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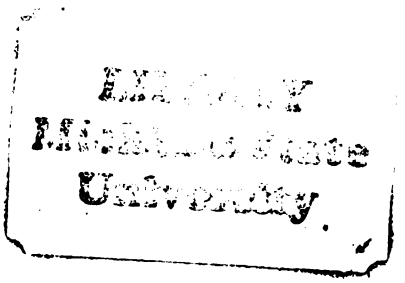

This is to certify that the

thesis entitled

PHENOTYPIC VARIATION IN PROTOPLAST-DERIVED PLANTS OF LYCOPERSICON PERUVIANUM (L.) Mill.

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JOCELYN ANN OZGA

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Major professor

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PHENOTYPIC VARIATION IN PROTOPLAST-DERIVED PLANTS OF *LYCOPERSICON PERUVIANUM* (L.) MILL.

By

Jocelyn Ann Ozga

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ABSTRACT

PHENOTYPIC VARIATION IN PROTOPLAST-DERIVED PLANTS OF LYCOPERSICON PERUVIANUM (L.) Mill.

By

Jocelyn Ann Ozga

Lycopersicon peruvianum protoplasts regenerated via callus-mediated organogenic (CMO) and somatic embryogenic (SE) pathways were compared relative to type and frequency of spontaneous variation in the resultant plants. More variability was exhibited for 5 of 10 leaf and flower morphology characteristics in the CMO population than in the parent plant. Variation in the CMO and SE populations was compared to a seedling population (SD). Three of ten morphology characteristics were more variable in the SE and CMO populations than in the SD population. A large proportion of tetraploid plants (60%) were regenerated via CMO whereas only diploids were regenerated via SE. In contrast, light green leaf chimeras occurred in both populations. Plants regenerated via CMO had a zone of the peroxidase isozyme banding pattern identical to those plants from which each was derived. The type, frequency, and potential agronomic value of spontaneous variation from CMO and SE populations are discussed.

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Introduction

The aim of plant breeding is to improve agronomically important plants by producing varieties which are resistant to insects and diseases, tolerant to adverse climatic conditions, improved in nutrient composition, and are physiologically more efficient, and thus, consequently more productive in relation to human needs. A basic requirement for such crop improvement strategies is the availability of a large pool of genetic variation within the population of a given species. Plant protoplasts are receiving increased interest as one technique which has the potential to further increase the pool of genetic variation in these species (Thomas et al., 1979; Bhojwani et al., 1977; Shepard, 1981).

Isolated protoplasts have the following useful properties: (1) fusability; including interspecies fusion (Cocking, 1972; Keller and Melchers, 1973); (2) the ability to take up macromolecules (Ohyama et al., 1972), virus particles (Takebe, 1975), cell organelles (Potrykus and Lorz, 1976), and microorganisms (Davey and Cocking, 1972); (3) the ability to divide after regeneration of the cell wall and to form colonies of callus cells, or somatic embryos, which for several species can subsequently produce whole plants (Nagata and Takebe, 1971). These

possibilities, coupled with the totipotency of plant cell cultures, lends protoplast techniques applicable to agricultural genetics and selection.

The potential that protoplast culture has for plant genetics and plant breeding can be realized firstly through large homogeneous populations of totipotent protoplasts of crop plants. Such populations can be used as targets for mutagens and specific selection systems or for cell fusion or the introduction of foreign genetic material. The second avenue is through the evaluation of spontaneous variation occurring in plant populations regenerated from protoplasts (p-clones). While the tissue culture systems of some model species (Nicotiana and Petunia) meet many of the criteria for application in agriculture or horticulture, still much research is needed to improve our ability to work at the protoplast level, and subsequently regenerate to whole plants, all important crops.

Plant protoplasts are readily isolated by enzymatic methods from various cell sources; however, at the present time only a limited number of plant species are capable of sustained division to produce cell colonies and protoplast calli (p-calli). Furthermore, the number of species from which whole plants can be regenerated from the p-calli is even more restricted (Vasil and Vasil, 1980a). Sometimes, when plants can be regenerated from p-calli, the regeneration is inconsistent (Bhojwani et al., 1977).

Although progress has already been made in obtaining plantlet formation in cultures of some important crop

plants, this has generally been from tissue explants; virtually nothing is known of the basic biochemical factors controlling morphogenesis from protoplasts (Thomas et al., 1979).

As protoplasts first develop into cell colonies the mitotic process is subject to disruptions in the genome such as changes in chromosome number during culture (D'Amato, 1977), chromosomal rearrangements (Cummings et al., 1976), and nuclear fragmentation and endomitosis (Cionini et al., 1978). These cryptic cytological events may eventually lead to genotypic and phenotypic variation in p-calli and in the regenerated population of p-clones.

Upon regeneration into whole plants evaluation of the variant regenerated plants as well as genotypic and phenotypic progeny analysis is necessary to assess the nature, cause, and possible use of the variant features.

Tomato, an important horticultural crop, has been greatly improved by plant breeders making extensive use of the wild Lycopersicon species to develop various desirable traits (Rick, 1978). Yet, they have not been able to transfer all available desirable traits due to interspecific incompatibilities (Hogenboom, 1972). The use of somatic cell manipulation or somatic hybridization may be an alternative approach for tomato improvement.

When using somatic cell techniques as a system to create homogeneous populations of transformed plants by genetic manipulation, spontaneous variation must be

minimized. On the other hand, spontaneous and induced genetic changes arising in culture may provide novel forms of variation useful to the plant breeder. Both applications depend on understanding the origin of spontaneous variation expressed in protoplast regenerated plants and learning how to regulate it. In the genus Lycopersicon high frequency regeneration from protoplasts has only been observed in L. peruvianum, making it a good model system with which to study variation induced by protoplast culture. This species, a wild relative of the cultivated tomato, has also been reported to be capable of two forms of morphogenesis from leaf protoplasts: 1) somatic embryogenesis (SE) forming embryogenic-like structures (Zapata and Sink, 1981), and 2) callus-mediated organogenesis (CMO) forming adventitious shoots (Zapata et al., 1977). This study was conducted to obtain an estimate of the relative type and frequency of spontaneous variation in plants regenerated via CMO and SE pathways. Variation in L. peruvianum was assessed in plants regeneratedfrom protoplasts via CMO and compared to that of the parent plant. Variation was also analyzed in a SE population derived from a previous study (Zapata and Sink, 1981). In addition, variation in the CMO and SE populations was compared to variation in a seedling (SD) population . The variation was characterized by using 4 leaf and 7 flower morphology characters, chromosome number, and peroxidase (Px) isozyme patterns.

Plant regeneration from protoplasts

Plant regeneration from protoplasts via CMO occurs when p-calli are induced to undergo shoot organization. This was first accomplished in tobacco by Takebe et al. (1971) and since than, many species have been reported capable of protoplast regeneration via CMO including Asparagus, Atropa, Bassica, Branus, Datura, Daucas, Hyoscyamus, Lycopersicon, Nicotiana, Pennisetum, Petunia, Solanum, and Ranunculus (Vasil and Vasil, 1980a).

In the morphogenic pathway of SE, dedifferentiated cells recapitulate stages similar to normal embryogenesis. Steward (1963) and Wetherall and Halperin (1963) demonstrated totipotency of somatic cells when single and small clumps of carrot cells were spread on a agar plate (basal medium + coconut milk) and they were shown to give rise to embryos that exhibited all the developmental stages of normal carrot sexual embryogenesis such as the globular, heart-shaped, and torpedo-shaped stages.

The formation of embryoid-like structures directly from isolated protoplasts has been described in only a few species to date. These embryoid structures were derived from protoplasts isolated from callus and cell suspension cultures of the amphi-haploid and diploid Nicotiana tabacum cv. Samsum (Lorz et al., 1977), leaf mesophyll and suspension cultures of haploid N. sylvestris (Facciotti and Pilet, 1979), suspension cultures of Atropa belladonna (Gosch et al., 1975) and Daucus carota L. cv. Royal Chantenay (Grambow et al., 1972), non-differentiating ovular

callus of Citrus sinensis (Vardi et al., 1975), embryogenic suspension cultures derived from immature embryos of Pennisetum americanum (Vasil and Vasil, 1980b), leaf mesophyll protoplasts of Medicago sativa (Kao and Michayluk, 1980) and leaf mesophyll protoplasts of Lycopersicon peruvianum (Zapata and Sink, 1981).

Many factors are involved in the determination of a cell or groups of cells to embark on a specific morphogenic pathway. Firstly, there is evidence that morphogenesis has a genetic basis. Frankenberger and Tigchelaar (1981) examined shoot morphogenesis in tomato genotypes having different regeneration potentials from cultured leaf discs. An analysis of diallel F_1 hybrids indicated a genetic control basis for shoot morphogenesis. In addition, media components and the in vitro environment are also factors that influence cells to express their morphological competence.

Halperin (1967) introduced, in relation to carrot cultures, the concept that the achievement of embryogenic competence occurred during the initiation of the culture from the primary explant, and that the embryos developed exclusively from cell clumps derived from such competent cells. The primary cultures were therefore thought to contain mixed populations of competent cells (capable of expressing totipotency) and non-competent cells (unable to express totipotency). This concept offered an explanation of why different cultures even from the same plant and type

of explant, differed in their yield of embryos in morphogenic media and suggested that active proliferation of the competent cells is essential for retention of embryogenic capacity.

Support for this concept of intercellular competition comes from Smith and Street (1974), who showed that suspension cultures declining in their ability to yield embryos, could, by plating, be made to yield cultures of fully restored yield and cultures incapable of yielding embryos. They hypothesized that such cells exhibit a degree of cytological instability which leads to changes in their ploidy and to losses and translocations of genetic information. This instability sooner or later gives rise to cells whose totipotency is impaired, as evidenced by abnormal plantlets, or lost. Some of these impaired cells may be eliminated because they are at a selective disadvantage, others may be components of a more or less stable mixed cell population, and still others may be at a selective advantage and hence replace the totipotent cells of the population. If some totipotent cells remain, restoration of embryogenic potential will be possible if, by altering the cultural conditions, the totipotent cells are favored over the non-embryogenic cells.

The generalization that conversion of non-competent into competent cells does not occur during culture initiation, must be treated with some reservation. There is considerable evidence for the importance of induced division in altering the potential of cells for cytodifferentiation

(Street, 1977; Evans et al., 1981), and it is possible that bringing tissue cells out of a G_0 state back into G_1 and hence into a mitotic cycle may offer an opportunity to establish new patterns of gene transcription. The difficulty has been to find any clear-cut examples of an experimental treatment at the time of culture initiation which has unambiguously modified the competence of the resulting cell culture.

Evidence also exists to suggest that cells responsible for different forms of morphogenesis are distinct and stable in their state of competence at the time of initiation of the culture. There are many instances where a culture is only rhizogenic or only embryogenic, and where cultures persistently lack morphogenic capacity under a wide range of culture conditions (Street et al., 1977).

The first type of culture can be regarded as containing cells with only one state of competence, the second as containing only non-competent cells. There are also cultures initially showing more than one form of morphogenesis but upon continuous culture either show a marked quantitative shift between the two forms of expression or retain only one type of morphogenesis.

Smith (1973) studied morphogenesis in two carrot cultures, one predominantly showing, in subculture 3, rhizogenesis and the other embryogenesis. By subculture 15 the initially rhizogenic culture showed predominantly embryogenesis; whereas, the culture showing only occasional

roots remained more stable. The rhizogenic culture showing two forms of morphogenesis was found to contain two types of cell populations capable of separation, large aggregates and a fine cell suspension. The embryogenic fraction, the fine cell suspension, had exhibited no root formation. From this data it was concluded that the subculturing method employed selected for the fine cell suspension in the initially rhizogenic culture and gradually yielded predominantly embryos by the 15th subculture.

Horner and Street (1978) also showed morphogenesis to be limited to certain pollen grains in tobacco. During the ripening of pollen in the tobacco anther, two kinds of pollen grains could be distinguished at the binucleate stage: normal grains (N) characterized by their high frequency, large size, deeply staining cytoplasm, and high starch content; and small grains (S) characterized by their low frequency and weakly staining cytoplasm. It was shown that only the S grains participated in embryogenesis. Studies by Dale (1975) on a similar natural dimorphism in barley anthers also pointed to the origin of pollen callus from only the infrequent S-type grains.

On the other hand, there are numerous cases in the literature where growth regulators appear to induce cells to develop along the embryogenic pathway. Auxin or auxin/cytokinin concentration in the primary culture medium or conditioning medium have been observed to be not only critical to the initiation of mitotic activity in non-mitotic differentiated cells but to the epigenetic

redetermination of these cells to the embryogenic state of development (Evans et al., 1981). The cellular redetermination to embryogenic cells is characterized as usually occurring on the primary or conditioning medium. Thereafter, cells need to be subcultured onto a secondary medium for induction or continued development of the embryogenic cells. The latter medium is usually referred to as an induction medium. Examples of this pattern of embryogenic induction have been reported in many taxa (Evans et al., 1981).

Variation in plants regenerated from protoplasts

Phenotypic variation in plants derived from leaf mesophyll protoplasts of Solanum tuberosum have been widely documented (Shepard et al., 1980; Secor and Shepard, 1981; Thomas et al., 1982). The phenomenon was first reported in the tetraploid cultivar 'Russet Burbank' for the agronomically important characters of tuber development, yield, and resistance to certain pathogens (Matern et al., 1978; Shepard et al., 1980; Secor and Shepard, 1981). Matern et al. (1978) and Secor and Shepard (1981) found that the majority of the potato p-clones appeared normal, but a substantial number of variants appeared without exposure of the protoplasts or calli to any form of mutagen. Thomas et al. (1982) obtained plants from protoplasts of shoot cultures of potato (Solanum tuberosum L. cv. 'Maris Bard'). None of these p-clones resembled each other in all of ten morphological characteristics scored and only one resembled

the parent 'Maris Bard'.

There is evidence that the basis of heritable variation following plant regeneration from protoplasts can involve changes in chromosome number (Karp et al., 1982; Prat, 1983) and single gene mutations (Prat, 1983). Chromosomal rearrangement is also a possible mechanism for generating variation, in cell cultures (Cummings et al., 1982).

Physiological and epigenetic changes may also contribute to the observed phenotypic variability in p-clones. Phenotypic changes which are designated "epigenetic" are transmitted stably to daughter cells produced by mitosis in culture and sometimes manifested in regenerated plants but not in their sexual progeny (Binns and Meins, 1973). Epigenetic changes generally are assumed to reflect changes in the expression of genes, rather than in the information contained within the DNA sequence. Genetic analysis of the p-clones and their progeny can permit the sources of the variability, genetic or epigenetic, to be identified.

Meins and Binns (1978) give strong evidence that cytokinin habituation of tobacco pith tissues results from epigenetic changes rather than from rare, random, genetic mutations. First, both the habituation process and the reversion of habituated cells to the cytokinin-requiring state are directed changes that occur at rates 100-1000 times more frequently than the rates expected for mutations. Secondly, fertile plants can regularly be regenerated from

the progeny of habituated cells. Pith tissue obtained from habituated cell-derived regenerated plants was able to grow profusely on a complete basal medium, but not without cytokinin. In this regard they were indistinguishable from non-habituated pith obtained from normal clones or seed-grown plants. Since tissues derived from cytokinin-habituated cells once again required cytokinin for growth in culture, it was concluded that cytokinin habituation is reversible and, therefore, not due to permanent genetic changes (Binns and Meins, 1973).

Other examples of non-genetic variation have been reported. Maliga et al. (1976) recovered cycloheximide resistant cell lines from haploid cells of Nicotiana tabacum exposed to cycloheximide. A detailed examination of callus growth on cycloheximide-containing and drug-free media showed cycloheximide-resistance as transient in nature, expressed only in shoot forming calli, therefore, apparently caused by an alteration in gene expression, rather than by a mutation. Chaleff and Keil (1981) found tricotyly observed in their experiments to not have a genetic basis. They scored the frequency of tricotyledenous seedlings of Nicotiana tabacum present among progeny of regenerated mutant plants and subsequent generations. It was concluded that tricotyly was induced directly or indirectly by picloram or by a picloram derivative.

While protoplasts are in culture, genomic modifications can occur before, during, and/or after the first cell division (Landgren, 1976). As a consequence, analysis of

derived p-calli or p-clones is insufficient to conclude that genetic alterations existed in leaves prior to protoplast preparation.

Evidence that contributions to the frequency of p-clone variation occur prior to isolation of protoplasts is only circumstantial. Wenzel et al. (1979) suggested that the variation reported in potato p-clones for resistance to A. solani (Matern et al., 1978) might be attributable to chimeral plants as protoplast donors. Chimeral potato plants have long been recognized, but none have yet been identified as a genetic mosaic (Shepard, 1981). Also, establishing whether a particular source tissue (i.e. a leaf) contains a population of variant cell lines is very difficult in the absence of well-defined genetic markers. Direct DNA measurements have suggested gross ploidy alterations in some leaf cells (Warden and Catarino, 1977), but more subtle forms of variation such as chromosomal aberrations are not detectable in interphase cells.

Although there is no direct evidence of mutation induction during protoplast isolation, this stage exhibits a particularly high rate of metabolic activity (Robenek and Peveling, 1977) with changes in protein synthesis (Fleck et al., 1979) and also in internal DNA precursor pools (Zelcer and Galun, 1976). This situation makes it possible for mutational events to occur within the protoplast.

Contributions to the frequency of protoclonal variation during culture have been documented. In one series of

experiments, Shepard (1981) showed that the auxin source used in protoplast and colony culture of potato significantly influenced shoot morphogenesis and the percentage of severely aberrant p-clones. Barbier and Dulieu (1980) also showed that, for their marked heterozygous genes in protoplast-derived Nicotiana tabacum cells, the frequency of mutation significantly increases with the duration of the cell culture stage. Additionally, severe mitotic disturbances such as nuclear fragmentation, endomitosis (Cionini et al., 1978), and lagging chromosomes (Cooper et al., 1964) are known to alter the karotype of cultured cells at the very onset of callus initiation. Polyploidy and aneuploidy can modify plant phenotype (Karp et al., 1982; Prat, 1983). Several reports have also described a variety of chromosome structural alterations in cultured plant cells in addition to the gain or loss of chromosomes (Cummings et al., 1976; Sunderland, 1977) which also may contribute to variation in p-clones.

MATERIALS AND METHODS

Plant materials

Seeds were obtained by sibing two plants of Lycopersicon peruvianum (L.) Mill. (P.I. 128657 Ames, Iowa) L-23. They were germinated on a soil-less planting medium (PPV; 50% peat, 25% perlite, and 25% vermiculite) under greenhouse conditions of 25±2°C minimum night temperature (NT), fluctuating day temperatures, and natural photoperiodic conditions present at East Lansing, MI during the month of May. One week old seedlings were subsequently transplanted into 4X6 cm plastic trays using the same cultural procedures. Three plants (L-23-1,2,3) were selected for different banding in one zone (designated region 3) of the peroxidase (Px) banding pattern. These (Px) banding patterns were determined to be stable in plants grown in different environments: a) greenhouse environment having 25±2°C minimum (NT) and fluctuating day temperature (DT); b) a controlled environment chamber (CEC) providing 200 uE m^{-2} s^{-1} light intensity (Sylvania F36T12-CW), 16 h photoperiod, with 27-29°C DT and 18-21°C NT; and c) 2 oz. glass culture vessels kept at 25±2°C under 21-42 uE $m^{-2}s^{-1}$ light intensity (GE F96T12-CW) on a 16 h photoperiod in a culture room. This region 3 Px banding pattern was also stable through shoot regeneration from leaf sections via

direct stimulation of adventitious shoots or callus-mediated shoot regeneration.

Cuttings from L-23-C-1,2,3 plants and one plant (L-23-SE-6) derived from a somatic embryogenic-like structure study (Zapata and Sink, 1981) were rooted in a mist chamber and subsequently placed in a CEC under 200 uE $m^{-2}s^{-1}$ light intensity (16 h photoperiod; 28±2°C DT, 20±2°C NT) from fluorescent lamps (Sylvania F36T12-CW). At a plant height of 10-15 cm, the first and second fully expanded leaves were harvested for protoplast isolation.

Leaf protoplast isolation and culture

Fully expanded leaflets were surface sterilized in an aqueous solution of 0.5% NaOCl (diluted commercial Clorox 5.25%) for 20 min. followed by 4 separate sterile distilled water rinses. All further steps were carried out using aseptic techniques.

To isolate protoplasts, the leaflets were cut perpendicularly to the midvein into approx. 1 mm wide strips and floated on an enzyme solution containing 1% w/v Cellulysin, 0.5% w/v Macerase, 0.5% w/v potassium dextran sulfate (all Calbiochem-Behring Corp., La jolla, CA) in the presence of 9% w/v mannitol, and CPW salts (Cocking and Peberdy, 1974) mg/l: KH_2PO_4 (27.2), KNO_3 (100), $CaCl\ 2H_2O$ (1480), $MgSO_4\ 7H_2O$ (246), KI (0.16), and $CuSO_4\ 5H_2O$ (0.025), adjusted to pH 5.6-5.8 using 0.1 N HCl.

After 16-18 h incubation on a gyrotory shaker (25 rpm) in the dark at 25°C, digestion was completed. The

protoplasts were pelleted by centrifugation at 35g for 10 minutes. The supernatant was removed and replaced by culture medium A : Murashige and Skoog (1962) salts and vitamins (MS) containing 3% sucrose, 9% mannitol, 1.0 mg/l kinetin (Kin) and 1.0 mg/l naphthaleneacetic acid (NAA) at pH 5.6-5.8. The protoplasts were pelleted (35g) and resuspended in 2 ml of culture medium. Subsequently, the suspension was layered on a 4 ml solution of CPW salts containing 30% w/v sucrose, pH 5.6-5.8, centrifuged in 16 ml culture tubes (100g for 15 min.) and the protoplasts which floated were collected, diluted and counted on a haemocytometer (Modified Fuchs Rosenthal).

The protoplasts were cultured in culture medium A at a density of $1.0\text{-}1.5X10^5$ per ml in plastic petri dishes (Falcon 1007, 60X15 mm), and wrapped with Parafilm. Plated protoplasts were incubated in the dark at 25°C for 9 days and then transferred to a light intensity of 15 uE $m^{-2}s^{-1}$ (16 h photoperiod) at 25°C. Every 7-9 days fresh medium A with progressively lower mannitol concentrations (6,3,0%) was added in order to reduce the osmotic concentration.

Fifty to sixty days after isolation, p-calli approx. 2 mm in diameter were transferred from liquid to solid medium (MS salts and vitamins containing 3% sucrose, 0.8% agar, and 1 mg/l zeatin) for shoot regeneration and kept at 25 ± 2°C under 21-42 uE $m^{-2}s^{-1}$ (GE F96T12-CW) on a 16 h photoperiod.

Plant regeneration

After 50-70 days on shoot regeneration medium, 25 shoot cuttings from regenerating calli of L-23-C-2 were randomly excised, transferred to perlite medium in 4X6 cm plastic trays for rooting, and covered with clear polyethylene for humidity control. Such cuttings were kept under the same conditions as the shoot regenerating cultures.

Rooted cuttings were transferred to PPV soil-less planting medium and maintained in the same manner for humidity control. After 1 week, the plants were transferred to a greenhouse for 1 week. Subsequently, plants were transplanted into 10 cm clay pots using the same planting medium and were given 150 ppm 20N-8.6P-16.6K at each watering. The greenhouse environment was the natural photoperiodic conditions present at East Lansing, MI, during the months of Febuary-May. A minimum NT of 22$\pm$2^{o}C was maintained; DT fluctuated with daily conditions (22-28^{o}C). These 25 plants made up the CMO population.

Plant populations analyzed for variation

Ten stem cuttings of the CMO parent plant (PP population) were taken on Febuary 12, 1983 and rooted in a mist chamber under greenhouse conditions. After 1 week, the rooted cuttings were transferred into 10 cm clay pots using the PPV soil-less planting medium.

<u>L. peruvianum</u> seeds were planted in PPV soil-less planting medium on March 1, 1983 and always kept under the same greenhouse conditions as the CMO population. After 1

week, 10 seedlings were randomly choosen for the seedling population (SD), and transferred into 4X6 cm plastic trays using the same planting medium. Subsequently, plants were transplanted into 10 cm clay pots using the same planting medium.

Ten p-clones regenerated via somatic embryogenesis (SE population) by Zapata and Sink (1981) were cut back to one 10 cm stem per 10 cm pot on Febuary 12, 1983 and were maintained as a one branched stem throughout the study.

At the 10 cm pot stage, the PP, SD, SE populations were maintained under the same greenhouse conditions and treated in the same manner as the CMO population.

Plants in the CMO, SE, PP, and SD populations were distributed into 5 randomized blocks, 13 plants per block, and assessment of phenotypic variation took place 2 weeks to 2 months after randomization. Variation in the CMO population was compared to that in the PP and SD populations. Variation in the SE population was compared to the SD population.

Variation in leaf and floral traits

Variation in floral traits was measured during the months of April and May using the characters, number of flowers per inflorescence, % of 2-branched inflorescence, petal length, petal fusion length, and sepal length. Measurements of leaf and stem traits were taken at a plant height of 35-40 cm during the month of April. Leaf and stem characteristics were evaluated using internode length under

the first fully expanded leaf, number of leaflets on the first fully expanded leaf, terminal leaflet length on the first fully expanded leaf, and the number of plants showing light green leaf chimeric sectors.

Statistical methods

Barletts test (Steel and Torrie, 1980) was used to test the homogeneity of variance between the measured population characteristics. The coefficients of variation (CV) and the corresponding standard errors (S_v) (Sokal and Rohlf, 1981) were calculated for all characteristics measured in each population.

Cytology

The chromosome number of plants derived from protoplasts via SE and CMO pathways was determined by chromosome counts of root-tip cell squashes. Root-tips (1-2 mm) were obtained from adventitious roots of approx. 2 inch cuttings, rooted in a mist chamber under greenhouse conditions. The excised root-tips were treated with 0.02% o-dichlorobenzene for 45 min at 24$\pm$2°C and stored in 3:1 v/v absolute ethanol: glacial acetic acid at 5°C. For chromosome counting, fixed roots were hydrolyzed enzymatically with 4% Macerase and 4% Cellulase (Hizume et al., 1980) for approx. 30 min. followed by rinsing with water. Subsequently, acetocarmine stain was applied to macerated root tips then flushed with 45% acetic acid. Microphotography to document the chromosome counts was done with a Zeiss microscope with phase contrast optics,

magnification 1250X, using Panatomic-X film.

Pollen viability

Pollen grains were placed on a microscope slide and subsequently stained using 1-2 drops of 2% lacto-phenol analine blue stain (Clark, 1981). The number of pollen grains viable or non-viable (considered viable if they took up stain, appeared round, and were fully expanded) was counted in random areas of the microscope slide. Approximately 600 pollen grains were scanned from each plant (200 grains/flower;3 flowers/plant).

Gel electrophoresis

Leaf tissue from 3 seedling plants (L-23-C-1,2,3) and 1 SE-derived plant (L-23-SE-6) and protoplast-regenerated plants derived from these plants were assayed for their anionic Px isozyme pattern. A crude extract was obtained by macerating approximately 0.5 g of leaf tissue in a few drops of 0.2M tris-citrate buffer (pH 8.3) containing 0.06% mercaptoethanol.

Electrophoresis of the leaf tissue extracts was carried out in horizonal starch gels (16X18X0.6 cm) as outlined by Scandalios (1969). A discontinuous buffer system was used: 270 ml 0.2M tris-citrate (pH 8.3); 30 ml 0.2M lithium-borate (pH 8.3) gel buffer, 0.2M lithium-borate electrode buffer (pH 8.3). Starch (hydrolyzed for electrophoresis; Sigma Chemical Co., St. Louis, MO) concentration was 12%. Electrophoresis at 200V (45 ma) was applied for an initial

period of 1 h, after which the wicks (Whatman chromatography paper, thickness 0.33 mm; W & R Balston, Ltd., England) were removed. Electrophoresis was then continued at 300 V (40 ma) for an additional 4-5 hours.

The entire electrophoretic run was conducted inside a modified insulated cooler in which the gel tray bottom was cooled by a constant stream of 0°C water. At the end of the run, the gels were sliced horizontally (2 mm thick) and stained for peroxidase activity using 3-amino-9-ethylcarbozole (Shaw and Prasad, 1970). The gels were developed in the staining solution overnight at 40°C and fixed in 50% ethanol.

Results

Variation in leaf and flower morphology

Callus-mediated organogenic population (CMO) compared to stem cuttings of the parent plant (PP population)

CMO population variances for terminal leaflet length on the first fully expanded leaf, number of leaflets on the first fully expanded leaf, internode length, petal fusion length, and sepal length were homogeneous (i.e. vary to the extent) with respect to those of the parent plant ($X^2 P < 0.01$) (Table 5). Non-homogeneous variance (i.e. vary to a different extent) was observed in the CMO population when compared to the parent plant for number of flowers per inflorescence, petal length, % of 2-branched inflorescence, style length, and % pollen viability (Table 5).

All characters showing non-homogeneous variance in the CMO population were more variable when compared to the parent plant. Variation in % pollen viabiltiy (CV=101.7$\pm$14.4) was 50.9 times greater, % of 2-branched inflorescence (CV=23.2$\pm$3.3) 2.6 times greater, style length (CV=6.1$\pm$0.3) 1.9 times greater, number of flowers per inflorescence (CV=29.7$\pm$0.7) 1.8 times greater, and petal length (CV=12.3$\pm$0.6) 1.1 times greater in the CMO population than in the parent plant (Tables 1,2; Figure 1).

Table 1. Mean, coefficient of variation (CV), and variance (V) for ten characters of the callus-mediated organogenic (CMO) population (25 plants) of L. peruvianum.

Character	CMO Population			
	No. of obs./plant	Mean±SD	CV±S_v	V
Terminal leaflet length(cm)	1	5.1±0.7	14.4±2.0	0.527
No. of leaflets	1	11.4±3.4	30.0±4.3	11.500
Internode length(cm)	1	3.7±1.3	34.9±4.9	1.659
No. of flowers/ inflorescence	20	25.8±7.7	29.7±0.7	58.980
Petal fusion (cm)	10	0.7±0.1	17.2±0.8	0.016
Sepal length (cm)	10	0.5±0.04	8.8±0.4	0.002
Petal length (cm)	10	1.6±0.2	12.3±0.6	0.039
% 2 branched inflorescence	20	73.2±16.8	23.2±3.3	281.95
Style length (cm)	10	1.1±0.1	6.1±0.3	0.005
% Pollen viability	3	36.3±36.6	101.7±14.4	1337.7

Table 2. Mean, coefficient of variation (CV), and variance (V) for ten characters of stem cuttings from the CMO protoplast source plant (PP population; 10 plants) of *L. peruvianum*.

Character	PP Population			
	No. of obs./plant	Mean±SD	CV±S_v	V
Terminal leaflet length(cm)	1	5.6±0.7	12.0±2.7	0.426
No. of leaflets	1	12.8±2.0	16.4±3.7	4.178
Internode length(cm)	1	5.0±2.0	40.3±9.0	3.897
No. of flowers/ inflorescence	20	27.3±4.6	16.8±0.8	20.940
Petal fusion length(cm)	10	0.7±0.1	16.1±1.1	0.012
Sepal length (cm)	10	0.5±0.04	8.2±0.6	0.002
Petal length (cm)	10	1.5±0.2	11.1±0.8	0.029
% 2 branched inflorescence	20	92.0±7.9	8.8±2.0	62.222
Style length (cm)	10	1.1±0.04	3.2±0.2	0.001
% Pollen viability	3	93.8±1.8	2.0±0.4	3.207

Figure 1. A comparison of the coefficients of variation (CV) between the CMO and SD populations and CMO and PP populations for sepal length, number of flowers per inflorescence,petal length, % 2-branched inflorescence, style length, and % pollen viability.

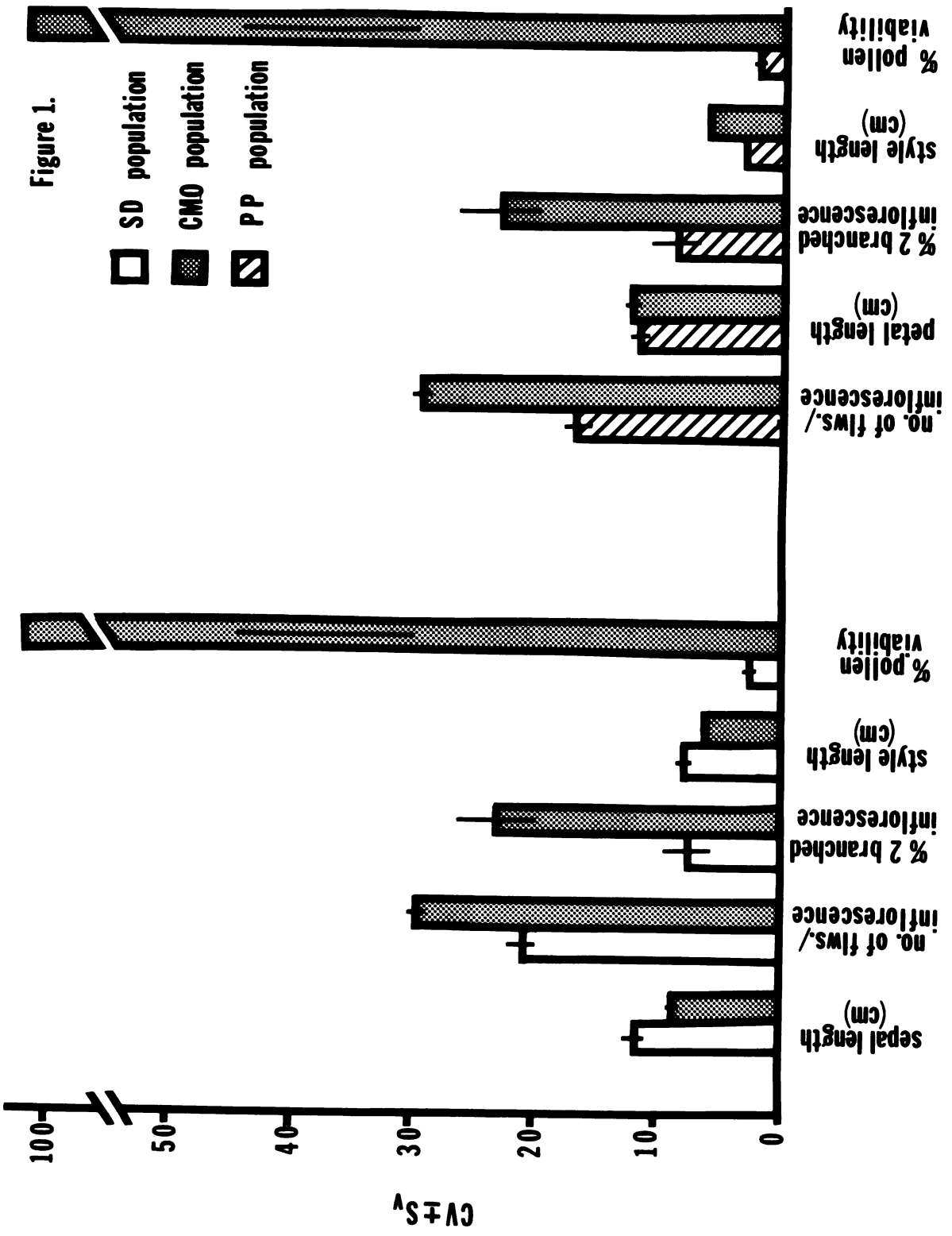
CV±SV
100
50
40
30
20
10
0
sepal length (cm)
no. of flws./ inflorescence
% 2 branched inflorescence
style length (cm)
% pollen viability
no. of flws./ inflorescence
petal length (cm)
% 2 branched inflorescence
style length (cm)
% pollen viability
SD population
CMO population
PP population

Figure 1.

Callus-mediated organogenic (CMO) population compared to the seedling population (SD)

CMO population variances for terminal leaflet length on the first fully expanded leaf, number of leaflets on the first fully expanded leaf, internode length, petal fusion length, and petal length were homogeneous with those of the SD population (X^2 $P < 0.01$) (Table 5). Non-homogeneous variance was observed between the CMO and the SD populations for sepal length, number of flowers per inflorescence, % of 2-branched inflorescence, style length, and % pollen viability (Table 5).

The % pollen viability, % of 2-branched inflorescence, and number of flowers per inflorescence were more variable in the CMO than in the SD population. Variation in % pollen viability (CV=101.7$\pm$14.4) was 40.7 times greater, % of 2-branched inflorescence (CV=23.2$\pm$3.3) 3.1 times greater, and number of flowers per inflorescence 1.4 times greater in the CMO than in the SD population. Sepal length and style length characters were observed to be less variable in the CMO than in the SD population. Variation in sepal length (CV=8.8$\pm$0.4) and style length (CV=6.1$\pm$0.3) was 1.3 times smaller in the CMO than in the SD population (Tables 1,3; Figure 1).

CMO tetraploids compared to CMO diploid plants

The CMO population was composed of 10 diploid and 15 tetraploid plants. Both ploidy types varied to the same extent in 9 of the 10 characters measured. The number of flowers per inflorescence was the only trait in which the

Table 3. Mean, coefficient of variation (CV), and variance (V) for ten characters of the L. peruvianum seedling population (SD; 10 plants).

Character	SD Population			
	No. of obs./plant	Mean±SD	CV±S_v	V
Terminal leaflet length (cm)	1	5.1±0.5	10.2±2.3	0.260
No. of leaflets	1	16.6±3.6	22.2±5.0	12.933
Internode length (cm)	1	2.9±1.2	40.3±9.0	1.325
No. of flowers/ inflorescence	20	28.2±6.0	21.0±1.1	35.160
Petal fusion length (cm)	10	0.7±0.1	18.7±1.3	0.016
Sepal length (cm)	10	0.5±0.1	11.8±0.8	0.004
Petal length (cm)	10	1.6±0.2	13.5±1.0	0.047
% 2 branched inflorescence	20	89.5±6.4	7.4±1.7	41.389
Style length (cm)	10	1.2±0.1	7.8±0.6	0.008
% Pollen viability	3	97.9±2.3	2.5±0.6	5.498

tetraploid plants showed non-homogeneity of variance when compared to the diploid plants. The tetraploids were more variable (CV=30.8±1.3) in this character than the diploids (CV=26.0±1.3). The tetraploids also showed a marked reduction in pollen viability (X=9.9%±14.2) when compared to the diploids (X=76.0%±18.4). The tetraploids did, however, have much thicker, dark green leaves and thicker, more pubescent stems. In addition, three 4n plants had a crumpled leaf morphology and one 4n plant appeared to be self-fertile.

Somatic embryogenic (SE) population compared to the seedling population (SD)

SE variances for terminal leaflet length on the first fully expanded leaf, internode length, number of flowers per inflorescence, petal fusion length, and petal length were homogeneous (X^2 $P < 0.01$) with respect to the SD population (Table 5). In contrast, non-homogeneous variance (X^2 $P < 0.01$) was observed for the SE when compared to the SD population for the number of leaflets on the first fully expanded leaf, sepal length, % of 2-branched inflorescence, style length, and % pollen viability (Table 5).

Sepal length, % of 2-branched inflorescence and % pollen viability were more variable in the SE than in the SD population. Variation in the % pollen viability (CV=17.6±3.9) was 7.0 times greater, % of 2-branched inflorescence (CV=46.8±10.5) 6.3 times greater, and the sepal length (CV= 19.0±1.4) 1.6 times greater in the SE than

in the SD population. The number of leaflets on the first fully expanded leaf and style length characters were less variable in the SE than in the SD population. Variation in number of leaflets on the first fully expanded leaf (CV=12.4±2.8) was 1.8 times smaller and the style length (CV=6.5±0.5) 1.2 times smaller in the SE than in the SD population (Tables 3,4; Figure 2).

Leaf chimeras

Forty percent of the plants in the CMO population (10 out of 25) and 10 % in the SE population (1 out of 10) had light green leaf sectoring (Figure 3). No leaf chimeras were observed in the SD or PP populations.

Cytology

Protoplast regeneration via CMO led to a large proportion (60%:15 out of 25) of tetraploids (4n=48+2) (Figure 4). The plants regenerated via somatic embryogenesis (Figure 4) were all diploid (2n=24).

Peroxidase (Px) isozyme patterns

Three seedling plants and one plant from the SE population (SE-6) were selected for their different leaf Px isozyme patterns in region #3 of a starch gel profile and their stability in different environmental conditions. The selected band(s) were; for seedling #1 R_f=0.53, seedling #2 Rf=0.51 & 0.47, seedling #3 R_f=0.44, and the SE-derived plant R_f=0.44 (Figure 5). Plants regenerated via CMO from protoplasts of the seedlings and SE-6 had banding patterns

identical to those in the plants from which each was derived.

Table 4. Mean, coefficient of variation (CV), and variance (V) for ten characters of the somatic embryogenic population (SE; 10 plants) of *L. peruvianum*.

Character	SE Population			
	No. of obs./plant	Mean±SD	CV±S_v	V
Terminal leaflet length(cm)	1	5.1±0.5	9.6±2.2	0.225
No. of leaflets	1	11.0±1.3	12.4±2.8	1.778
Internode length(cm)	1	5.3±2.1	39.8±8.9	4.245
No. of flowers/ inflorescence	20	16.2±5.7	35.0+1.8	31.917
Petal fusion length(cm)	10	0.6±0.1	18.7±1.3	0.012
Sepal length (cm)	10	0.6±0.1	19.0±1.4	0.011
Petal length (cm)	10	1.4±0.2	14.6±1.0	0.042
% 2 branched inflorescence	20	41.4±18.9	46.8±10.5	357.60
Style length (cm)	10	1.1±0.1	6.5±0.5	0.005
% Pollen viability	3	88.4±15.2	17.6±3.9	230.80

Figure 2. A comparison of the coefficients of variation (CV) between the SE and SD populations for the number of leaflets, sepal length, % 2-branched inflorescence, style length, and % pollen viability characters.

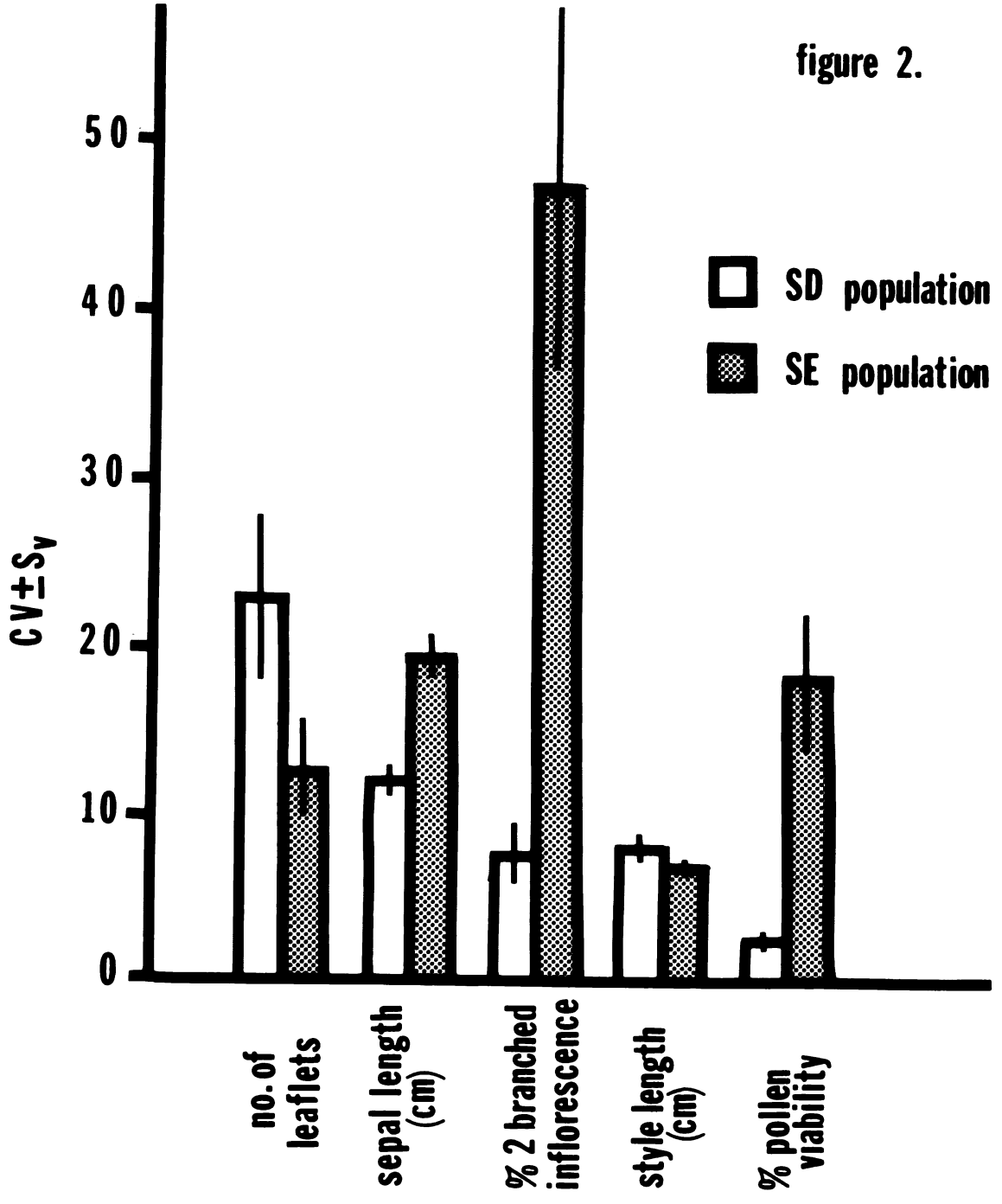
50
40
30
20
10
0
CV±Sv
SD population
SE population
no. of leaflets
sepal length (cm)
% 2 branched inflorescence
style length (cm)
% pollen viability

figure 2.

Table 5. Chi-square values for homogeneity of variance comparisons of seedling (SD) -vs- somatic embyrogenic (SE), SD -vs- callus-mediated organogenic (CMO), and CMO -vs- stem cuttings of the parent plant (PP) populations of *L. peruvianum*.

Character	SD-VS-SE	SD-VS-CMO	PP-VS-CMO
	X^2	X^2	X^2
Terminal leaflet length(cm)	0.11	3.34	0.33
No. of leaflets	17.73*	0.11	6.49
Internode length(cm)	6.66	0.37	6.11
No. of flowers/ inflorescence	1.07	40.28*	147.52*
Petal fusion length(cm)	4.62	0.03	5.85
Sepal length (cm)	67.93*	36.04*	0.20
Petal length (cm)	0.80	2.61	10.10*
% 2 branched inflorescence	20.50*	19.67*	13.18*
Style length (cm)	17.60*	26.70*	115.26*
% Pollen viability	46.69*	89.78*	101.05*

(*) Non-homogeneous variance; X^2 significant at $p<0.01$

Figure 3. A light green chlorophyll leaf chimera appearing on a plant regenerated from protoplasts of L. peruvianum.

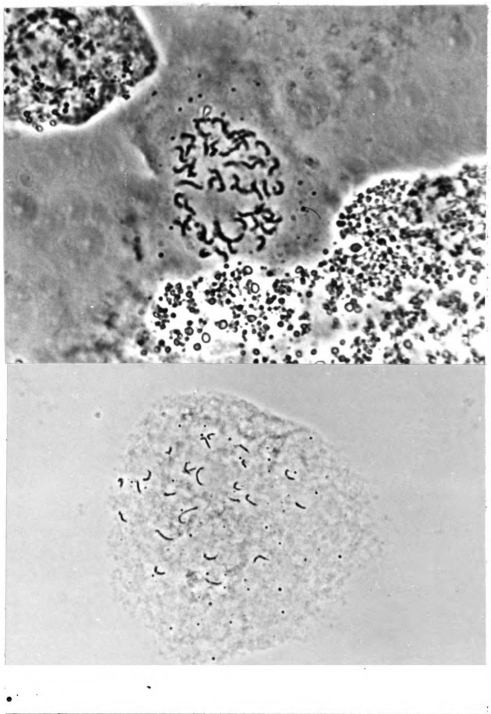

Figure 4. Root-tip chromosomes of L. peruvianum plants regenerated from protoplasts: a) a cell from a plant with double the normal diploid number (4n=48) b) a cell from a plant with the correct chromosome number (2n=24).

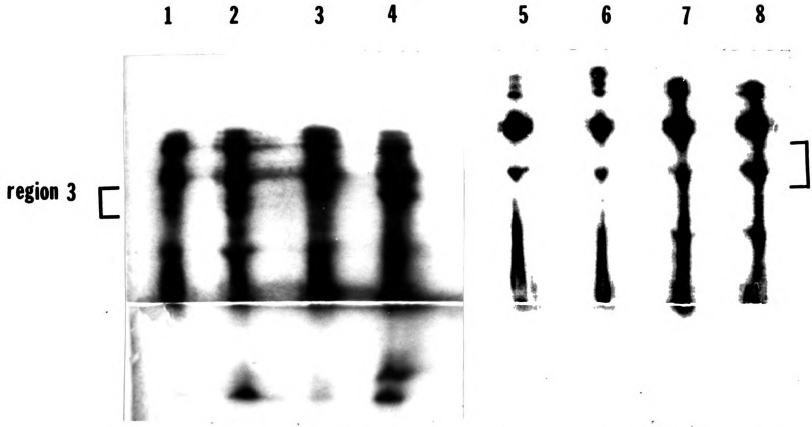

Figure 5. Leaf peroxidase (Px) isozyme patterns for 3 seedlings, lanes 1,3,5, 1 SE-derived plant, lane 7, and their protoplast regenerated plants, from seedlings, lanes 2,4,6, from the SE-derived plant, lane 8.

DISCUSSION

Plant tissue, cell, and protoplast cultures transmit genetic variation to their regenerated plant populations (Prat, 1983; Chaleff and Keil, 1981; Barbier and Dulieu, 1980). The mode of regeneration and type of variation in such plants are of particular interest because of their practical importance in crop improvement. L. peruvianum is one of the few species with high protoplast regeneration capacity (Vasil and Vasil, 1980a) and the only species with this capacity in the genus Lycopersicon (Zapata et al., 1977). In addition, along with Nicotiana tabacum (Takebe et al., 1971; Lorz et al. 1977) and Nicotiana sylvestris (Nagy and Maliga, 1976; Facciotti and Pilet, 1979), L. peruvianum is capable of protoplast regeneration via organogenesis wherein shoots arise from callus (CMO pathway) (Zapata, et al., 1977) and somatic embryogenesis (SE) wherein the cell(s) or calli form embryo-like structures (Zapata and Sink, 1981). These characteristics make L. peruvianum a good model system for comparing the CMO and the SE pathways relative to the type and frequency of spontaneous variation in the regenerated plants.

Protoplast culture can induce, at high frequency, a doubling of the chromosome number in the regenerated plants. Protoplast culture of L. peruvianum diploid plants via CMO

led to a large proportion of tetraploids. Facciotti and Pilet (1979) showed that, from haploid protoplasts of Nicotiana sylvestris, only diploids occurred in the regenerated plants. Also a large number of tetraploid plants were regenerated from protoplasts of diploid lines of Nicotiana sylvestris (Prat, 1983).

The tetraploid plants may have their origin at several steps in the cultural sequence. Firstly, they may have originated from spontaneous dinucleate fusion products. These products could occur during protoplast isolation (Reinert and Hellmann, 1971). Endopolyploidy during callus division may also have occurred (Cionini et al., 1978). The chromosomal constitution could have been affected by culture conditions including hormonal composition of the culture medium (Sacristan, 1971) and duration of the culture period prior to morphogenesis (Barbier and Dulieu, 1981). The tendency to vary also depends on the epigenetic state of the cultured material. Tissues with a high degree of tissue organization, such as shoot meristems, usually give rise to plants with normal chromosome numbers, whereas callus cultures from the same plant may not (D'Amato, 1977). This holds true for this study, for tetraploid plants were only regenerated from the CMO regeneration pathway. It is not known, however, whether these ploidy changes resulted from changes in cells during protoplast culture or from regeneration of pre-existent 4n karyotypes in the leaf cell population (Winkler, 1916; D'Amato, 1977).

Appearance of morphological variability in protoplast-derived plants has been described in Solanum tuberosum (Shepard et al., 1980) and Nicotiana sylvestris (Prat, 1983). In this study , similar phenotypic variation was observed among 25 plants regenerated via CMO from leaf protoplasts taken from a single plant of L. peruvianum. The increased variability exhibited for 5 of 10 flower morphology characters in the CMO population, when compared to the parent plant, could result from genetic mutation, epigenetic change, or a combination of both processes. Genetic changes such as single gene mutation (Prat, 1983), deletion (Barbier and Dulieu, 1980), rearrangements of the genetic material (Cummings et al., 1976), as well as changes in chromosome number (Karp et al., 1982; Prat, 1983), chromosome bridges and lagging chromosomes (Cooper et al., 1964), all have been documented in plant tissue culture systems. The ploidy changes observed in the CMO-derived plants in this study and the observations of genetic changes by other researchers cited above suggest that some of the variant phenotypes observed in the CMO regenerated plants may be genetically based. However, physiological and epigenetic changes also can be expected to contribute to the observed variability (Binns and Meins, 1973; Maliga et al., 1976; Meins, 1983). Genetic analysis of the observed variation in the CMO regenerates is needed to determine the exact source of variation. The line of L. peruvianum used in this study is self-incompatible, therefore, limiting genetic analysis of the variant traits observed in the regenerated

population.

Doubling of the chromosomal complement, is at least partially responsible for the increased variation in the number of flowers per inflorescence character in the CMO population, and may suggest that the number of alleles present affects expression of this character. Sources of variation in % pollen viability may be two fold. Firstly, passage through protoplast culture can cause a reduction in viable pollen (Schnabelrauch, 1982) which could be brought about by genetic or epigenetic mechanisms previously cited. Secondly, irregular meiotic distribution of chromosomes caused by the additional chromosomal complement in tetraploid plant pollen can result in a marked reduction in pollen fertility (Rao and Rao, 1978) and a concomitant increase in variation for this character.

Whether the variability observed in style length, type of inflorescence, and petal length is epigenetic or genetic is unknown although heritable mutations for such characters as long styles (Dorossiev, 1972), variable branched inflorescence , and slender petals (Stubbe, 1965) have been reported in the Lycopersicon genus.

Tomato leaf and stem morphology characters measured were not affected by passage through protoplast culture, although differences between the diploid and tetraploid plants for leaf thickness and color were evident. Variability has been reported for terminal leaflet length and number of primary and tertiary leaflet pairs but

constancy in internode length and secondary leaflet pairs in 65 protoplast-regenerated plants of Solanum tuberosum when compared to their protoplast source line (Secor and Shepard, 1981). Constancy was also observed for number of leaflets on the first fully expanded leaf, and for internode length in the CMO regenerated plants when compared to the single parent plant. The difference in the variability for terminal leaflet length between L. peruvianum and S. tuberosum could be due to species specific responses to protoplast culture or variability in this character for L. peruvianum was not detected due to a low plant population.

L. peruvianum is known as a strictly self-incompatible species (Lamm, 1950; McGuire and Rick, 1954). This self-incompatibility is gametophytic, governed by S-alleles. The failure of self-fertilization, resulting in complete outcrossing, leads to a high level of heterozygosity in L. peruvianum seed progeny. Even when compared to this large amount of natural variation (the SD population), the CMO regenerates, as a function of protoplast culture, showed more variation in 3 flower morphology characters. Conversely, style length and sepal length were less variable in the CMO regenerates than in the SD population; therefore, it appears that events which occur during protoplast culture leading to variation had less affect on these characters, or eliminated variants, or the SD population was simply more variable.

As with the CMO regenerates, when SE were compared to the SD plants, 3 flower morphology characters were more

variable. Since the L. peruvianum somatic embryoid structures did not fully resemble normal seedling embryos, events which disrupt the organizational patterns of a developing embryo in culture may also be a possible cause for variation in the SE-derived plants. For example, Sium suave (water parsnip) somatic embryos grown from cultured cells could be markedly altered by light (green cotyledons, expanded leaves, and short hypocotyls) or darkness (shoots were reduced and hypocotyls more elongated) (Ammirato and Steward, 1971). Addition of the growth regulator zeatin to the culture medium resulted in embryonic aberrations in Carum carvi L. such as multiple shoots, leafy and abnormal cotyledons, and enlarged hypocotyls (Ammirato, 1977). In addition, the total concentration of the nutrient medium (MS+sucrose) in which somatic carrot cells developed into embryos is a potent factor in determining their final form. In dilute solutions, embryos became large, the roots were much elongated, and the shoots expanded precociously and became leafy (Ammirato and Steward, 1971). The previously cited genetic and epigenetic mechanisms which may play a role in creating the variation in the CMO regenerates, except for tetraploidy and the callus organizational state, may also play a part in increasing the observed variability in the SE-derived plants.

Light green, leaf chimeral sectors occurred in both CMO and SD populations. Chlorophyll chimeras have been reported in plants regenerated from tissue culture in Lycopersicon

esculentum (Seeni and Gnanam, 1981) and Nicotiana tabacum (Opatrny and Landa, 1974), and from protoplasts in Solanum tuberosum (Thomas et al., 1982). Seeni and Gnanam (1981) found varigated chlorophyll leaf sectors in 10-15% of plants regenerated from tissue cultures of Lycopersicon esculentum heterozygous for the xanthophyllic-2 gene. They concluded that the leaf chimeras in heterozygous L. esculentum arose from somatic segregation in a mitotically dividing population of cells. Chlorophyll chimeral events can also originate from mutations in the nuclear genome (Epp, 1973). It seems feasible that if mutations affecting the chlorophyll molecule or chloroplast structure occurred as a result of protoplast isolation or culture, then somatic segregation of cells for this mutation could lead to chimeral sectoring. Somatic sectors can also arise in chimeric plants consisting of a mixture of genetically different cells (Stewart, 1978). Because it is not known whether or not regenerated plants arise from single cells (Broertjes and Keen, 1980), the sectors may simply reflect heterogeneity of the leaf tissue from which these plants were derived.

Protoplast isolation and regeneration via CMO did not result in changes of region #3 of their leaf peroxidase isozyme patterns. Due to the stability of these banding patterns, they may be useful as biochemical markers for L. peruvianum in interspecific fusions.

Clearly, morphogenesis via CMO and SE pathways are complex processes. Variation in morphological characters

may increase, decrease, or remain constant following organ or embryo morphogenesis. Variabilities such as altered pollen fertility, type of inflorescence, number of flowers per inflorescence, petal length, and sepal length, occurring after passage of standard lines through tissue, cell, or protoplast culture may be of value when quantitative characters of agronomic importance such as yield and certain forms of disease resistance are screened. This variation may also prove valuable for crop improvement in achieving the goal of germplasm diversification, providing an extra hedge against germplasm vulnerability that is rapidly occurring with the use of restricted genotypes. In addition, assessment of variation in p-clones is a necessary prelude for experiments which attempt to use somatic cell techniques as tools to manipulate the plant genome.

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