Means	
Smallest Subclass	CEC	IV
$u + CD_{0,0}$		26.845 ± 1.416
$u + CD_{0,24}$		25.720 ± 1.606
$u + CD_{0,48}$		23.912 ± 2.041
$u + CD_{12,0}$		31.187 ± 1.967
$u + CD_{12,24}$		30.062 ± 2.125
$u + CD_{12,48}$		28.254 ± 2.327
$u + CD_{24,0}$		26.348 ± 1.688
$u + CD_{24,24}$		25.223 ± 2.041
$u + CD_{24,48}$		23.415 ± 2.041
$u + C_0 + D_0$	41.389 ± 1.769	
$u + C_0 + D_{24}$	43.686 ± 1.274	
$u + C_0 + D_{48}$	43.420 ± 1.573	
$u + C_{12} + D_0$	-	
$u + C_{12} + D_{24}$	36.594 ± 1.626	
$u + C_{12} + D_{48}$	36.328 ± 1.628	
$u + C_{24} + D_0$	-	
$u + C_{24} + D_{24}$	36.068 ± 1.793	
$u + C_{24} + D_{48}$	35.802 ± 1.671	

Table A13. Interaction effects.

Interactions		Yield	Viability	Microcalli diameter	Shoot morphogenesis
source x cold x dark		1%	1%	1%	n.s.
cold x dark	CEC	5%	1%	n.s.	n.s.
	IV	5%	5%	1%	n.s.

Table A14. Contrasts for microcalli diameter.

Source	h_o		F value	Sign.
CEC	$C_{12} - C_{24}$	$= 0$	<1	n.s.
	$D_{24} - D_{48}$	$= 0$	<1	n.s.
	$C_0 = C_{24} = C_{48}$		58.528	1%
	$D_0 = D_{24} = D_{48}$		2.329	n.s.
IV	$2CD_{12,0} - (CD_{0,0} + CD_{0,24})$	$= 0$	13.182	1%
	$(CD_{0,24} + CD_{12,24}) - 2CD_{24,24}$	$= 0$	4.231	n.s.
	$CD_{12,48} - CD_{0,48}$	$= 0$	5.407	5%

Table A15. Smallest subclass means for shoot morphogenesis.

Smallest Subclass	Means	
	CEC	IV
$u + C_0 + D_0$	33.460 ± 1.271	33.436 ± 0.986
$u + C_0 + D_{24}$	36.041 ± 1.189	33.757 ± 1.040
$u + C_0 + D_{48}$	39.578 ± 1.318	34.091 ± 1.178
$u + C_{12} + D_0$	-	34.450 ± 1.180
$u + C_{12} + D_{24}$	32.133 ± 1.332	34.771 ± 1.115
$u + C_{12} + D_{48}$	35.670 ± 1.254	35.105 ± 1.138
$u + C_{24} + D_0$	-	27.206 ± 1.211
$u + C_{24} + D_{24}$	32.915 ± 1.259	27.528 ± 1.226
$u + C_{24} + D_{48}$	36.453 ± 1.239	27.861 ± 1.310

Table A16. Contrasts for shoot morphogenesis.

Source	h_o	F value	Sign.
CEC	$C_0=C_{12}=C_{24}$	7.865	1%
	$D_0=D_{24}=D_{48}$	14.991	1%
	$C_{24} - C_{12} = 0$	2.782	n.s.
	$2C_0 - (C_{12} + C_{24}) = 0$	15.334	1%
	$C_{12} - C_{24} = 0$	<1	n.s.
	$2D_{48} - (D_0 + D_{24}) = 0$	29.963	1%
	$D_0 - D_{24} = 0$	4.879	1%
IV	$(C_{12} + C_0) - 2C_{24} = 0$	>1000	1%
	$C_0 - C_{12} = 0$	17.660	1%
	$C_{12} - C_{24} = 0$	701.365	1%
	$D_0 - D_{24} = 0$	1.749	n.s.
	$D_{24} - D_{48} = 0$	1.654	n.s.

Table A17. Pre-conditioning treatments with highest means for protoplast yield, viability, microcalli diameter and shoot morphogenesis.

	Source	
Variable	**CEC**	**IV**
Yield	$CD_{0,0}$, $CD_{0,24}$ $CD_{0,48}$ $CD_{12,48}$	$CD_{0,24}$ $CD_{24,0}$ $CD_{12,0}$
Viability	$CD_{0,48}$	$CD_{12,0}$
Microcalli diameter	$C_0 + D_{24}$ $C_0 + D_{48}$	$CD_{12,0}$
Shoot morphogenesis	$C_0 + D_{48}$	$C_0 + D_{0,24\ \&\ 48}$ $C_{12} + D_{0,24\ \&\ 48}$

Table A18. Tomato protoplast culture media

Components	KM-T	A4
KNO_3	1875	1875
MS salts w/o NH_4NO_3		
myo-Inositol	2000	2000
Thiamine HCl	10	10
Nicotinic acid	1	0.5
Pyridoxine HCl	1	0.5
L-glutamine	100	100
D-Ca Pantothenate	1	-
Folic acid	0.4	-
p-ABA	0.02	-
1/2 V-KM organic acids[2]	+	-
Biotin	0.01	-
Choline Chloride	1	-
Ascorbic acid	2	-
V-KM sugars and sugar alcohols[2]	+	-
V-KM supplements[2]	+	-
2,4-D	1	1
NAA	1	1
6-BAP	0.5	0.5
Sucrose	17100	17100
Mannitol	63700	31850
Glucose	-	31500
Purified agar[1]	-	6000
pH	5.8	5.8

[1] See Table 2.
[2] ref. 17.

Table A19. Purification of agar

1. Suspend 1/2 pound of Bacto Agar in ddH_2O and wash by drawing of the supernatent.
2. Resuspend agar in ddH_2O and allow it to stand overnight at $4^{\circ}C$.
3. The resultant cake is resuspended in acetone and filtered through 2 ply cheesecloth.
4. The resultant cake is resuspended in 100% ethanol and filtered through 2 ply cheesecloth.
5. The cake is dried.

APPENDIX B

Least Squares Approach

In cell and tissue culture experiments the data collected is commonly unbalanced. In other words, there are unequal numbers of observations in the individual treatment classes. The problem with unbalanced data is the sums of squares due to the individual factors do not sum to the total sum of squares due to all of the factors. Hence, the factors are not independent and so must be considered simultaniously. The calculations involved in this type of analysis are usually more complicated than those of traditional analysis of variance for balanced data. Prior to the era of computers only limited demand existed for analyzing unbalanced data. Now, however, because of the availability of computers, analysis of unbalanced data is increasingly more common. Analysis of unbalanced data cannot be made just by means of some minor adjustments to the traditional analysis of variance of balanced data. Unbalanced data analyses have their own analysis of variance techniques, and those for balanced data are merely special cases of the techniques for unbalanced data. Unbalanced data analyses use matrix expressions, many of which do not simplify in terms of summation formulas. When the data is balanced or, in other words, the number of observations are all the same, these matrix expressions simplify considerably. They reduce, in fact, to the well-known summation formula of traditional analysis of variance of designed experiments. It is easy then to think of balanced

designed experiments as special cases of the more basic analysis of variance for unbalanced data. The method of analysis used in these experiments is the Least Squares Approach, also known as the method of fitting constants of the linear model.

1. General fixed model

A simplified example will best introduce the idea of the general fixed model used in these experiments and the matrices necessary to manipulate and solve them.

Example. Numbers of protoplasts isolated per gram of fresh tissue was obtained for 47 individual protoplast isolation experiments. Protoplasts were isolated from seedlings preconditioned by 0, 24 or 48 hours in the dark prior to protoplast isolation. I will consider just 9 isolation experiments for illustrative purposes, 4 from 0 hours, 3 from 24 hours and 2 from 48 hours dark preconditioning. For the entries in TableB1 let y_{ij} represent the protoplast yield of the j^{th} plant of the i^{th} dark treatment, i having a value of 1, 2 or 3 for 0, 24 and 48 hours respectively, and $j=1,2,\ldots,n_i$, where n_i is the number of observations in the i^{th} dark treatment. The problem is to estimate the effect of dark preconditioning on protoplast yield. To do this the observation y_{ij} is considered as the sum of three parts

$$y_{ij} = u + d_i + e_{ij}, \qquad (1)$$

Table B1. Protoplast yield ($x10^6$) of dark preconditioned seedlings.

	0 hrs.	24 hrs.	48 hrs.
	1.073	1.002	0.884
	0.992	1.202	0.973
	1.053	1.136	
	1.023		
Totals	4.106	3.393	1.857

where u represents the population mean or the constant common to all observations, d_i is the effect of dark preconditioning i on protoplast yield, and e_{ij} is the random error associated with each y_{ij}. The e_{ij}'s are assumed to be independent and distributed around a mean of zero. Also, it is assumed each e_{ij} has the same variance, σ^2, so that the variance-covariance matrix of the vector of e_{ij}'s is $\sigma^2 I$.

$$\sigma^2 I = \begin{bmatrix} \sigma^2 & 0 & \cdots & 0 \\ 0 & \sigma^2 & \cdots & 0 \\ \vdots & \vdots & & \vdots \\ 0 & 0 & \cdots & \sigma^2 \end{bmatrix} = \sigma^2 \begin{bmatrix} 1 & 0 & \cdots & 0 \\ 0 & 1 & \cdots & 0 \\ \vdots & \vdots & & \vdots \\ 0 & 0 & \cdots & 1 \end{bmatrix}$$

The problem is to estimate u and the three d_i terms, and also σ^2, the variance of the error terms. To provide an estimation of these parameters each observation is written in terms of the above 3-part equation (1):

$$\begin{aligned}
1.038 &= y_{11} = u + 1d_1 &&&&&&+ e_{11} \\
0.992 &= y_{12} = u + 1d_1 &&&&&&+ e_{12} \\
1.053 &= y_{13} = u + 1d_1 &&&&&&+ e_{13} \\
1.023 &= y_{14} = u + 1d_1 &&&&&&+ e_{14} \\
1.055 &= y_{21} = u &&+ 1d_2 &&&&+ e_{21} \\
1.202 &= y_{22} = u &&+ 1d_2 &&&&+ e_{22} \\
1.136 &= y_{23} = u &&+ 1d_2 &&&&+ e_{23} \\
0.884 &= y_{31} = u &&&&+ 1d_3 &&+ e_{31} \\
0.973 &= y_{32} = u &&&&+ 1d_3 &&+ e_{32}
\end{aligned}$$

The equations are easily converted into matrix forms

$$\begin{bmatrix} 1.038 \\ 0.992 \\ 1.053 \\ 1.023 \\ 1.055 \\ 1.202 \\ 1.136 \\ 0.884 \\ 0.973 \end{bmatrix} = \begin{bmatrix} y_{11} \\ y_{12} \\ y_{13} \\ y_{14} \\ y_{21} \\ y_{22} \\ y_{23} \\ y_{31} \\ y_{32} \end{bmatrix} = \begin{bmatrix} 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 0 & 1 \\ 1 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} u \\ d_1 \\ d_2 \\ d_3 \end{bmatrix} + \begin{bmatrix} e_{11} \\ e_{12} \\ e_{13} \\ e_{14} \\ e_{21} \\ e_{22} \\ e_{23} \\ e_{31} \\ e_{32} \end{bmatrix}$$

representing the general form of the fixed model

$$\mathbf{y = Xb + e}$$

where **y** is the vector of individual observations, the **X** is termed the incidence matrix, because the presence of 0's and 1's describes the incidence of the terms in the model (u, d_1, d_2 and d_3), and **b** is the vector of parameters to be estimated,

$$\mathbf{b} = \begin{bmatrix} u \\ d_1 \\ d_2 \\ d_3 \end{bmatrix},$$

and **e** is the vector of error terms.

2. Estimation

The problem is to estimate **b** as all computational procedures for estimation and hypothesis testing requires estimates of **b**. Applying the method of "Least Squares" results in an estimator of **b** that minimizes the squared errors (i.e. sum of squared deviations)

$$\sum_{i=1}^{n} [y_i - E(y_i)]^2$$

$$= \sum_{i=1}^{n} e_i^2$$

$$= e'e.$$

Differentiating e'e with respect to b minimizes e'e. The resultant equations are termed the normal equations.

$$X'X\hat{b} = X'y$$

where the "$\wedge$" represents the solutions of $\hat{b}$. Therefore, $\hat{b}$ is solved for algebraically from:

$$\hat{b} = (X'X)^{-1}X'X$$

where $(X'X)^{-1}$ is the inverse of the coefficient matrix (X'X).

3. The normal equations

The coefficient matrix, X'X, in the normal equations is obtained from the incidence matrix X in the model, y = Xb + e. The incidence matrix, X, can be thought of as a 2-way table with the parameters as headings to the columns and the observations as headings to the rows (Table B2). From the example,

Table B2. **X matrix as a 2-way table.**

	Parameters			
Observations	u	d_1	d_2	d_3
y_{11}	1	1	0	0
y_{12}	1	1	0	0
y_{13}	1	1	0	0
y_{14}	1	1	0	0
y_{21}	1	0	1	0
y_{22}	1	0	1	0
y_{23}	1	0	1	0
y_{31}	1	0	0	1
y_{32}	1	0	0	1

$$X'X = \begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 \end{bmatrix} \begin{bmatrix} 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 0 & 1 \\ 1 & 0 & 0 & 1 \end{bmatrix} = \left[\begin{array}{c|ccc} 9 & 4 & 3 & 2 \\ \hline 4 & 4 & 0 & 0 \\ 3 & 0 & 3 & 0 \\ 2 & 0 & 0 & 2 \end{array}\right]$$

The top left-hand element, 9, is the total number of observations with the next three elements, 4, 3 and 2 being the number of observations in those particular classes, 0, 24 and 48 hours dark preconditioning, repectively. The submatrix is composed of the number of observations in each subclass. So, the intersection of 24 hrs. and 48 hrs. is 0 as the seedlings were preconditioned in one of the three treatments. Likewise, the X'Y matrix is obtained from the incidence matrix X and the y observation vector from the model $y = X'b + e$. In the X'Y 9.356 is the top element and is the sum of all nine observations, and the following elements are the corresponding sums of the individual classes 0, 24 and 48 hrs dark (d_1, d_2 and d_3), respectively.

$$\mathbf{X'y} = \begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 0 & 0 \\ 1 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 \end{bmatrix} \begin{bmatrix} 1.038 \\ 0.992 \\ 1.053 \\ 1.023 \\ 1.055 \\ 1.202 \\ 1.136 \\ 0.884 \\ 0.973 \end{bmatrix} = \begin{bmatrix} 9.356 \\ 4.106 \\ 3.393 \\ 1.857 \end{bmatrix}$$

Writing the normal equations from the example out in full is the following:

$$\left[\begin{array}{c|ccc} 9 & 4 & 3 & 2 \\ \hline 4 & 4 & 0 & 0 \\ 3 & 0 & 3 & 0 \\ 2 & 0 & 0 & 2 \end{array}\right] \left[\begin{array}{c} u \\ \hline d_1 \\ d_2 \\ d_3 \end{array}\right] = \left[\begin{array}{c} 9.356 \\ \hline 4.106 \\ 3.393 \\ 1.857 \end{array}\right]$$

The normal equations are constructed using numbers of observations in the X'X and their sums in the X'y.

4. Solving for b

Unlike the regression model the fixed classification model is a non-full rank model. The result is the inverse of X'X, $(X'X)^{-1}$, does not exist and, estimates cannot therefore be calculated by $b = (X'X)^{-1}X'y$. For the fixed

classification model a generalized inverse, $(X'X)^-$, is used. One of the properties of a generalized inverse is that solutions can be found for **b** but those solutions are not unique as with a regression model. In fact there are an infinite number of solutions for **b**. Therefore, the normal equations are now written as

$$(X'X)b^o = X'X$$

with b^o signifying these equations have no single solution for b^o. None of these solutions estimates **b**, the solutions only solve the normal equations. So, for the example Table B3 lists four of the many solutions for **b**.

5. Estimable functions

As they stand none of the solutions for b^o in Table B3 estimate **b**, but certain linear functions of the elements of b^o can be used. Suitable linear functions yield the same estimate regardless of the solution for b^o. This set of linear functions is called "estimable". Table B4 lists four linear functions and their respective solutions using the solutions for b^o from Table B3. Linear functions 1 and 2 give a different solution for each b^o solution, but linear functions 3 and 4 are invariant to the solutions for b^o. Hence, linear functions 3 and 4 are estimable linear functions and can be used to estimate the parameters of the linear model.

To determine the estimability of a given linear function it is not necessary to test its invariance with several b_o

Table B3. Four solutions for b^o.

	b_1	b_2	b_3	b_4
u	0	.929	1.131	1.027
0 hrs (d_1)	1.027	.098	-.105	0
24 hrs (d_2)	1.131	.203	0	.1045
48 hrs (d_3)	.929	0	-.203	-.098

solutions. There are two methods to determine the estimability of a function.

1) The smallest subclass means are estimable as are any linear combination of them. In the example, the smallest subclass means are $(u + d_1)$, $(u + d_2)$ and $(u + d_3)$. So, linear function 3 in Table B4 is estimable as it is a linear combination of the smallest subclass means $(u + d_1)$ and $(u + d_2)$.

$$
\begin{aligned}
&(u + d_1) - (u + d_3) \\
&= u + d_1 - u - d_3 \\
&= d_1 - d_3
\end{aligned}
$$

2) A linear function $\mathbf{k'b}$ is estimable if it satisfies the following equation:

$$\mathbf{k'(X'X)^{-}X'X = k'}$$

where $\mathbf{k}$ is a vector containing the values of the interested contrast. So, linear function 3, $(d_1 - d_3)$, would be written as follows:

$$\mathbf{k'b} = \begin{bmatrix} 0 & 1 & 0 & 1 \end{bmatrix} \begin{bmatrix} u \\ d_1 \\ d_2 \\ d_3 \end{bmatrix}$$

and,

Table B4. Estimates of four linear functions.

	Solutions			
linear function	b_1	b_2	b_3	b_4
1) $1/2\ (d_1 + d_3)$	.978	.049	-.154	-.049
2) $(u + d_1 + d_2 + d_3)/3$	1.029	.410	.274	1.034
3) $d_1 - d_3$	.098	.098	.098	.098
4) $(d_1 + d_2) - 2d_3$	.3	.3	.3	.3

$$k(X'X)^{-}X'X = \begin{bmatrix} 0 & 1 & 0 & 1 \end{bmatrix} \left[\begin{array}{c|ccc} 0 & 0 & 0 & 0 \\ \hline 0 & 1/4 & 0 & 0 \\ 0 & 0 & 1/3 & 0 \\ 0 & 0 & 0 & 1/2 \end{array}\right] \left[\begin{array}{c|ccc} 9 & 4 & 3 & 2 \\ \hline 4 & 4 & 0 & 0 \\ 3 & 0 & 3 & 0 \\ 2 & 0 & 0 & 2 \end{array}\right]$$

$$= \begin{bmatrix} 0 & 1 & 0 & 1 \end{bmatrix} \begin{bmatrix} 0 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 0 & 1 \end{bmatrix}$$

$$= \begin{bmatrix} 0 & 1 & 0 & 1 \end{bmatrix}$$

$$= k'$$

hence, $d_1 - d_3$ is estimable.

6. Other calculations

With solutions for b^o and estimable functions suitable to answer the research questions of interest sums of squares are calculated and hypotheses tested. The following are the equations used in this study:

1) Sums of squares (SS) of the full model

$$= R(\text{full})$$

$$= b^{o\prime}X'X$$

2) Sums of squares minus the mean

$$= R(\text{factors}|u)$$

$$= b^{o\prime}X'X - ny^2$$

3) Residual SS

$$= y'y - b^{o}'X'X$$

4) $\hat{\sigma}^2$

$$= \text{residual SS}/(n-r)$$

where, n is the number of observations and
r is the rank of the X'X.

5) Proportion of variation explained by the model

$$= R^2$$

$$= R(\text{factors}|u)/(y'y - ny^2)$$

6) Contribution of individual factors

$$= R^2_f$$

$$= R(\text{factor}|\text{other})/[y'y - R(\text{others})]$$

7) Confidence intervals for an estimable function, k'b

$$k'b^{o} \pm \hat{\sigma} t_{n-r,\, \alpha/2} \qquad k'(X'X)^{-}k$$

8) Test for goodness of model

$$= F = R(\text{factors}|u)/(r-1)\hat{\sigma}^2$$

9) Test for partial contribution of factors

$$= F = R(\text{factors}|\text{other})/(r-r_{\text{others}})^2$$

10) Numerator sum of squares (Q-value) for specific hypotheses.

$$Q = (k'b^{o} - m)[k'(X'X)^{-}k]^{-1}(k'b^{o} - m)$$

where, m is a vector of specified constants of order s x 1 and s is the number of simultaneous hypotheses being considered.

11) Test for specific hypotheses (F-ratio).

$$F(H) = Q/s\hat{\sigma}^2$$

7. Method of calculation

Matrix manipulations were preformed on an IBM Personal Computer using software written in BASIC. Matrix inversion was done by a modified Gause-Jordan Elimination Method.

(X'X) for colony diameter with dependencies removed.

	C 2	C 3	D 2	D 3	S 1	S 2	CS 22	CS 32	DS 22	DS 32	CD 32	CD 33
C 2	64	0	26	24	28	36	36	0	12	10	0	0
C 3	0	70	29	27	24	46	0	46	19	13	29	27
D 2	26	29	104	0	52	52	12	19	52	0	29	0
D 3	24	27	0	78	42	36	10	13	0	36	0	27
S 1	28	24	52	42	112	0	0	0	0	0	10	14
S 2	36	46	52	36	0	143	36	46	52	36	19	13
CS22	36	0	12	10	0	36	36	0	12	10	0	0
CS32	0	46	19	13	0	46	0	46	19	13	19	13
DS22	12	19	52	0	0	52	12	19	52	0	19	0
DS32	10	13	0	36	0	36	10	13	0	36	0	13
CD32	0	29	29	0	10	19	0	19	19	0	29	0
CD33	0	27	0	27	14	13	0	13	0	13	0	27

$(X'X)^{-}$ for colony diameter.

	u	C 1	C 2	C 3	D 1	D 2	D 3	S 1	S 2	CS 11	CS 21	CS 31	CS 12	CS 22	CS 32	DS 11	DS 21	DS 31	DS 12	DS 22	DS 32	CD 11	CD 12	CD 13	CD 21	CD 22	CD 32	CD 23	CD 33
	*	*			*					*	*	*	*			*	*	*	*			*	*	*	*	*	*		
u *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C 1 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C 2	0	0	.0610	.0252	0	-.0209	-.0297	0	0	0	0	0	0	-.0609	-.0251	0	0	0	0	.0216	.0286	0	0	0	0	0	0	-.0019	.0028
C 3	0	0	.0252	.2147	0	-.0211	-.0292	0	-.0236	0	0	0	0	-.0276	-.1197	0	0	0	0	.0617	.0741	0	0	0	0	0	0	-.1402	-.1522
D 1 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D 2	0	0	-.0209	-.0211	0	.0852	.0677	-.0556	-.0001	0	0	0	0	.0210	.0212	0	0	0	0	-.0838	-.0697	0	0	0	0	0	0	-.0039	.0056
D 3	0	0	-.0297	-.0292	0	.0677	.1028	-.0556	.0001	0	0	0	0	.0294	.0291	0	0	0	0	-.0705	-.0987	0	0	0	0	0	0	.0078	-.0113
S 1	0	0	0	0	0	-.0556	-.0556	.0556	0	0	0	0	0	0	0	0	0	0	0	.0556	.0556	0	0	0	0	0	0	0	0
S 2	0	0	0	-.0236	0	-.0001	.0001	0	.0296	0	0	0	0	-.0151	-.0060	0	0	0	0	-.0239	-.0232	0	0	0	0	0	0	.0238	.0233
CS 11 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CS 21 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CS 31 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CS 12 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CS 22	0	0	-.0609	-.0276	0	.0210	.0294	0	-.0151	0	0	0	0	.1052	.0428	0	0	0	0	-.0229	-.0322	0	0	0	0	0	0	.0035	.0004
CS 32	0	0	-.0251	-.1197	0	.0212	.0291	0	-.0060	0	0	0	0	.0428	.1257	0	0	0	0	-.0378	-.0509	0	0	0	0	0	0	.0449	.0575
DS 11 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DS 21 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DS 31 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DS 12 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DS 22	0	0	.0216	.0617	0	-.0838	-.0705	.0556	-.0239	0	0	0	0	-.0229	-.0378	0	0	0	0	.1350	.1001	0	0	0	0	0	0	-.0451	-.0385
DS 32	0	0	.0286	.0741	0	-.0697	-.0987	.0556	-.0232	0	0	0	0	-.0322	-.0509	0	0	0	0	.1001	.1584	0	0	0	0	0	0	-.0406	-.0449
CD 11 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 12 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 13 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 21 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 22 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 23 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 31 *	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CD 32	0	0	-.0019	-.1402	0	-.0039	-.0078	0	.0238	0	0	0	0	.0035	.0449	0	0	0	0	-.0451	-.0406	0	0	0	0	0	0	.1630	.1188
CD 33	0	0	.0028	-.1522	0	.0056	-.0113	0	.0233	0	0	0	0	.0004	.0575	0	0	0	0	-.0385	-.0449	0	0	0	0	0	0	.1188	.1832

(X'Y) for colony diameter.

u	8267.1
C 1	4151.9
C 2	2100.8
C 3	2014.4
D 1	2275.3
D 2	3476.3
D 3	2514.5
S 1	4458.9
S 2	3808.2
CS 11	2576.1
CS 21	1020.9
CS 31	861.9
CS 12	1575.8
CS 22	1079.9
CS 32	1152.5
DS 11	745.0
DS 21	2096.2
DS 31	1617.7
DS 12	1530.3
DS 22	1380.1
DS 32	897.8
CD 11	1438.9
CD 12	1785.9
CD 13	927.1
CD 21	475.3
CD 22	858.6
CD 32	766.9
CD 13	361.1
CD 23	831.8
CD 33	821.5

$(X'X)^{-}(X'Y) = b$

0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8267.1	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4151.9	0
0	0	.0610	.0252	0	-.0209	-.0297	0	0	0	0	0	0	-.0609	-.0251	0	0	0	0	.0216	.0286	0	0	0	0	0	0	0	-.0019	.0028	2100.8	-5.0408
0	0	.0252	.2147	0	-.0211	-.0292	0	-.0236	0	0	0	0	-.0276	-.1197	0	0	0	0	.0617	.0741	0	0	0	0	0	0	0	-.1402	-.1522	2014.4	-6.2138
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2275.3	0
0	0	-.0209	-.0211	0	.0852	.0677	-.0556	-.0001	0	0	0	0	.0210	.0212	0	0	0	0	-.0838	-.0697	0	0	0	0	0	0	0	-.0039	.0056	3476.3	.6016
0	0	-.0297	-.0292	0	.0677	.1028	-.0556	.0001	0	0	0	0	.0294	.0291	0	0	0	0	-.0705	-.0987	0	0	0	0	0	0	0	.0078	-.0113	2514.5	-4.4129
0	0	0	0	0	-.0556	-.0556	.0556	0	0	0	0	0	0	0	0	0	0	0	.0556	.0556	0	0	0	0	0	0	0	0	0	4458.9	43.4069
0	0	0	-.0236	0	-.0001	.0001	0	.0296	0	0	0	0	-.0151	-.0060	0	0	0	0	-.0239	-.0232	0	0	0	0	0	0	0	.0238	.0233	3808.2	29.3400
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2576.1	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1020.9	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	861.9	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1575.8	0
0	0	-.0609	-.0276	0	.0210	.0294	0	-.0151	0	0	0	0	.1052	.0428	0	0	0	0	-.0229	-.0322	0	0	0	0	0	0	0	.0035	.0004	1079.9	7.5640
0	0	-.0251	-.1197	0	.0212	.0291	0	-.0060	0	0	0	0	.0428	.1257	0	0	0	0	-.0378	-.0509	0	0	0	0	0	0	0	.0449	.0575	1152.5	= 2.6680
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	745.0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2096.2	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1617.7	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1530.3	0
0	0	.0216	.0617	0	-.0838	-.0705	.0556	-.0239	0	0	0	0	-.0229	-.0378	0	0	0	0	.1350	.1001	0	0	0	0	0	0	0	-.0451	-.0385	1380.1	-2.7411
0	0	.0286	.0741	0	-.0697	-.0987	.0556	-.0232	0	0	0	0	-.0322	-.0509	0	0	0	0	.1001	.1584	0	0	0	0	0	0	0	-.0406	-.0449	897.8	-3.2724
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1438.9	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1785.9	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	927.1	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	475.3	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	858.6	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	766.9	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	361.1	0
0	0	-.0019	-.1402	0	-.0039	-.0078	0	.0238	0	0	0	0	.0035	.0449	0	0	0	0	-.0451	-.0406	0	0	0	0	0	0	0	.1630	.1188	831.8	.1619
0	0	.0028	-.1522	0	.0056	-.0113	0	.0233	0	0	0	0	.0004	.0575	0	0	0	0	-.0385	-.0449	0	0	0	0	0	0	0	.1188	.1832	821.5	1.6745

For CEC data:
(X'X) with dependencies removed.

	C 1	C 2	C 3	D 1	D 2
C 1	60	0	0	18	28
C 2	0	28	0	0	14
C 3	0	0	24	0	10
D 1	18	0	0	18	0
D 2	28	14	10	0	52

$(X'X)^{-}(X'Y) = b$

$$\begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & .0439 & .015 & .0125 & -.0439 & -.0301 & 0 \\ 0 & .015 & .047 & .0094 & -.015 & -.0226 & 0 \\ 0 & .0125 & .0094 & .0495 & -.0125 & -.0188 & 0 \\ 0 & -.0439 & -.015 & -.0125 & .0994 & .0301 & 0 \\ 0 & -.0301 & -.0226 & -.0188 & .0301 & .0451 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} 4458.9 \\ 2576.1 \\ 1020.9 \\ 861.9 \\ 745.0 \\ 2096.2 \\ 1617.7 \end{bmatrix} = \begin{bmatrix} 0 \\ 43.4204 \\ 36.3278 \\ 35.8018 \\ -2.0315 \\ .2658 \\ 0 \end{bmatrix}$$

For IV data:
(X'X) with dependencies removed.

	C 1	C 2	C 3	D 1	D 2
C 1	61	0	0	27	21
C 2	0	36	0	14	12
C 3	0	0	46	14	19
D 1	27	14	14	55	0
D 2	21	12	19	0	52

$(X'X)^{-}(X'Y) = b$

$$\begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & .0396 & .0213 & .021 & -.0302 & -.0286 & 0 \\ 0 & .0213 & .0473 & .0193 & -.0274 & -.0265 & 0 \\ 0 & .021 & .0193 & .0411 & -.0257 & -.0279 & 0 \\ 0 & -.0302 & -.0274 & -.0257 & .0465 & .0279 & 0 \\ 0 & -.0286 & -.0265 & -.0279 & .0279 & .0471 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \begin{bmatrix} 3808.2 \\ 1575.8 \\ 1079.9 \\ 1152.5 \\ 1530.3 \\ 1380.1 \\ 897.8 \end{bmatrix} = \begin{bmatrix} 0 \\ 23.9124 \\ 28.2542 \\ 23.4151 \\ 2.9326 \\ 1.8077 \\ 0 \end{bmatrix}$$

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