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CROSSABILITY STUDIES & THE ISOLATION, CULTURE AND REGENERATION OF PROTOPLASTS OF PETUNIA ALPICOLA

By

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ABSTRACT

CROSSABILITY STUDIES & THE ISOLATION, CULTURE AND REGENERATION OF PROTOPLASTS OF PETUNIA ALPICOLA

By

Jane Laverne Ford-Logan

In order to expand the diversity of germplasm available for improvement of petunia, Petunia alpicola was investigated for its use as a potential genetic resource for introgression into the cultivated P. hybrida Hort. This study was conducted to determine the breeding behavior of P. alpicola with selected Petunia species, to determine the stage(s) where failure occurs in the reproductive cycle between crosses of P. alpicola and other Petunia species, and to develop a procedure for the isolation, culture and regeneration of plants of this species from protoplasts.

Self-pollinations of different P. alpicola plants and reciprocal cross-pollinations of P. alpicola with P. parviflora and P. hybrida were performed under greenhouse conditions to assess self-compatibility and crossability relationships. The degree of crossability among the species was determined by the number of seeds set, seed germination and the number of successful intercrosses between the species. A fluorescence technique was used to monitor growth of pollen tubes in each of these self- and cross-pollinations. P. alpicola was found to be self-incompatible and all interspecific crosses failed to produce hybrids, the failures being caused by pre- and/or post-zygotic incompatibility.

Procedures were developed for the regeneration to plants of P. alpicola from callus and suspension culture protoplasts. Protoplasts were released from plasmolyzed cells in a defined enzyme mixture, plated in liquid culture medium and plating efficiency was determined. Growth of macroscopic colonies was enhanced by plating cells between layers of semi-solid agar. On transfer of protoplast-derived calli to

regeneration medium, numerous adventitious shoots were formed from which rooted plantlets were regenerated.

To My Mother and Oscar,

for their never-ending love, support and encouragement

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Guidance Committee:

This dissertation is organized in journal style format in accordance with the Department of Horticulture and Michigan State University requirements. Two papers were prepared following the journal style formats of HortScience and Journal Plant Physiology.

INTRODUCTION

In 1803 A. L. Jussieu founded the genus Petunia to describe plants which he collected on the banks of the Plata River. He delineated Petunia nyctaginiflora and P. parviflora, but the former species had already been decribed as Nicotiana axillaris by Lamarck in 1793. It was not until 1888 that the earliest trivial name, axillaris, was associated with the generic name Petunia when Britton, Sterns and Poggenburg listed Petunia axillaris (Lam.) in their Preliminary Catalogue of Anthophyta and Pteridophyta. At present there are approximately 30 recognized species of Petunia (Sink, 1984). They are indigenous to Central and South America and extend north into southern parts of the United States.

The precise genetic background of the cultivated Petunia hybrida has not been established, although P. axillaris and P. violacea are considered to be progenitors (Ferguson and Ottley, 1932; Gleason and Cronquist, 1963). Steere (1930), based on cytological studies, suggested that P. inflata as well as P. axillaris and P. violacea have contributed to the development of P. hybrida.

The species investigated in this research, P. alpicola, has generated interest since it is the second Petunia species now known to have a $2n = 2x = 18$ chromosome number. The other one is P. parviflora (Ferguson and Coolidge, 1932). Except for these two species, the diploid chromosome number of all other species and cultivated types reported to date is $2n = 2x = 14$.

The phylogenetic origin of P. alpicola is unknown, although morphological observations suggest its closest affinity may be to P. parviflora. In addition to sharing the same chromosome number ($2n = 18$), they both have a prostrate or creeping

growth habit, short-petioled succulent leaves and small magenta flowers. All of these features are in distinct contrast to the 2n = 14 Petunia species.

Since the first hybridization of Petunia species in the early 1800's to create P. hybrida Hort., there has been no further improvement in it based on wild species germplasm. The bedding plant industry, to which petunias are of considerable economic importance, is presently experiencing a decrease in sales primarily due to increased sales of other competing species. It is felt that improvement in botrytis resistance, floral features, growth forms and cultural management could renew the commercial demand for petunias. P. parviflora is a species which could serve as a potential germplasm resource for these traits, but it is sexually incompatible with the cultivated petunia (Sink and Power, 1977). Thus, integration of desirable genes into P. hybrida may require using novel tissue culture techniques such as protoplast fusion (Sink, 1980).

An objective of somatic hybridization is to combine species that exhibit incongruity at the interspecific or intergeneric level in order to expand the diversity of germplasm available for crop improvement. Based on research to date, it could be expected that P. alpicola is also a potential genetic resource for introgression into P. hybrida. A prerequisite for somatic hybridization would be to evaluate the type of incongruity that exists between two potential species by identifying the stage where reproductive failure occurs, and the methodology for regenerating plants from the protoplasts of at least one of the species to be used in cell fusion. Thus, this investigation was conducted to: 1) determine the breeding behavior of P. alpicola with selected Petunia species, 2) determine the stage(s) where failure occurs in the reproductive cycle of crosses between P. alpicola and other Petunia species, and 3) develop a procedure for the isolation, culture and regeneration of plants of this species from protoplasts.

REVIEW OF LITERATURE

Before Darwin called attention to the discontinuous intraspecific variation characteristic of cultivated plants, taxonomists of the period often elevated these variants to the rank of species. With the rediscovery of Mendelian genetics, many of these species were correctly evaluated as lines differing in only one or a few major genes. The development of genecology focussed attention on the process of speciation, particularly the role of reproductive isolation in the differentiation of species. This led to the recognition of fewer, but more variable, species.

I. ORIGIN, TAXONOMY AND EVOLUTION OF PETUNIA

Origin: The species Petunia axillaris (Lam.) B.S.P. and P. violacea Lindl. are considered to be the progenitors of the cultivated Petunia hybrida Hort. According to Ferguson and Ottley (1932), seeds of these two species were transported from their indigenous South American countries to European countries about 1820-1830; subsequently, hybridization between these species produced the germplasm base for all further breeding and selection. By 1937 a number of ornamental strains had been developed. Mather (1943) related a similar occurrence in accounting for the origin of P. hybrida.

Taxonomy and Evolution: The early literature concerning the taxonomic status and nomenclature of P. axillaris and P. violacea is confusing. Early taxonomic workers on the genus used P. violacea to describe much of the plant material under study, including both species types and cultivated types. Even today there exists an unsettled taxonomic status with respect to P. inflata and P. violacea. For many years, P. axillaris was referred to as P. nyctaginiflora Juss. as first illustrated by Sims (1825), although earlier it had been described by Lamarck (1793) as Nicotiana axillaris. There

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1.2 Problem Statement and Motivation

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is also the possibility that two other species, P. inflata and P. parodii, could have entered into the evolution of the cultivated types. Steere (1930) described P. parodii as similar to P. axillaris with the exception of a longer corolla tube, smaller limb, pointed lobes, and deep purple veining of the throat. Their similarities may have caused them to be mislabeled due to both having a white corolla. The characteristic long corolla tube of P. parodii should have resolved the identity confusion because it would have been transmitted to the offspring, as shown by Sink (1981). By reason of the fact that this obvious trait was not indicated in early figures describing P. hybrida and the fact that P. parodii was not taxonomically classified until 1930 by Steere, argues against it playing an early role in the evolution of cultivated types (Sink, 1981). Using floristic data plus flower color requirements and breeding behavior, Sink (1981) excluded P. parodii as a possible ancestor.

Petunia inflata, which was described by Fries (1911), is quite similar morphologically to P. violacea except for minor differences in corolla characteristics; it may easily have been mistaken for the latter. Smith and Downs (1966) combined both P. violacea and P. inflata under P. integrifolia (Hook.) Schinz and Tellung var. integrifolia. After comparing the taxonomic delineating characters of P. axillaris, P. inflata and P. violacea, Lamprecht (1953) considered them all to be P. axillaris. His decision was based on an evaluation of morphological characters as well as the genetics of flower color which was determined by Mather and Edwardes (1943).

Based on cytological studies, Steere (1930) reported that P. hybrida may be a composite of the three species P. axillaris, P. inflata and P. violacea. Natarella and Sink (1974), using thin-layer chromatographic analyses of phenolic leaf extracts, concluded that P. axillaris and P. violacea were most likely the immediate ancestors of P. hybrida. In contrast, analyses by electrophoresis (Natarella and Sink, 1975) suggested that P. inflata may have been a progenitor of P. hybrida.

Cytogenetic Studies in Petunia: The basic chromosome number in Petunia is x = 7. The diploid chromosome number for all Petunia species and cultivated types reported to date is 2n = 2x = 14, with the exception of P. parviflora (Ferguson and Coolidge, 1932) having 2n = 2x = 18. Chromosome counts of P. axillaris (2n = 14) were made by Steere (1932), Ferguson and Coolidge (1932), and Sullivan (1947); on P. parodii (2n = 14) by Steere (1932) and Sullivan (1947); and on P. violacea (2n = 14) by Kostoff and Kendall (1931), Ferguson and Coolidge (1932), and Sullivan (1947).

Steere (1932) determined that chromosome pairing was synaptic in meiosis of P. parodii and likewise, Kostoff and Kendall (1931) found normal pairing of the 2n = 2x = 14 P. violacea chromosomes and about 2% abortive pollen. Dermen (1931) indicated a 2n = 2x = 14 chromosome number for P. hybrida and observed very loose pairing of the chromosomes at diakinesis, so much so that some pairs showed almost no connection between members. Rick (1971) found every possible aneuploid in the progeny of a 3x x 2x P. hybrida cross; the plants were comparable in viability and had phenotypic morphology almost identical to diploids. This observation was interpreted as an indication of the large degree of genetic redundancy and tolerance to aneuploidy which exists in Petunia.

Skalinska and Cuchtma (1927) studied a number of varieties of Petunia and reported differences in chromosome size in different varieties. Malinowski (1928) also reported chromosome size differences in a strain of variegated Petunia and found on average the chromosomes of large purple flowers were larger than those of small lilac ones. Contrary to this, Dermen (1931) found no significant difference in size of chromosomes between the small flowered and large flowered diploids that were used in connection with his work on polyploidy in Petunia.

II. INCOMPATIBILITY IN PETUNIA

Incompatibility - An Overview: Incompatibility may be defined as the failure, following self- or cross-pollination, of a male and female gamete to achieve fertilization, whereas each of them is capable of uniting with other gametes of the breeding group after similar mating or pollination. Pandey (1960) indicated that self-incompatibility has been found in 78 angiosperm families and occurs in every major phylogenetic line (East, 1940; Fryxell, 1957; Brewbaker, 1957). Pandey has provided excellent review articles on the evolution and description of self-incompatibility (Pandey, 1960; Pandey, 1968; Pandey, 1977).

The operation of self-incompatibility centers on the fact that pollen that is incompatible with the stylar tissue will either not germinate on the style or will produce very slow pollen tube growth.

Incompatibility, or compatibility, is dictated by a genetic system operating in both the male and female. The functionality of the system is based on a physiological interaction between the pollen (1n) and the style (2n). A multiple allelic series, designated S, governs the system (Brewbaker, 1957). Traditionally, a single locus has been proposed to control the mechanism, while Pandey (1977) indicated the possible involvement of two loci in some species.

Incompatibility can be expressed in one of two systems, either gametophytic or sporophytic. In each of these systems there are variations, particularly with regard to the number of S loci, and the interactive relationship between the alleles at the same or different loci (Pandey, 1957). The gametophytic system was proposed by Prell (1921) with the first supporting data obtained by East and Mangelsdorf (1925, 1926) in Nicotiana hybrids and by Lehman (1926) in Veronica. Incompatibility results when a pollen grain and the stigma have an allele in common. Thus, the incompatibility is determined gametophytically by the particular allele in the pollen grain. Dominance

among gametophytic S alleles cannot occur in the haploid pollen and is not known to occur in the diploid style (Brewbaker, 1957). In contrast, S allele interactions do occur in the heterogenic diploid pollen grain in autotetraploids (Brewbaker, 1957). One of these is the competition interaction in which neither allele is fully active, with the result that such grains are not inhibited or are only partially inhibited and progress through pistillate tissue (Maheshwari, 1949). The second interaction observed in tetraploids is that of dominance, in which one allele partially or completely suppresses the action of the other allele in the heterogenic pollen (Brewbaker, 1957).

The sporophytic system was first described with supporting data by Gerstel (1950). The system contains a form of dominance in which S_1 is dominant over all other alleles, S_2 is dominant over all but S_1, and so on. In microsporogenesis all pollen, regardless of genotype, retains the phenotypic response of the dominant allele in the male diploid tissue (Brewbaker, 1957). There is also some evidence for dominance in the pistil of plants having the sporophytic system (Brewbaker, 1957).

The difference between the gametophytic and sporophytic mechanisms is suggested to be based on the time at which S alleles act to produce incompatibility substances or their precursors which later change into incompatibility substances (Lewis, 1956; Pandey, 1958). Pandey (1958) suggested that the time of the S allele action in the sporophytic system is after anaphase II in the pollen mother cell, before the separation of the four microspores from the common cytoplasm. The specific substances in the sporophytic system are already present in the cytoplasmic material which forms the microspore wall. These substances produce the incompatibility reaction on contact of the pollen grains with the incompatible stigma; thus, inhibiting pollen germination (Pandey, 1960). In the gametophytic system the time of S allele action is after cytokinesis. Therefore, the specific substances are produced internally within each microspore whose wall is free of specific substances. In gametophytic species, pollen grains germinate and penetrate the incompatible style,

but the growth of pollen tubes is stopped after the specific substances of the pollen tube and the corresponding substances of the style are brought together through diffusion or other processes (Pandey, 1960).

The Role of Glycoproteins in Incompatibility: In the crucifer Brassica, the self-incompatibility reaction is localized at the stigma surface, and occurs within minutes after the initial contact between the pollen and the papillar cells on the outer surface of the stigma. In this genus, self-incompatibility is under the control of a single genetic locus, the S locus, which is highly polymorphic, some 50 alleles having been identified.

A molecular analysis of the genetic control of incompatibility may be performed by detecting antigens specific to various S-locus alleles in stigma homogenates from different Brassica strains (Nasrallah and Wallace, 1967). These antigens have been shown to correspond to glycoproteins that may be resolved in various electrophoretic systems (Nasrallah et al., 1970; Nasrallah et al., 1972; Nishio and Hinata, 1977; Nasrallah and Nasrallah, 1984). Several lines of evidence suggest that these glycoproteins play an important role in incompatibility. (1) The mobilities of these molecules vary in stigma extracts derived from Brassica strains with different S-locus alleles (Nasrallah and Nasrallah, 1984). (2) These molecules are found in the stigma but not in stylar or seedling tissue (Nasrallah et al., 1985a). (3) The increased rate of synthesis of these S-locus-specific glycoproteins (SLSGs) in the developing stigma correlates with the onset of the incompatibility reaction in the stigma (Nasrallah et al., 1985a). (4) Mutations in genes unlinked to the S locus which result in self-compatibility are also associated with reduced levels of these molecules (Nasrallah, 1974). (5) The inheritance of the various forms of SLSG correlates with the segregation of S alleles in genetic crosses, indicating that the gene responsible for this polymorphism must be genetically located at or closely linked to the S locus (Nasrallah et al., 1972).

Nasrallah et al. (1985b) reported the isolation of a complementary DNA clone

containing sequences encoding an S-locus-specific glycoprotein from Brassica oleracea in which they show that the spatial and temporal distribution of the messenger RNA homologous to these sequences mirrors the appearance of the S-locus-specific glycoprotein. Several fragments of the B. oleracea genomic DNA, generated by restriction endonucleases, hybridize with the SLSG cDNA clone; polymorphisms in certain of these fragments segregate precisely with alleles of the S locus.

The Callose Response in Incompatibility: Evidence that the callose response may be used as an indicator of biocommunication between pollen and stigma, and a diagnostic tool for rapid assessment of the nature of a pollination in the biotechnology of seed production has been reviewed by Dumas and Knox (1983). Callose, a cell wall polysaccharide composed generally of 1,3 ß-glucans, which can be localized by the decolorized analine blue fluorescence (ABF) method (Currier, 1957; Linskens and Esser, 1957), provides a useful phenotypic bioassay: to determine the site of rejection of pollen tubes in incompatibility phenomena (Linskens and Esser, 1957); for estimates of the dynamics of pollen tube growth in gametophytic competition (Mulcahy, 1975); and in estimating the viability of the pistil in determining the effective pollination period (Anvari and Stösser, 1978).

Callose, in appearance, is uncoloured and gelatinuous, amorphous and isotrophic (Herth et al., 1974), and is characterized by its solubility properties. The chemical nature of callose has been discussed by Clarke and Stone (1963). Callose produced in pollen tubes of rye after self-pollination proved to comprise a mixture of 1,3 and 1,4 ß-linked glucans in the proportion of 9:77 (Vithanage et al., 1980). Reynolds and Dashek (1976) found that lily pollen tube callose stained with the ABF method, but not following protease treatment, suggesting the callose may be a glycoprotein. Dickinson and Lewis (1973) could not detect any protein-staining of callose in stigmas of Raphanus.

In several differentiation programmes in plant tissues, callose is rapidly

synthesized, especially after wounding and during plant host/parasite interactions, particularly during pollination (Aist, 1976; Heslop-Harrison, 1978). The callose response during pollination may be highly specific, occurring in stigma cells in contact with incompatible, but not compatible pollen in genera such as Brassica, Raphanus, Cosmos and Helianthus which have well-developed sporophytic self-incompatibility systems (de Nettancourt, 1977). Wall-held pollen proteins elicit the response (Heslop-Harrison et al., 1974, 1975) and its specificity has been explored using cell surface probes (Kerhoas et al., 1983). Sood et al. (1982) found that the response may be induced not only by pollen grains, but by macerates of somatic tissues.

There are numerous hypotheses on the role of callose since it is so strategically sited at the pollen-stigma interface.

- that it prevents tissue dehydration through control of cell wall equilibrium by the intervention of calcium and potassium ions. Calcium ions block water molecules on the surface of callose; potassium ions liberate these water molecules (Vithanage et al., 1980).
- it mobilizes reserve carbohydrate, according to the transitory nature of callose deposits (Currier, 1957).
- that it takes part in defense reactions. Callose plays both an active and passive role in incompatibility; is related to stress responses, both trauma and environment (Vithanage and Knox, 1977; Aist, 1976; Lewis, 1980), by isolating or sealing pollen from the stigma (Heslop-Harrison, 1975; de Nettancourt, 1977).
- it has a trophic role. Callose formation utilizes substrate that would otherwise be available for tube growth (Sedgley, 1977).
- a physiological role in pollen tube growth: in vitro growth activated by 1,3 ß-glucanases (Reynolds and Dashek, 1976). These enzymes may act during growth in vivo to maintain tip growth through control of balance of wall-synthesizing

and degrading enzymes. Callose accumulation in incompatible tubes could be due to a change in balance.

Self-Incompatibility in Petunia: The self-incompatibility reaction in Petunia is gametophytically controlled by one locus with a series of S alleles (Lewis, 1944). All P. inflata accessions studied to date exhibit self-incompatibility, and sib-matings are required for seed production. Only on rare occasions have seedling derived plants been found that set a very low quantity of seed from a few self-pollinated flowers (Sink, 1981). Physiological studies of self-incompatibility in P. inflata have been conducted by Brewbaker and Majumder (1961).

Both P. parodii and P. axillaris are self-fertile and fruit abundantly, both in the greenhouse and in open culture (Ferguson and Ottley, 1932). In general, more than 95 percent of the flowers form large capsules. A given capsule may contain from 800 to 1000 or more seeds. P. parviflora, another self-compatible species, produces approximately one-hundred seeds per capsule which are smaller but otherwise similar to the seeds of P. axillaris (Ferguson and Ottley, 1932). P. violacea accessions, while not readily producing the self-seed quantity per capsule or per plant as P. axillaris and P. parodii, does set seed following self-pollination (Sink and Power, 1978).

P. hybrida exhibits a functional self-incompatible system when selfed, but Flaschenriem and Ascher (1979) found plants which produced varying amounts of seed when used as the seed parent in crosses with unrelated individuals homozygous for the same S allele. This phenomenon has been termed pseudo-self-compatibility (PSC) and is attributed to the action of non-allelic genes which affect normal S-gene activity and result in self-seed (Mather, 1943). Takahashi (1973) found the PSC in P. hybrida to be the result of a stylar reaction which resulted in faster pollen tube growth in styles of plants which expressed higher levels of PSC and also to the increased vitality of some pollen.

Interspecific Cross-Incompatibility in Petunia: Petunia axillaris, P. inflata, P. parodii and P. violacea have all been readily intercrossed with P. hybrida (Sink, 1975). Intercrosses among these selected Petunia species have been successful using standard pollination techniques with the exception of the cross between P. parodii and P. inflata (Sink et al., 1978). Small quantities of hybrid seed were obtained by bud-pollination of P. parodii, although the reciprocal cross failed. It was later shown that these two species exhibit a unilateral cross-incompatibility with a pre-zygotic mode of reproductive isolation preventing hybridizations with P. inflata as the maternal parent (Sink et al., 1978). The interspecific hybrids obtained from all of these crosses set abundant seed by self-fertilization and backcrossed readily with each of the parental species (Sink, 1975).

Because only the P. parodii by P. inflata hybridization failed using standard pollination techniques and fertile F_1 offspring can be obtained which cross easily with each parental species, a high degree of genetic homology between both species is indicated (Sink, 1975). It is suspected that a minor portion of the chromosome complement of these two species is responsible for the reproductive isolation.

Sink and Power (1978) reported reproductive isolation between P. parviflora and the four previously mentioned Petunia species plus P. hybrida cv. Comanche, using standard and bud-pollination procedures. Reciprocal attempts at the crosses were also not successful. The incongruity of P. parviflora with the 5 Petunia species was established by the failure of approximately 1000 pollinations. A later study showed that the Fraction 1 protein patterns of P. parviflora differs in having a single small subunit polypeptide located between the two polypeptides found in the other species and cultivars (Gatenby and Cocking, 1977b). The small subunit composition may represent the point of divergence of P. parviflora from the other petunias. This lends support to the theory that although P. axillaris and P. violacea may have given rise to the fourteen chromosome petunias by allopolyploidy, they were probably not

immediately related to P. parviflora or its progenitor. This is further supported by the distinct growth habit, plant and flower morphology of P. parviflora ($2n = 2x = 18$) compared to the aforementioned petunias (Ferguson and Ottley, 1932) and its record of no intercrossing (Sink and Power, 1978). Subsequently, Power et al. (1980) reported the production of somatic hybrid plants between P. parodii and P. parviflora in an attempt to affect morphological change in ornamental petunias by the transfer of the different growth habit of parviflora to P. parodii.

III. PROTOPLAST ISOLATION, CULTURE AND REGENERATION

Plant protoplasts are routinely isolated through the use of cell wall degrading enzymes. With the appropriate enzyme treatment it is possible to isolate protoplasts from virtually any plant species or any type of plant tissue. However, the ability to isolate protoplasts capable of sustained cell division with subsequent callus or plant regeneration is limited to a small, but increasing, list of plant species.

Protoplast Isolation: Protoplasts were first isolated using mechanical methods (Klercker, 1892). In most cases, the yield was small, and only large and highly vacuolated cells could be used for isolation. The use of cell wall degrading enzymes (Cocking, 1960) was soon recognized as the preferred method to release large numbers of uniform plant protoplasts.

Enzymes for protoplast isolation are dissolved in an osmoticum which usually consists of a sugar such as glucose or sucrose or a sugar alcohol such as mannitol or sorbitol. Mannitol and sorbitol, separately or in combination, have been used most often with mannitol preferred for the isolation of leaf mesophyll protoplasts. Glucose has been used successfully as an alternative to these hexitols for cultured cells (Kao and Michayluk, 1974). In some cases mineral salts, particularly KCl and $CaCl_2$, are added

to increase protoplast membrane stability (Gamborg et al., 1973; Rose, 1980). Magnesium chloride has also had a positive effect on the release of stable protoplasts. The effective osmotic concentration depends on the cell osmotic pressures at the time of isolation. Endogenous cell osmotic pressures are influenced by environmental conditions (Shepard and Totten, 1975) and can be manipulated by dark pretreatment of plants, use of young leaf tissue, etc. Agents such as potassium dextran sulfate (Takebe et al., 1968; Passiatore and Sink, 1981) and polyamines (Galston et al., 1978) have been added to counteract the effect of toxic substances which are present as contaminants or released by the cells during protoplast isolation. Minimal enzyme concentrations are used to obtain viable protoplasts, depending on factors such as enzyme type, protoplast source, and incubation temperature. Enzyme preparations also exhibit specific pH and temperature optima but these parameters must be adjusted to levels that are not deleterious to the plant cells. The pH of the enzyme isolation solution has been varied, usually between 5.4-6.2. It has been suggested that higher pH, 6.0-7.0, is most favorable to release mesophyll protoplasts of Phaseolus (Pelcher et al., 1974). However, a lower pH, 5.8, has been used to release mesophyll protoplasts of Glycine (Schwenk et al., 1981), a closely related seed legume. In some cases buffering agents such as a phosphate or MES [2-(N-morpholino)-ethanesulfonic acid] are added for pH stabilization (Kao and Michayluk, 1975). These compounds minimize the shift to acidic pH that may occur during protoplast isolation (Gamborg, 1976). Incubation temperatures of 20-27^{0}C are commonly employed but extremes such as 2^{0}C (De La Roche et al., 1977) and 36^{0}C (Othman and Paranjothy, 1980) have been used. The time required for isolation can range from 30 min (Nagata and Ishii, 1979) to 24 h (Kao et al., 1974) depending on protoplast source, enzymes, pH, and temperature. While the effect of light on isolation of protoplasts has not been studied in detail, protoplasts are usually isolated in the dark (Gill et al., 1981), or in low-light intensity (Chellappan et al., 1980).

Protoplasts may be isolated from a wide range of tissues or cell types (Vasil and Vasil, 1980). Leaf tissue and cell suspension cultures have been used as protoplast sources in many studies because of their availability and the satisfactory yields that can be obtained from them. Leaf protoplasts have been obtained by a two-step method involving treatment with pectinase to release cells from the mesophyll tissue followed by treatment with cellulase to convert the cells into protoplasts (Takebe et al., 1968). A single step system involving the use of mixed enzyme solutions is more frequently used in protoplast isolation. Solutions of different enzyme combinations may be used in sequence (Kartha et al., 1974; Gamborg et al., 1975) or the initial enzyme solution is discarded along with cellular debris and dead protoplasts which are often released during the early period of incubation (Gresshoff, 1980). Tissues derived directly from plants generally require surface sterilization, although a procedure for obtaining sterile protoplast preparations from non-sterile leaves has been described (Wilson et al., 1980). Leaf tissue can be mixed with the enzymes or floated on the surface of the enzyme solution. In the case of suspension cultures, specific volumes of cells in liquid medium are mixed with the enzymes or the medium is discarded after centrifugation and replaced by the enzyme solution.

Procedural modifications can facilitate protoplast isolation. These include peeling the lower epidermal layer (Power et al., 1976; Zapata et al., 1977) or brushing the leaf with carborundum to expose the mesophyll cells (Hughes et al., 1978), slicing leaf tissues into thin strips to facilitate enzyme entry (Chin and Scott, 1979), drawing the enzyme into intercellular spaces through vacuum treatment (Chin and Scott, 1979) and agitating the enzyme system (Chin and Scott, 1979).

Several factors or conditions influence the rate of release, final yield obtained, and stability of the isolated protoplasts. The physiological condition of the donor tissue prior to enzyme treatment as well as the isolation process are significant factors. The growth conditions of the donor plants critically affect both yield and stability of leaf

protoplasts. In many instances shoots or plantlets to be used for protoplast isolation have been grown aseptically in vitro in order to control growth parameters more effectively (Durand, 1979; Schenk and Hoffman, 1979).

Following isolation, protoplast preparations must be washed to remove the hydrolytic enzymes, cell debris and toxic products released from the donor tissue. Most purification procedures include pelleting via centrifugation followed by resuspension in wash or culture medium. In some studies, protoplasts have been washed by flotation in concentrated osmotica such as mannitol (Gatenby and Cocking, 1977a), sucrose (Shepard and Totten, 1977) or ficoll (Larkin, 1976). In addition, discontinuous gradient centrifugation and two phase separation techniques have been very effective in removing cell debris and contaminating organelles (Piwowarczyk, 1979; Slabas et al., 1980). Cellular debris has also been removed by binding to an anti-galactan-sepharose conjugate (Keller and Stone, 1978).

Protoplast Culture: Following isolation and purification, protoplasts are suspended in medium for culture. A minimal density in the order of 10^4/ml is generally required for culturing protoplasts. Viable protoplasts will respond by regenerating a cell wall and undergoing cell division (Vasil and Vasil, 1980). Maximizing plating efficiency is an important goal in protoplast culture. Many factors influence the viability and ultimate plating efficiency. These include the physiological condition of the donor cells prior to protoplast isolation, the procedures used in the isolation process, the composition of the culture medium, and the environmental conditions established for culture maintenance.

The composition of protoplast culture media varies with the plant species studied. Detailed descriptions of the components of protoplast culture media have been published (Gamborg, 1977; Eriksson, 1977). As the nutritional requirements of cultured plant cells and protoplasts are very similar, protoplast media are usually modifications of frequently used cell culture media. Gamborg's B5 (Gamborg et al., 1968) and

Murashige and Skoog (1962) cell culture media are most commonly used as a basis for protoplast media. Alterations in these and other cell culture media have been used for optimum growth of protoplasts.

It has been proposed that concentrations of iron, zinc, and ammonium in the standard cell culture medium may be too high for some protoplasts (Von Arnold and Eriksson, 1977). Ammonium has been found to be detrimental to protoplast survival, and media have been devised for many species, such as tomato (Zapata et al., 1981), that are devoid of ammonium. Calcium concentration is increased 2-4 times over the concentrations normally used for cell cultures (Eriksson, 1977).

While glucose may be the preferred carbon source for most protoplasts (Gamborg, 1977), other carbon sources, including sucrose, may be preferred or necessary for some species. Uchimiya and Murashige (1976) have shown that tobacco protoplasts grow equally well on sucrose, cellobiose, or glucose. Most protoplast media contain a mixture of carbon sources. For tomato, sucrose and glucose are mixed in a 2:1 ratio (Zapata et al., 1981). Kao and Michayluk (1974) showed that the preferred carbon source (in this case, glucose) can also be the preferred osmoticum. On the other hand, in some cases a nonmetabolizible osmoticum may be necessary, such as for pea mesophyll protoplasts where only mannitol and sorbitol could be used as osmotica (Von Arnold and Eriksson, 1977).

Numerous organic nutrients have been added to protoplast culture media. In most cases, vitamin requirements are the same for plant cells and protoplasts. Kao and Michayluk (1974) have suggested that addition of several vitamins, organic acids, sugar, sugar alcohols, and undefined nutrients such as casamino acids and coconut water for culture of protoplasts in very low densities. More often than not, many of these components are unnecessary for culture of protoplasts, as no benefit can be attributed from their use.

Types and concentrations of growth regulators are the media components that have been varied most frequently. Changes in growth regulators have been shown to have dramatic effects on cultured cells. Nearly all media contain an auxin and for some species the addition of a cytokinin may be necessary. 2,4-D is the growth regulator most commonly used in protoplast media; however, in some species, other growth regulators are preferred. For tobacco protoplasts, Uchimiya and Murashige (1976) observed a higher rate of cell division in cultures with NAA than in cultures with 2,4-D or IAA. Also, in tobacco, cytokinin is unnecessary to induce cell division in cultured protoplasts. Von Arnold and Eriksson (1977) reported the requirement for both auxin (2,4-D) and a cytokinin (2iP) to induce cell division in pea mesophyll protoplasts.

In some instances conditioned medium obtained from cell suspension cultures has been utilized to supplement protoplast culture media (Durand, 1979). Nurse tissue techniques including culture on an underlayer of irradiated cells (Cella and Galun, 1980) or co-culture with albino cells (Menczel et al., 1978; Evans, 1979) have been used to increase plating efficiency in low density cultures. Gleba (1978) and Caboche (1980) were also able to achieve high plating efficiencies in low density protoplast populations after an initial culture period at high densities.

The physical aspects of protoplast culture can influence plating efficiency and a number of techniques for establishing cultures have therefore been developed. Protoplasts are commonly suspended in liquid medium and plated either as droplets or thin layers in petri dishes. Microdrop techniques have been developed to permit the culture of small numbers of protoplasts (Gleba, 1978) and multiple drop arrays have been used to test large numbers of media modifications (Harms et al., 1979). Protoplasts have also been embedded in agar and in some cases sustained division could only be obtained in solid medium (Gill et al., 1979). Pipetting protoplast suspensions onto filter paper placed on agar medium has led to improved plating efficiency in some species (Partanen, 1981). Other modifications have included transfer from liquid to

agar media after short culture periods (Li et al., 1980) and use of reservoir media in quadrant dishes (Bidney and Shepard, 1980). After successful culture establishment, the dividing cells require the addition of fresh medium. During such feedings the concentration of the osmoticum is generally reduced in a sequential manner.

Plant Regeneration: The regeneration of plants from protoplasts has been achieved in a number of species with the greatest success obtained with members of the Solanaceae. These include Nicotiana species, Petunia species, and Solanum species. Unfortunately, even among the Solanaceae where most effort on protoplast regeneration has been directed, an economic food crop, Lycopersicon esculentum, cannot be efficiently regenerated from protoplasts. Tomato does not seem to be as amenable to protoplast regeneration as other solanaceous species (Niedz et al., 1985).

Protoplasts isolated from callus, cell suspension, leaf, and flower petal have all been regenerated. Most of the methods for protoplast regeneration vary between species and donor tissue. Regeneration is generally achieved through organogenesis (Power et al., 1976; Bourgin et al., 1979), although somatic embryogenesis has been induced in protoplasts of a few species (Dudits et al., 1976; Zapata and Sink, 1981).

Several problems remain unresolved in the area of protoplast culture, one of which is the general lack of success in cereal protoplast culture (Potrykus, 1980). Cell division has been observed in protoplast cultures of some species, but plating efficiencies have generally remained low and morphogenesis is still very limited. Legume protoplast cultures have been of limited value for the induction of morphogenesis. In the seed legumes such as peas and soybeans, protoplast-derived calli have often been obtained (Gamborg et al., 1975; Oelck et al., 1983), but plants have thus far not been consistently regenerated. In the case of forage legumes successes in plant regeneration have been reported for alfalfa (Dos Santos et al., 1980; Kao and Michayluk, 1980; Johnson et al., 1981) and white clover (Gresshoff, 1980).

Protoplast cultures of a number of species lack morphogenic capacity although plants can be regenerated from callus, cell suspensions, or cultured explants. In these species, plants have previously been regenerated from the same tissues utilized for protoplast isolation. It is not clear whether reduced morphogenic capacity is related to exposure to the enzymes, disruption of tissue organization during protoplast isolation or to irreversible effects induced by the protoplast culture conditions.

The list of species other than solanaceous capable of plant regeneration from protoplasts has been steadily expanding to include both monocots and dicots, and a number of economically important crops. As this list increases, it is anticipated that the use of protoplasts in somatic hybridization and genetic manipulation experiments will be extended to include other economically important crops such as the legumes and cereals.

Regeneration of Petunia From Protoplasts: Hess and Potrykus (1972) observed division of isolated Petunia hybrida protoplasts and Potrykus and Durand induced callus formation in 1972. In 1973, Durand et al. recovered intact plants of petunia - thus completing the entire sequence from isolated protoplasts to whole plants. Since that time, within the genus Petunia, other species and breeding lines have been found to be amenable for regeneration into plants from isolated protoplasts (Binding and Krumbegel-Schroeren, 1984. There are several reports of regeneration in both haploid (Binding, 1974) and diploid (Frearson et al., 1973; Vasil and Vasil, 1974) protoplast systems of P. hybrida Hort. and for some other species of Petunia; P. axillaris (Power et al., 1976), P. inflata (Power et al., 1976), P. parodii (Hayward and Power, 1975; Patnaik et al., 1981), P. parviflora (Sink and Power, 1977) and P. violacea (Power et al., 1976).

All the above cited reports, with the exception of P. parviflora (Sink and Power, 1977), have utilized Petunia species possessing an $n = 7$ haploid or $2n = 2x = 14$ diploid chromosome number. P. parviflora is a species documented to have a $2n = 2x = 18$

chromosome number. Even though the Petunia species studied to date all have an x = 7 base number, they have varied with respect to protoplast isolation procedures, composition of protoplast culture media, and shoot and root regeneration media (Table 1). This distinct variability in cultural requirements indicates that taxonomic differences are reflected in in vitro culture systems.

IV. PROTOPLAST TECHNOLOGY

Fusion of Protoplasts: One of the most important uses of protoplast culture is for somatic hybridization. Somatic cell fusion leading to the formation of viable cell hybrids has been developed primarily as a method for the genetic manipulation of plant cells. This technique enables the construction of hybrids between taxonomically distant plant species beyond the limits of sexual crossability, and also creates cells with new genetic, nuclear as well as cytoplasmic, constitutions that otherwise are unobtainable. The experimental establishment of new combinations of nuclei, chloroplasts, and mitochondria provides a novel and potent tool to study the genetic and physiological interaction between these organelles.

The spontaneous fusion of mechanically isolated protoplasts was first observed by Küster as early as 1909. The first induced protoplast fusion was produced by Cocking and collaborators using sodium nitrate as the fusogen (Power et al., 1970). However, the efficiency of this technique was found to be low. During subsequent searches for a more suitable fusogen, treatment with gelatin (Kameya, 1973), concanavalin (Hartmann et al., 1973; Glimelius et al., 1974), and different salt solutions (Eriksson, 1971; Kameya and Takahashi, 1972) were tried. Also, Kameya (1975, 1979, 1982; Kameya et al., 1981) found that high molecular weight dextrans in the presence of high concentrations of inorganic salts cause protoplast aggregation and fusion, which are enhanced by NaOH or by electrical treatment.

Table 1. Procedures for culture and plant regeneration from protoplasts of Petunia species.

Species	Isolation Mixture	Protoplast Culture Medium (μM)[1]	Protoplast Culture Conditions	Regeneration Medium (μM)	Reference
P. axillaris (mesophyll)	Meicelase P 2%, Macerozyme 0.2%, Mannitol 0.71 M	MS, 2,4-D 4.5, 6BA 2.2	25 C, 1000 lux	MS, NAA 0.27, 6BA 2.2	Power et al., 1976
P. hybrida (mesophyll)	(Preplasmolysis, Mannitol 0.4 M) Pectinase 2% then, Cellulase 2%, Mannitol 0.4 M	DPD, 2,4-D 6.3, 6BA 1.8	28 C, 700 lux 16 hr light	NT, NAA 11.0, 6BA 2.3	Durand et al., 1973
P. hybrida (mesophyll)	(Preplasmolysis, Mannitol 0.71 M) Cellulase P1500 1.2% or Macerozyme 0.4%, Mannitol 0.71 M	F5, NAA 8.0, 6BA 4.4	22 C, 700 lux	MS, KIN 11.9, IAA 22.8	Frearson et al., 1973
P. hybrida Haploid (mesophyll)	(Preplasmolysis, Mannitol 0.3-0.4 M) then, Cellulase 3%, Macerozyme 2%, Mannitol 0.4-0.6 M	V47, NAA 6.0, 6BA 2.0	27 C, 50-300 lux	NT, 6BA 5.0, NAA 2.0	Binding, 1974
P. hybrida (stem callus)	Cellulase 3%, Macerozyme 3%, Mannitol 0.4 M	NT, NAA 2.7, 6BA 0.88	Not reported	MS, NAA 0.54, 6BA 14-56.7	Vasil & Vasil, 1974
P. inflata	Meicelase P 3%, Macerozyme 0.3%, Mannitol 0.71 M	MS, NAA 11.0, 6BA 2.2	25 C, 1000 lux	MS, KIN 2.3	Power et al., 1976

Table 1 (cont'd)

Table 1 (cont'd).

Species	Isolation Mixture	Protoplast Culture Medium (μM)[1]	Protoplast Culture Conditions	Regeneration Medium (μM)	Reference
P. parodii (mesophyll)	Meicelase 3%, Macerozyme 0.3%, Mannitol 0.7 M	F5, NAA 8.0, 6BA 4.4	25 C, 1000 lux	MS, IAA 8.6	Hayward & Power, 1975
P. parodii (mesophyll)	Meicelase 5%, Macerozyme 0.5%, Mannitol 0.71 M	MS or 8p, NAA 11.0, 6BA 2.2	25 C, dark	MS, 6BA 4.4, IAA 11.0	Patnaik et al., 1981
P. parviflora (mesophyll)	(Preplasmolysis, Mannitol 0.49 M) Rohament P 1%, Mannitol 0.71 M then, Meicelase P 4%, Macerozyme 0.4%, Mannitol 0.71 M	MS or 8p, NAA 11.0, 6BA 2.2	25 C, 1000 lux	MS, ZEA 4.6	Sink & Power, 1977
P. violacea (mesophyll)	Meicelase P 3%, Macerozyme 0.3%, Mannitol 0.71 M	MS, NAA 27.0, 6BA 4.4	25 C, 1000 lux	MS, ZEA 4.6	Power et al., 1976

[1]Culture media for Table 1:

DPD = Durand et al., 1973
F5 = Frearson et al., 1973
MS = Murashige & Skoog, 1962
NT = Nagata & Takebe, 1971
8p = Kao & Michayluk, 1975
V47 = Binding, 1974

Keller and Melchers (1973) introduced an effective fusion technique based on the treatment of protoplasts with Ca^{2+} ions. The ability of Ca^{2+} to induce fusions could be increased by incubating the protoplast in media containing Ca^{2+} ions at high temperature (37°C) and at the highly alkaline pH of 10.5.

Another very successful and more popular method for the fusion of protoplasts was developed by Kao and associates (Kao and Michayluk, 1974; Constabel and Kao, 1974) and by Wallin et al. (1974); also based on the use of Ca^{2+} ions but with lower concentrations. This method involves the agglutination of protoplasts with the aid of high molecular weight (MW) polyethylene glycol (PEG, MW ca. 6000). Protoplasts treated with PEG solutions containing Ca^{2+} fuse during the elution and/or dilution of PEG in the presence of, or by eluting with solutions containing high Ca^{2+} at high pH and high temperature (Burgess and Fleming, 1974; Kao et al., 1974; Wallin et al., 1974; Schieder, 1977).

Zimmermann and Scheurich (1981a, b; Zimmerman, 1982) described a completely new approach to fusion, the application of an electric field for protoplast agglutination and fusion. Protoplasts from different tissues and species have been fused via this method which has also been utilized for the production of viable hybrids of animal cells and of yeast. There are no reports of the application of this method for the production of somatic hybrids of higher plants. This technique has also been used to release individual chloroplasts from mesophyll protoplasts of Avena sativa (Zimmerman et al., 1982) and may prove suitable for the isolation of small numbers of pure plastids.

In addition to the above mentioned techniques, a wide range of additives such as poly-L-ornithine, poly-D-lysine, poly-L-lysine, cytocholasin B and protamine sulfate (Grout and Coutts, 1974), lysozyme (Potrykus, 1971), glycerols and dimethyl sulfoxide (Ahkong et al., 1975) have been employed.

The existing techniques of protoplast fusion are suitably efficient and appropriate for most applications in parasexual plant hybridization. These and developing technologies may in the future become an efficient complement to the classical methods of plant breeding.

Protoplasts for Studies of Cell Organelles: Plant protoplasts provide a unique system for studying the structure, chemistry, and function of cell organelles. Organelles can be isolated without harsh mechanical methods necessary for disrupting plant cells. Isolation of many organelles has been achieved using plant protoplasts (Fowke and Gamborg, 1980; Galun, 1981). Experiments using isolated cell organelles such as nuclei (Lörz and Potrykus, 1978), chloroplasts (Potrykus, 1973), or mitochondria have been described and have also been successful with respect to physical uptake of the organelles into protoplasts.

By means of isolated chromosomes, a promising new scheme for genetic manipulation called chromosome-mediated gene transfer has been developed (Klobutcher and Ruddle, 1981). The use of plant material for such studies has been hindered, until recently, by the lack of reliable procedures for mass isolation of plant chromosomes. Recent developments in protoplast and cell culture of plants may soon change this situation. Although considerable efforts have been made in this field (Malmberg and Griesbach, 1980; Szabados et al., 1981; Griesbach et al., 1982), the isolation of plant chromosomes is still not well developed.

Hadlaczky et al., (1982, 1983) have developed a procedure for mass isolation of plant chromosomes, in milligram quantities, from protoplasts. Plant chromosomes isolated by this method exhibit excellent preservation of morphology, and the purity of the chromosomes has made them suitable for structural and biochemical studies.

In studies on somatic genetics, there is interest in transplantation of chloroplasts and their extrachromosomal genetic information into protoplasts. Such studies are of importance in understanding developmental biology and how the development of the

chloroplast is controlled through nuclear versus chloroplastic DNA. Such procedures are of interest when considering the potential for improving photosynthetic efficiency within or between species. A first step in developing the transfer of chloroplasts from one species into protoplasts of another is to isolate pure, intact, functional chloroplasts.

Until the early 1970s, the only established means for isolation of chloroplasts was to disrupt the plant cell wall by mechanically grinding the leaf material. This was a limitation, since most species are very resistant to mechanical grinding. However, in the 1970s, the procedures for isolation of protoplasts from various species became well established. It was found that chloroplasts could be efficiently isolated from protoplasts by mild lysis of the plasmalemma (Gutierrez et al., 1975). Since then, the list of species from which intact, functional chloroplasts can be isolated has grown dramatically, but the full potential has not been realized.

Protoplast isolation allows a much wider range of species from which intact chloroplasts can be isolated, including C_4 and Crassulacean acid metabolism (CAM) plants (Huber and Edwards, 1975; Edwards et al., 1978). However, among the leaf materials of species examined, not all are susceptible to digestion by the commonly used commercial cellulase and pectinase. Also, isolation of protoplasts is a more difficult and time-consuming process, and the yields are often relatively low. Nonetheless, protoplast isolation is an excellent procedure by which intact chloroplasts can be isolated from many species and has allowed a number of studies, including intracellular compartmentation of enzymes, metabolite transport, metabolic activity, and the isolation and study of the properties of chloroplast envelopes (Robinson et al., 1979).

Protoplasts have also been utilized for studies of cytoskeletal elements of plant cells. Emphasis has primarily focused on microtubules, particularly regarding their relationship to cell wall formation and cell shaping (Lloyd et al., 1980; Gunning and Hardham, 1982; Robinson and Quader, 1982). Information concerning other

cytoskeletal components such as contractile elements and intermediate filaments is still very limited. The work of Yamaguchi and Nagai (1981) illustrates the potential of protoplasts for microfilament isolation and identification.

Plant protoplasts also provide an excellent system to probe the plant plasma membrane which normally is inaccessible due to the presence of a cell wall and provides direct access to this vital cellular component and associated cell organelles (Fowke et al., 1983).

Coated vesicles and pits are numerous in cells which are involved in active cell wall formation. Very little is known about the function of coated vesicles in plants. The idea that they are exocytotic and are responsible for contributing material to the growing cell wall has received wide support (Fowke et al., 1983). Until recently it has not been possible to determine the direction of movement of coated vesicles and arguments for exocytosis rather than endocytosis have been based on circumstantial evidence. Protoplasts derived from rapidly growing cultured plant cells contain numerous coated vesicles and thus are particularly well suited to studies of this cell organelle (Mersey et al., 1982). Ultrastructural investigations of thin sections of protoplasts (Van der Valk and Fowke, 1981) and isolated plasma membrane fragments (Doohan and Palevitz, 1980; Van der Valk and Fowke, 1981) have provided valuable information regarding the distribution and morphology of plant coated vesicles. Protoplasts also offer advantages for the isolation of these (Fowke et al., 1983). Fractions highly enriched in coated vesicles have been obtained from soybean protoplasts and biochemical characterization of these organelles is being pursued (Mersey et al., 1983).

Research with plant protoplasts has provided the only clear demonstration of the direction of movement of coated vesicles in plant cells. The experiments with soybean protoplasts indicate that endocytosis of cationized ferritin (CF) can occur via coated pits and coated vesicles (Tanchak et al., 1983, 1984). Further research is required to

characterize the process and to determine whether such a mechanism operates in intact plant cells.

Enucleation of Protoplasts: The analysis of somatic hybridization products with respect to nuclear-cytoplasmic interaction is rather complicated. To avoid some of the problems associated with the combination of both nuclear genomes and mixed cytoplasmic material, subprotoplasts (protoplast fragments) can be used to replace one or even both of the fusion partners. Subprotoplasts can experimentally be prepared by the fragmentation of isolated protoplasts into miniprotoplasts and enucleated cytoplasts (Wallin et al., 1978; Lörz et al., 1981; Bradley, 1983). In general, protoplasts without green chloroplasts isolated from cell suspension or callus cultures are more suitable for enucleation than mesophyll protoplasts.

The fragmentation of protoplasts is achieved by centrifugal forces during centrifugation. Different specific densities of the cellular components (nuclei versus cytoplasmic material) allow the enucleation of protoplasts into iso-osmotic density gradients (Lörz et al., 1981). Additional exposure of isolated protoplasts to cytochalasin B in combination with centrifugation was also found to be beneficial for enucleation (Wallin et al., 1978). Suitable components for establishing gradients for protoplast centrifugation are inorganic salts, sugars, and modified silica gels such as Percoll (Harms and Potrykus, 1978; Lörz et al., 1981; Lesney et al., 1983).

Cytoplasts are very fragile structures and are metabolically less active than nucleated protoplasts (Lörz et al., 1981). More important, miniprotoplasts and enucleated protoplasts are suitable for fusion experiments, and cytoplasts are especially useful experimental tools for transfer of chloroplasts and mitochondria (Bracha and Sher, 1981; Maliga et al., 1982).

Plant Protoplast Transformation: Conventional plant breeding programs have introduced numerous improvements in agronomic crops during the past centuries. However, plant breeders may have reached a limit in the ability to introduce new

genetic information into plants and to create new plant varieties through conventional plant breeding techniques. The introduction of exogenous DNA into cells can result in a stable and heritable change in phenotype. This process known as transformation, is well established in several bacterial genera. In higher plants numerous transformation experiments have been reported.

Protoplasts are often the material of choice in genetic transformation studies, because the absence of the cell wall should presumably remove one barrier to DNA entry. Protoplasts are being used as a single-cell crown gall transformation system instead of the traditional wound infection procedure of whole plants, seedlings, or different parts of a plant. The advantages of a single-cell transformation system are in facilitating controlled conditions, and also in the possibility of obtaining a large number of simultaneously transformed cells (cell lines) derived from individual transformation events, which can be used in comparative studies (Ooms et al., 1982).

Rapid advances in recombinant DNA technology have permitted the transfer, integration, and expression of foreign genes in plants. Much of the success, to date, has resulted from the use of the tumor-inducing (Ti) plasmid of Agrobacterium tumefaciens, a soil bacterium, as a gene vector (Chilton et al., 1980; Thomashow et al., 1980). The transformation of protoplasts by foreign DNA necessitates a balance between maximizing the transformation frequency and maintaining an acceptable level of protoplast viability. Methods of DNA delivery to protoplasts include (1) infection (co-cultivation) of protoplast-derived cells with intact agrobacteria, (2) chemically stimulated uptake of isolated DNA into protoplasts, (3) fusion of bacterial spheroplasts with protoplasts, and (4) fusion and/or uptake of liposomes carrying DNA into protoplasts (Power et al., 1986).

Crown gall transformation of protoplasts requires the selection of transformants. Transformants can be selected by the tumorous character of growth substance independence, or the antibiotic resistance conferred by foreign genes (e.g., kanamycin

resistance). An indication of transformation to the tumorous condition is opine synthesis since the production of opines is encoded by the integrated sequence of Ti-plasmids in the plant cells. Unambigious evidence for the presence of foreign DNA in transformants is integrated Ti-plasmid sequences found in opine-negative clones by DNA-DNA hybridization techniques, as shown by Thomashow et al. (1980) and Ooms et al. (1982).

Methods of Plant Protoplast Transformation

1. Infection (Co-cultivation of Protoplast-Derived Cells with Agrobacterium - The coculture technique has become a procedure of general use in the molecular biology of the crown gall transformation of plant cells (Márton et al., 1979; Wullems et al., 1981; Ooms et al., 1982). This transformation procedure is less labor intensive than methods involving uptake of isolated plasmids, liposome delivery, or fusion of plant protoplasts with bacterial spheroplasts. It has been used with different strains of Agrobacterium and various plant species (Hasezawa et al., 1981; Wullems et al., 1981).

The high transformation frequency in cocultures and selection at the cultured cell level made possible significant progress in the field of plant cell genetic engineering. Achievements using the coculture techniques include the expression in plants of Ti-plasmids carrying chimeric resistance genes, thereby conferring drug resistance on the plant cells in culture (Caplan et al., 1983). The possibility of selection based on drug resistance of transformed plant cells allows the elimination of those genes from the Ti-plasmids that cause the tumorous growth of transformants after integration.

2. Chemically Stimulated Uptake of Isolated DNA into Protoplasts - Detailed procedures have been published for the isolation of Agrobacterium Ti-plasmid by buoyant density centrifugation (Davey et al., 1980; Draper et al., 1982). One of these involves a mechanical shearing step to fragment the bacterial chromosomal DNA (Davey et al., 1980; Draper et al., 1982); the other utilizes a high pH to denature the chromosomal DNA. Theoretically, the use of isolated Ti-plasmid should overcome any

host range limitations which may arise when attempting to transform plant cells with intact Agrobacterium. The methodology for transformation of protoplasts by isolated Ti-plasmid is based upon the use of chemical agents originally employed to stimulate virus uptake into protoplasts, e.g., poly-L-ornithine (PLO), or those used to induce protoplast fusion, e.g., polyethylene glycol (PEG). Krens et al. (1982) reported transformation of mesophyll protoplasts prepared from shoot cultures of Nicotiana tabacum v. Petit Havana SR1 by Ti-plasmid using PEG to stimulate uptake. A significant detail of the technique is the addition of calf thymus DNA to act as a carrier for the plasmid DNA.

3. Fusion of Bacterial Spheroplasts with Plant Protoplasts - The second approach to overcome host range limitations involves the fusion of Agrobacterium spheroplasts with plant protoplasts. Treatment of spheroplast-protoplast mixtures with a polyvinyl-alcohol resulted in the uptake of Agrobacterium spheroplasts into Vinca rosea cell suspension protoplasts, and expression of T-DNA in 0.1-0.2% of protoplast-derived cell colonies (Hasezawa et al., 1981). Since it is most convenient to perform genetic manipulations in E. coli, it is useful to be able to transfer genes directly from E. coli to higher plant protoplasts. This has been achieved by fusing E. coli spheroplasts with tobacco mesophyll protoplasts, giving a transformation frequency of 2.0 in 10^3.

4. Liposome-Encapsulated Delivery of DNA - Liposome-mediated delivery is a promising new technique for introducing macromolecules into plant protoplasts. These are small artificial lipid vesicles prepared (Uchimiya and Harada, 1981) for phosphatidyl choline and stearylamine by a process known as reverse phase evaporation (REV). Nucleic acid entrapped in such liposomes renders it highly tolerant to attack by nucleases. A number of studies established that incubation of liposomes with plant protoplasts resulted in their association with plant cells (Matthews et al., 1979; Lurquin and Sheehy, 1982; Fraley and Papahadjopoulos, 1982). It has been demonstrated by several laboratories that plant viral RNAs encapsulated in liposomes can be

used to infect protoplasts at high efficiency (Nagata et al., 1981; Watanabe et al., 1982; Fraley, 1983; Christen and Lurquin, 1983). It is likely that this method will also have application to DNA delivery experiments in studies on stable plant cell transformation or in short-term transient expression assays.

Protoplasts used in liposome studies have been prepared from a variety of plant species including carrot (Matthews et al., 1979), tobacco (Fraley et al., 1982), petunia (Fraley, 1983), and cowpea (Lurquin, 1979), using relatively standard enzymatic isolation methods. Complete removal of the cell wall is essential for maximum uptake (Nagata et al., 1981; Watanabe et al., 1982).

Optimal conditions for the uptake of nucleic acids into plant protoplasts have been reviewed (Ohgawara et al., 1983). In general, optimum delivery of plasmid DNA encapsulated in liposomes is achieved with negatively charged liposomes in the presence of 15% w/v PEG 6000. Maximum infection by TMV-RNA occurs using the same conditions. Currently, reports of transformation of plant cells by liposome-encapsulated Ti-plasmid exist, but are unsubstantiated. The transformation frequency is impractically low, probably reflecting the problems inherent in encapsulating such a large plasmid (90-150 MDa).

CROSSABILITY OF PETUNIA ALPICOLA

Additional index words. crossability, fluorescence technique, germplasm

Abstract. Self-pollinations of different P. alpicola plants and reciprocal cross-pollinations of P. alpicola with P. parviflora and P. hybrida were performed under greenhouse conditions to assess self-compatibility and crossability relationships. The fluorescence technique was used to monitor growth of pollen tubes in each of these self- and cross-pollinations. P. alpicola was found to be self-incompatible and caused by pre-zygotic incompatibility preventing the pollen tubes from growing beyond the stigmatic region. All interspecific crosses failed to produce hybrids. P. hybrida pollen germinated on the P. alpicola stigma, but there was no subsequent tube growth. In the reciprocal, nongerminating seeds were produced from this cross even though pollen tubes were only observed to extend into the lower half of the style without penetrating the embryo sac; thus, indicating the occurrence of pre- and/or post-zygotic incompatibility. Likewise, reciprocal pollinations between P. parviflora and P. alpicola were incompatible as confirmed by the inability of pollen tubes to grow past the stigmatic region of the style.

At present there are approximately 30 recognized species of Petunia (16, 19). They are indigenous to Central and South America and extend north into southern parts of the United States. Since the first hybridization of Petunia species in the early 1800's, which created the cultivated Petunia hybrida Hort., there has been no further breeding endeavors based on wild species germplasm. The bedding plant industry, of which petunias are of considerable economic importance, is presently experiencing a decrease in sales of petunia primarily due to increased sales of competing species such as impatiens and geranium. Improvement in botrytis resistance (7), floral features, and growth forms could renew the commercial demand for petunias.

The potential value of a wild Petunia species, Petunia parviflora Juss., which could serve as such a germplasm resource for these traits was recognized by Sink and Power (17). However, these authors reported P. parviflora to be sexually incompatible with the cultivated petunia (18); thus, Sink (15) proposed protoplast fusion to integrate desirable genes into P. hybrida. Petunia alpicola is another wild species which might also serve as a potential genetic resource for P. hybrida. It possesses small magenta flowers and a highly branched, prostrate growth habit very similar to that of P. parviflora. These two species, with $2n = 2x = 18$ chromosomes, are distinctly different from all other Petunia species and at present are the only available sources for potential genetic changes in cultivated petunias.

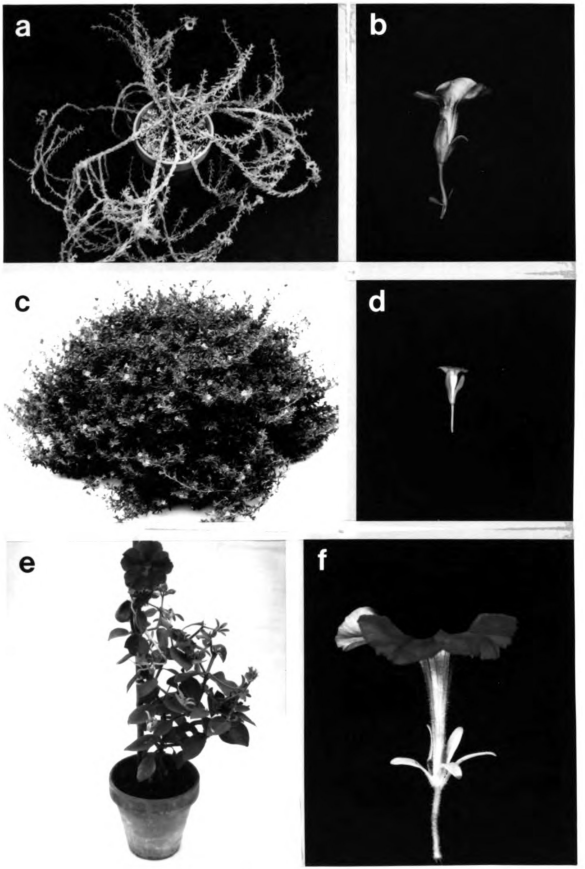
Before somatic hybridization is attempted, knowledge of the crossability and breeding behavior in selected interspecific Petunia crosses should first be assessed along with a determination of the stage(s) where failure occurs in the reproductive cycle between P. alpicola and other Petunia species.

Materials and Methods

Crossability studies. Plants of Petunia alpicola were obtained from Maureen Hanson, Cornell University, and subsequently taxonomically verified by Lyman B. Smith, Smithsonian Institute. Seeds of P. parviflora and P. hybrida 'Red Joy Improved' were germinated and plants grown to flowering (Fig. 1) in the greenhouse using standard cultural, disease and insect control practices. The greenhouse was maintained at 21-27^{0}C with a 16 hour photoperiod provided by incandescent lamps. At flowering, percent pollen viability was assessed by staining freshly dehisced pollen grains in analine blue. Pollen grains that exhibited a sharp and uniform stain were considered normal and viable. The number and percentage of normal and defective pollen grains were calculated from three replications, fifty fields per replication.

Self-pollinations were performed at anthesis on different P. alpicola plants and

Fig. 1. Plants and flowers of Petunia alpicola (a , b), P. parviflora (c , d) and P. hybrida cv. Red Joy Improved (e , f).


a
b
c
d
e
f

reciprocal cross-pollinations between alpicola and the other Petunia species were made using the standard procedure for emasculation and pollination. The immature corolla tube was slit open 24 hours prior to anthesis and pollination was performed the next day or when the stigmatic exudate appeared. Bud-pollinations were also carried out on P. alpicola by slitting open the corolla tube at various bud lengths prior to anthesis and emasculating anthers followed by immediate pollination. The degree of cross-ability among the species was determined by the number of seeds set, seed germination and the number of successful intercrosses between the species. Pollen grain germination and tube penetration in the style was observed in standard self- and reciprocal cross-pollinations of P. alpicola with P. parviflora and P. hybrida, 48 hours after pollination by use of the analine blue fluorescence technique (9). Pollen tube growth was rated using the following numerical system: 1) pollen grains present, but no germination; 2) pollen tubes in the stigmatic region; 3) tubes in upper half of style; 4) tubes in lower half of style; 5) tubes penetrated to the style base.

Electrophoretic hybrid identification. Axenic shoot cultures of P. alpicola and inbred lines of P. parviflora, P. hybrida 'Red Joy Imp.', P. parodii W.C.S., P. inflata Fries and P. axillaris (Lam.) B.S. P. were maintained on Linsmaier and Skoog (LS) salts (10) supplemented with the following (mg/liter): myo-inositol, 100; nicotinic acid, 0.5; pyridoxine HCl, 0.5; thiamine HCl, 0.1; glycine, 2; sucrose, 30000 and agar, 8000. Culture conditions were 28^{0}C under 16 hours of cool white fluorescent light of 32 $\mu Em^{-2}s^{-1}$. Leaf extracts for malate dehydrogenase (MDH) electrophoresis were prepared from the parental species and others by grinding approximately 1/2 g of leaf material in 10 drops of the extraction buffer (1.0 M Tris-citrate buffer, pH 7.0) plus 2 drops of cold mercaptoethanol between two plastic weighing dishes. The extract was absorbed into 6x8 mm filter paper wicks.

Horizontal slab starch gels were prepared using the modified system described by Meizel and Markert (12) and poured into a gel form to set. The gel was covered with

plastic wrap, refrigerated overnight, and trimmed of excess starch the next morning.

The paper wicks were inserted along a cut in the gel 5 cm from the cathodal end. The gel was placed between the electrode buffer trays and the electrode reservoirs filled with 500 ml of 5% 1.0 M Tris-citrate buffer (pH 7.0). Vinyl sheets over the wicks and thin sponges between the gel and buffer trays were used to establish contact. The wicks were removed after 1 hour (300 V/45mA); afterwhich, the run continued at 300 V at 4°C until the front moved about 8 cm from the origin (ca. 5 hours).

Upon termination of the run, the trimmed gel was cut horizontally into 3 slices and assayed with the substrate stain. The gels were stained at room temperature overnight, in the dark, with 50 mg ß-nicotinamide adenine dinucleotide (NAD), 20 mg nitro blue tetrazolium (NBT) and 5 mg phenazine methosulfate (PM) dissolved into 50 ml of 0.2 M Na-malate (pH 7.0) plus 50 ml of 0.2 M Tris-citrate buffer (pH 8.3) just prior to use. Three replications for each species was repeated four times with similar results.

Results and Discussion

P. alpicola had a pollen viability of 93.1%, but was found to be self-incompatible as shown by the inability to set seed following standard or bud-pollinations (Table 1). Pollen readily germinated on the surface of the stigma but the tubes only grew into the stigmatic region of the style with a mean growth rating of 2.6, 48 hours after pollination (Table 2). Observations of pollen grain germination and pollen tube growth suggest the self-incompatibility as probably pre-zygotic in nature. Previous studies have shown that the self-incompatibility reaction in Petunia is gametophytically controlled by one locus with a series of S alleles (2, 3, 8). Gametophytic self-incompatibility functions by regulating pollen tube growth in the style. Recognition between the pollen and style is mediated by the S gene, which has many allelic forms.

Table 1. Self- and reciprocal-crossability of P. alpicola with P. parviflora and with P. hybrida.

Pollination	No. of Flowers Pollinated	No. Seeds Set	Seed Set (%)	No. Seeds Sown	No. Seeds Germ	Seed Germ (%)	No. Plants Grown	No. Hybrid Plants	Cross-Ability (%)
P. alpicola ⊗	60[z]	0	0	-	-	-	-	-	0
P. alpicola x P. parviflora	50	0	0	-	-	-	-	-	0
P. parviflora x P. alpicola	50	40	80	30	29	96.7	25	0	0
P. alpicola x P. hybrida	50	0	0	-	-	-	-	-	0
P. hybrida x P. alpicola	50	74	148	65	0	0	-	-	0

[z]60 = 35 standard and 25 bud-pollinated.

Table 2. Pollen-tube growth 48 hr after self- and reciprocal cross-pollinations of P. alpicola with P. parviflora and with P. hybrida.

Pollination	No. of Pollinations	Mean Pollen Tube Growth Rating[z]
P. alpicola (x)	11	2.6
P. alpicola x P. parviflora	10	2.0
P. parviflora x P. alpicola	10	2.0
P. alpicola x P. hybrida	10	1.0
P. hybrida x P. alpicola	14	4.2

[z]Microscopic observation with 1 = pollen but no pollen tubes present; 2 = tubes in the stigmatic region; 3 = tubes in upper half of style; 4 = tubes in lower half of style; 5 = tubes penetrated to the style base.

When the pollen S allele matches an S allele in the style, as occurs in all self-pollinations, normal growth of pollen tubes is prevented. The action of incompatibility (S) genes has been shown to be a result of the interaction of the proteins released by the pollen with those of the stigma, similar to the antigen-antibody reaction, and is supported by evidence that specific glucoproteins form in the pistil following incompatible matings (6, 13).

One method of circumventing self-incompatibility is through the use of pseudo-self-compatibility (PSC), which has been reported in most species studied (1). PSC can be defined as limited or occasionally full seed set following incompatible pollination of a plant known to possess self-incompatibility. Bud-pollination is another form of PSC which is commonly used to inbreed plants in Cruciferae, Solanaceae and Scophulariaceae, but does not work on all individuals within a species or population. Sink and Power (18) were successful in producing a large quantity of self-seed from Nicotiana alata, which does not set seed following standard self-pollination due to the one-locus gametophytic type of incompatibility (5). Similarly, they produced abundant seed following bud-pollination of Petunia inflata, which also exhibits a gametophytic type of incompatibility (3). Unfortunately, similar success was not realized in trying to achieve self-pollination of P. alpicola by sib matings.

The most common form of PSC is the regular production of a few seeds from a small percentage of self-pollinations. Ascher (1) suggested that this form of PSC might explain the apparent self-compatibility of some individuals in Petunia. This probably explains the very limited seed production from P. alpicola bud-pollinations obtained by Jane Smith at Harvard University (Personal Communication to K. C. Sink). When seed was sown on moistened filter paper in petri dishes, viable seedlings were never recovered in this study. Susceptibility to environmental interaction and failure to respond to selection for the PSC character suggests quantitative inheritance (11, 20). Temperature is a major component of the environment and environmental interaction

with genetic factors appears to be the common explanation for PSC (4).

Crossability data for reciprocally crossed Petunia species are presented in Table 1. The term crossability, as used herein, denotes the relative ease with which hybrid seed could be obtained from a cross between two Petunia species, the germination ability of this hybrid seed, and the percentage of confirmed hybrids. Usually, lower frequency of crossability indicates a more distant genetic relationship and, in general, the more distantly related the parents, the more difficult it is to produce a hybrid between them. All interspecific crosses failed to produce seed with the exception of P. hybrida as the female parent crossed to P. alpicola. Out of 50 flowers pollinated, a total of 74 seeds were obtained from 3 capsules having 10, 2, and 62 seeds, respectively. Attempted germination of 65 of these seeds failed to produce viable seedlings. From the remaining 47 pollinated flowers, ovaries were harvested which contained dried chaff inside. Examination of P. hybrida styles pollinated with P. alpicola pollen resulted in a mean pollen tube growth rating of 4.2 (Table 2). Most tubes extended into the lower half of the style while a few penetrated as far as the bottom, but none were observed which had entered the embryo sac. In as much as entry into the ovules was not observed using the analine blue fluorescence technique, it is still uncertain whether fertilization occurred in obtaining the 74 nongerminating seeds from this interspecific cross. Pollen germination and pollen tube growth must occur for timely delivery of the male gamete to allow successful fertilization, and endosperm and embryo development must follow to produce viable hybrids capable of gene transfer. These results indicate pre-zygotic and/or post-zygotic barriers may be in effect. Conversely, in the reciprocal cross P. hybrida pollen, with 91% viability, germinated profusely on the P. alpicola stigma but subsequent pollen tube growth did not occur.

The interspecific cross between P. parviflora (98% viability) as female and P. alpicola as the pollen parent was initially believed to be a compatible mating, as indicated by the number of seeds set and the percentage of seeds that germinated

(Table 1). However, cross-incompatibility was confirmed since the plants were not hybrids. The plants from this mating were also electrophoretically examined and identified as being contaminants of P. parviflora by the identical malate dehydrogenase (MDH) isozyme pattern of the parental plant material (Fig. 2b). The migration distance and banding intensities of all isozymes found in the species examined are shown in Fig. 3. Similarly, the reciprocal cross proved to be cross-incompatible.

The answer to producing P. alpicola interspecific crosses may ultimately involve using techniques such as shortening the style length so that pollen tubes can reach the ovules. In vitro pollination and fertilization might also be attempted to circumvent these pre- and post-zygotic incompatibilities. However, in vitro fertilization techniques (14) are used primarily to overcome pre-zygotic self- and cross-incompatibility factors resulting from pollen-style interactions, and may not be the solution for the post-zygotic incompatibilities. Therefore, somatic hybridization may be a potential means to overcome both pre- and post-zygotic type incompatibility barriers existing between P. alpicola and other Petunia species. While most Petunia species can be readily regenerated to plants from protoplasts, somatic hybridization appears to be a viable alternative.

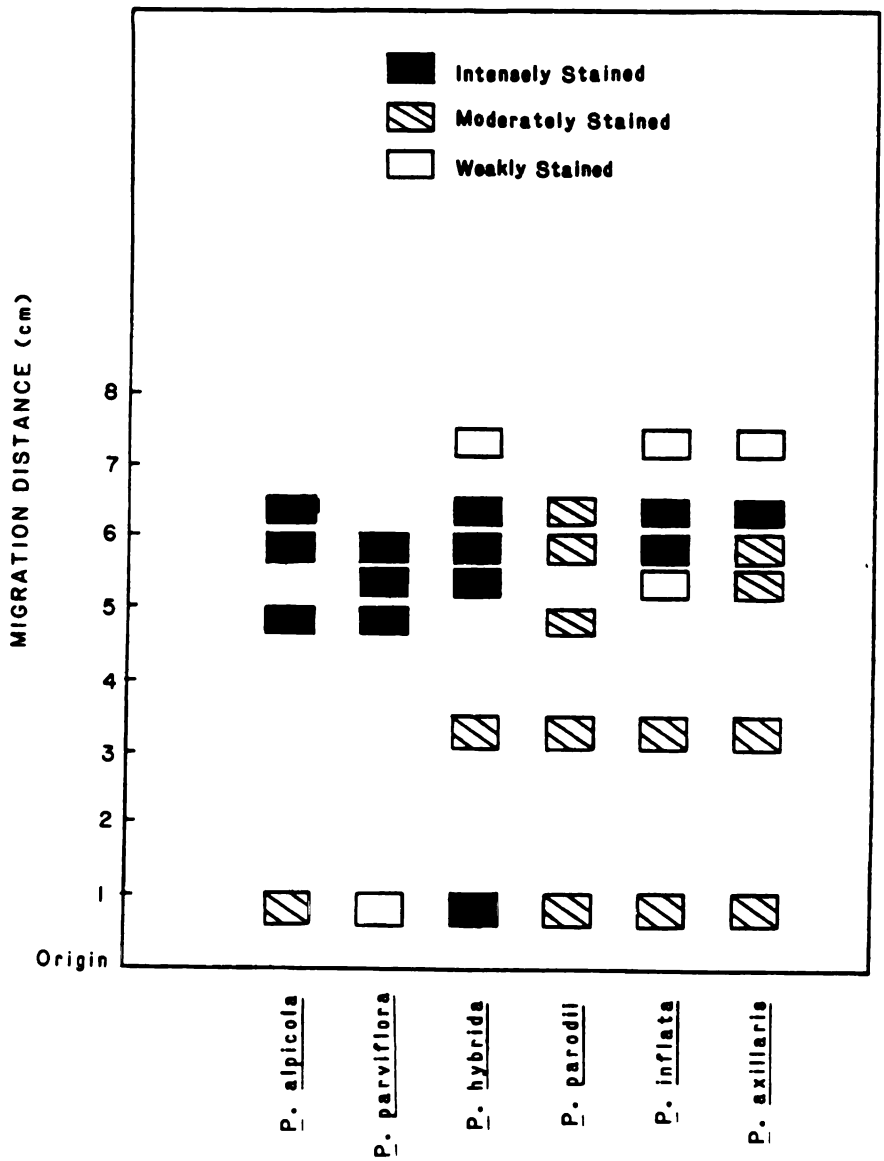
Table 1. [illegible] was continued since the plants were [illegible] hybrids. The plants from this mating were also electrophoretically examined and identified as being constituents of *P. parviflora* chemical mutant dehydrogenase (Gill) in some pattern of the parental plant material (fig. 1). The variation distance and banding intensities of all isozymes found in the species examined are shown in Fig. 3. Similarly the reciprocal cross pattern of the cross [illegible] is shown in Fig. 4.

The answer to producing *P. glycinicola* interspecific crosses may ultimately reside using techniques such as shortening the style length so that pollen tubes can reach the ovule sooner. Hybridization and fertilization may also be accomplished in vitro with these pre and post fertilization incompatibilities. However in vitro fertilization techniques [illegible] are used primarily to overcome pre zygotic and zygotic incompatibilities resulting from pollen style interactions and may not be the solution for the post zygotic incompatibilities. Therefore somatic hybridization may be a practical means to overcome both pre and post zygotic type incompatibility barriers existing between *P. glycinicola* and other *Physalis* species. While most *Physalis* species can be readily regenerated to plants from protoplasts, somatic hybridization appears a viable alternative

Fig. 2. Malate dehydrogenase electrophoretic pattern from Petunia alpicola (a), P. parviflora (b), P. hybrida (c), P. parodii (d), P. inflata (e) and P. axillaris (f).

a b c d e f

Fig. 3. MDH zymogram from leaf extracts of 6 Petunia species.


Intensely Stained
Moderately Stained
Weakly Stained
MIGRATION DISTANCE (cm)
8
7
6
5
4
3
2
1
Origin
P. alpicola
P. parviflora
P. hybrida
P. parodii
P. inflata
P. axillaris

Literature Cited

1. Ascher, P.D. 1976. Self-incompatibility systems in floriculture crops. Acta Hort. 63:205-215.

2. Ascher, P.D. 1984. Self-incompatibility, pp. 92-110. In: K.C. Sink (ed.). Petunia. Monographs on Theoretical and Applied Genetics 9. Springer-Verlag, Berlin Heidelberg.

3. Brewbaker, J.L. and S.K. Majumder. 1961. Cultural studies of the pollen population effect and the self-compatibility inhibition. Amer. J. Bot. 48:457-464.

4. East, E.M. 1923. Genetical aspects of self- and cross-sterility. Amer. J. Bot. 10:468-473.

5. East, E.M. 1928. The genetics of the genus Nicotiana. Bibliogr. Genetica 4:243-320.

6. Heslop-Harrison, J. 1975. Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Physiol. 26:403-425.

7. Laemmlen, F.F. and K.C. Sink. 1978. Evaluation of petunia cultivars for botrytis resistance. Plant Dis. Reptr. 62:361-365.

8. Lewis, D. 1944. Incompatibility in plants, its genetical and physiological synthesis. Nature 153:575-578.

9. Linskens, H.F. and K. Esser. 1957. Uber eine spezifische Anfarbung der Pollenschlauche im Griffel und die Zahl der Kallosepfropfen nach Selbstung und Fremdung. Naturwissenschaften 44:16.

10. Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.

11. Mather, K. 1943. Specific differences in Petunia. I. Incompatibility. J. Genet. 45:215-235.

12. Meizel, S. and C.L. Markert. 1967. Malate dehydrogenase isozymes of the marine snail Ilyanassa obsoleta. Arch. Biochem. Biophys. 122:753-765.

13. Nasrallah, M.E., J.T. Barber and D.H. Wallace. 1970. Self-incompatibility proteins in plants: detection, genetics, and possible mode of action. Heredity 25:23-27.

14. Rangaswamy, N.S. 1977. Applications of in vitro pollination and in vitro fertilization , p. 412-424. In: J. Reinert and Y.P.S. Bajaj (eds.). Applied and fundamental aspects of plant, cell, tissue, and organ culture. Springer-Verlag, Berlin.

15. Sink, K.C. 1980. Plant protoplasts - a novel method for producing new hybrids of horticultural crops. Part I. Flor. Rev. 167 (4323):22; 57-58. Part II. Flor. Rev. 167 (4324):18; 62.

16. Sink, K.C. 1984. Taxonomy, pp. 3-9. In: K.C. Sink (ed.). Petunia. Monographs on Theoretical and Applied Genetics 9. Springer-Verlag, Berlin Heidelberg.

17. Sink, K.C. and J.B. Power. 1977. The isolation, culture and regeneration of leaf protoplasts of Petunia parviflora Juss. Plant Sci. Lett. 10:335-340.

18. Sink, K.C. and J.B. Power. 1978. Incongruity of interspecific and intergeneric crosses involving Nicotiana and Petunia species that exhibit potential for somatic hybridization. Euphytica 27:725-730.

19. Smith, L.B. and R.J. Downs. 1966. Solanacea, p. 321. In: P.R. Reitz (ed.). Flora illustrada Catarinense. Itajai, S. Catarina, Brazil.

20. Takahashi, H. 1973. Genetical and physiological analysis of pseudo-self-compatibility in Petunia hybrida. Jap. J. Genet. 48:27-33.

THE ISOLATION, CULTURE AND REGENERATION OF PROTOPLASTS OF PETUNIA ALPICOLA

Summary

Petunia alpicola is recognized as a potential germplasm resource for enhancing the cultivated Petunia hybrida Hort. P. alpicola is sexually incompatible with the other species and thus somatic hybridization may be a viable alternative. The isolation and culture of protoplasts isolated from callus and suspension cultures of this species was achieved as a prerequisite to cell fusion. Efficient shoot regeneration was achieved from protoplast-derived calli on MS + zeatin (1.0 mg/l) and rooting on either MS + NAA (0.01 mg/l) or IBA (1.0 mg/l).

Key Words: Petunia, germplasm, protoplasts, regeneration.

Introduction

Regeneration of plants from protoplasts has been reported for many members of the Solanaceae family and for different species within the genus Petunia. Comparative studies on protoplast regeneration have been performed with different genotypes, species, and hybrids in regard to protoplast technology, taxonomic relationships, and somatic hybridization (Frearson et al., 1973; Power et al., 1976; Izhar and Power, 1977). Earlier protoplast work on Petunia has involved species possessing a $2n = 2x = 14$ diploid chromosome number, with one exception; that being P. parviflora Juss., the first species documented to have a $2n = 2x = 18$ chromosome number (Ferguson and Coolidge, 1932). The species investigated herein, Petunia alpicola, has the same chromosome number ($2n = 18$), and, although its phylogenetic origin is uncertain, morphological observations suggest its closest affinity may be to P. parviflora (Ford-Logan, unpublished). They both have a prostrate or creeping growth habit, short-petioled succulent leaves and small magenta flowers. All of these features are in

distinct contrast to the 2n = 14 Petunia species. The two 18-chromosome species have been recognized as potential germplasm resources for the integration of desirable genes into the cultivated P. hybrida Hort., but are known to be sexually incompatible with the cultivated petunia (Sink and Power, 1978; Ford-Logan, unpublished). Thus, novel tissue culture techniques such as protoplast fusion may be required before further exploitation can be realized. This study was conducted to develop a procedure for the isolation, culture and regeneration of plants of P. alpicola from protoplasts, based upon established protocols of plant protoplast isolation which have been either developed for Petunia, adapted to this species or utilized for it (Binding and Krumbegel-Schroeren, 1984).

Materials and Methods

Plants of P. alpicola were obtained from Maureen Hanson, Cornell University. Because P. alpicola is self-incompatible (Ford-Logan, unpublished) and does not set selfed seed, a micropropagation system was developed in order to maintain a constant supply of plant material. Excised shoot-tips, approximately 1 cm in length, were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 1.0 mg/l zeatin at 27°C for 16 h with 17 $\mu Em^{-2}s^{-1}$ from cool-white fluorescent tubes (G. E. F40CW-RS-WM). Every four weeks the multiple shoots that occurred were subcultured on the same medium with single stem growing plants maintained for experimental use as shoot-tip cultures on MS medium without growth regulators.

Leaf pieces (1-2 mm^2) from in vitro shoots were used to initiate friable callus when placed on MS + 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Dark green callus developed over the entire surface of the leaf explants and was subcultured after 5-6 weeks. These cultures were held at 28°C and 58 $\mu Em^{-2}s^{-1}$ for 16 h from cool-white fluorescent tubes (G. E. F96-T12-CW) and routinely subcultured every 21 days to maintain actively dividing callus. Suspension cultures were subsequently established

from this callus in liquid MS + 1.0 mg/l 2,4-D and maintained by subculturing every 10-14 days in the same liquid medium. These cultures were maintained in 125-ml flasks on a gyratory shaker at 90 rpm at 27°C in diffuse light.

Protoplasts were isolated from both callus and cell suspension cultures. With the aid of a spatula, callus (approx. 3 g) was gently separated by passage through a coarse sieve (35 μm) placed in a 100 x 15 mm plastic Petri dish and rinsed with a cell protoplast washing (CPW) solution (Frearson et al., 1973) containing 8% (w/v) mannitol (8M). The cell suspension cultures were handled differently in that the liquid medium was removed by transferring the cells to 16 x 125 mm culture tubes and pelleting them by centrifugation (80 x g; 5 min). The medium was replaced by CPW 8M afterwhich the cell slurry was pipetted onto a coarse sieve (35 μm) and separated in the same manner as callus. Hereafter, all cells, regardless of media source, were handled in the same manner. The cells were plasmolyzed in the CPW 8M solution for 1 h at room temperature in the light, without agitation. The plasmolyzed cell slurry was transferred to screw-capped culture tubes and pelleted by centrifugation (80 x g; 5 min). The supernatant was replaced by a filter sterilized enzyme solution which consisted of 2% Cellulysin, 2% Macerase, 2% Driselase and 8% (w/v) mannitol dissolved in CPW salt solution, pH 5.8. The cells suspended in the enzyme solution (approx. 5 ml packed; 20 ml) were transferred to 100 x 15 mm Petri dishes, which were wrapped with Parafilm® and incubated 17-19 h in dark at 27°C on a gyratory shaker (35 rpm). Following incubation, the cells were gently teased with a Pasteur pipette to release any protoplasts not liberated by enzyme action alone. The enzyme-protoplast mixture was passed through a fine sieve (61 μm) and collected in 100 x 15 mm Petri dishes before being transferred to culture tubes. The protoplasts were pelleted by centrifugation (100 x g; 5 min), and the supernatant was removed. The protoplasts were washed free of enzyme by resuspension in CPW 8M and centrifuging (100 x g; 5 min). The supernatant was replaced by 6 ml of a CPW solution containing 25% sucrose and

centrifuged (100 x g; 15 min). A second wash in CPW 25S removed any carryover of enzyme and debris. Protoplasts were removed with a Pasteur pipette to culture tubes and resuspended in liquid culture medium, counted and plated at test densities in 60 x 15 mm plastic Petri dishes. The components of the plating medium are listed in Table 1. Four ml of protoplasts were added to each dish, which were wrapped with Parafilm® and incubated at 25°C with constant illumination of 15 $\mu Em^{-2}s^{-1}$ provided by cool-white fluorescent tubes (G. E. F20-T12-CW). The effectiveness of the isolation procedure for obtaining high yields of viable protoplasts was monitored by fluorescein diacetate (FDA) (Widholm, 1972).

The dishes were replenished with the same medium with the mannitol level held constant during feeding at 2 and 4 week intervals after plating. The viability and division of the protoplast-derived cells were monitored by examining the culture dishes periodically under an inverted microscope. Plating efficiency (P.E.) was determined by the percentage of viable protoplasts 24 h after isolation that produced cell colonies after 7 days. After approximately 6 weeks in culture, the dishes were decanted of old culture medium and the macro-colonies were plated between two layers of semi-solid agar. This was done by adding the cell colonies plus 1 ml of fresh MS culture medium to a dish containing 2 ml of 0.4% agar cooled to 45°C and layering an additional 1 ml of cooled agar on top of the cells and swirling gently. When the proliferating cell colonies were of sufficient size, after 2 to 6 weeks, they were transferred to semi-solidified regeneration medium (Table 1) to produce shoots.

Results and Discussion

Leaves of P. alpicola proved to be technically unsuitable for protoplast isolation due to their small size and the difficulty involved in handling. Callus and suspension cultures proved excellent sources because these cells were already conditioned to growth in culture, and the requirements for their continued growth, differentiation

Table 1. Media used in P. alpicola protoplast culture and shoot regeneration.

Compound	Liquid Culture Medium (mg/l)	Shoot Regeneration Medium (mg/l)
MS salts	4627.93	4627.93
myo-Inositol	100.	100.
Thiamine-HCl	0.1	0.1
Glycine	2.0	2.0
Nicotinic acid	0.5	0.5
Pyridoxine-HCl	0.5	0.5
2,4-D	1.0	-
NAA	2.0	-
6-BAP	0.5	-
Zeatin	-	1.0
Coconut water	20 ml	-
Sucrose	30 000.	30 000.
Mannitol	130 000.	-
Agar	-	0.4
pH	5.8	5.8

Table 1 Media used in Eucalyptus protoplast culture and their preparation

Compound	Liquid Culture Medium (mg l⁻¹)	Shoot regeneration Medium (mg l⁻¹)
MS salts	4.3 g l⁻¹	4.3 g l⁻¹
myo-Inositol	100	100
Thiamine-HCl	0.1	0.1
Glycine	2.0	2.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
2,4-D	1.0	-
NAA	2.0	-
6-BAP	0.2	-
Zeatin	-	1.0
Coconut water	20 ml	-
Sucrose	30 g	30 g
Mannitol	136 g	-
Agar	-	0.4
pH	5.5	5.5

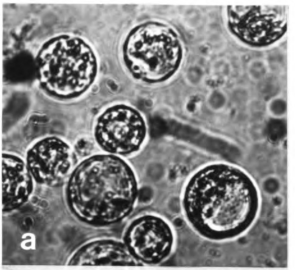
and organogenesis were established. There was no difference in the suitability or response of either of these cell sources when isolating and culturing protoplasts.

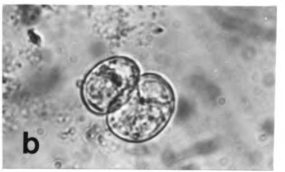
Protoplast yields were consistently 2-3 x 10^6 per gram of callus or suspension culture cells. These yields were considerably lower, approximately 6 x 10^5, when protoplasts were isolated from cultures nearing the time of subculture. Protoplasts isolated by this method were initially spherical in shape (Fig. 1a) and most exhibited normal ultrastructures when tested for their ability to hydrolyse fluorescein diacetate. After a day in culture, they became oval shaped, indicating cell wall synthesis (Kao et al., 1970), with active cytoplasmic streaming. Soon after protoplasts are cultured in an appropriate medium, an increase in cell organelles indicates a general activation of the metabolic activity of the protoplasts for the regeneration of a cell wall. With the increase in cell size, new cytoplasmic strands are formed, and most of the cell organelles, particularly the chloroplasts, aggregate around the nucleus (Vasil, 1976). First division occurred within 48-72 h (Fig. 1b). With repeated divisions (Fig. 1c) the number of chloroplasts per cell visibly decreased, and the cells became more vacuolated with micro-colony formation observed after 4 days (Fig. 1d). Pale green, visible macro-colonies (0.3-1 mm diam.) were formed in 3-4 weeks. A plating efficiency of 85% was recorded when protoplasts were plated at a density of 1 x 10^5 protoplasts/ml in the liquid culture medium. Prior to developing the medium which sustained division of P. alpicola protoplast-derived cells, several modifications of the MS culture medium were tested. The MS medium (from Table 1) with the same level of mannitol, naphtheleneacetic acid (NAA) and 6-benzylaminopurine (6-BAP), but lacking 2,4-D and coconut water (CW), only resulted in budding when plated at 1 x 10^5/ml; first division was never observed. It has been proposed that incomplete cell wall resynthesis, termed protoplast budding, occurs when pectin is not incorporated into the new cell wall (Hanke and Northcote, 1974) and is the result of weakened areas in the newly synthesized cell wall (Fowke and Gamborg, 1980). In the same culture

response of each of these cell sources when isolating and culturing protoplasts

Protoplast yields were consistently 2×10^6 per gram of callus or suspension culture cells. These yields were considerably lower, approximately 5×10^5, when protoplasts were isolated from cultures nearing the time of subculture. Protoplasts isolated by this method were initially spherical in shape (Fig. 1a) and most exhibited normal ultrastructures when tested for their ability to hydrolyze fluorescein diacetate. After a day in culture they became oval shaped, indicating cell wall synthesis (Lao et al., 1970) with active cytoplasmic streaming. Soon after protoplasts are cultured in an appropriate medium, an increase in cell organelles indicates a general activation of the metabolic activity of the protoplasts for the regeneration of a cell wall. With the increase in cell size, new cytoplasmic strands are formed, and most of the cell organelles, particularly the chloroplasts, migrate around the nucleus (Vasil, 1970). First division occurred within 48–72 h (Fig. 1b). With repeated divisions (Fig. 1c), the regenerated chloroplasts per cell gradually decreased, and the cells became more vacuolated with micro-colony formation observed after 4 days (Fig. 1d). Pale green, visible micro-colonies (0.5–1 mm diam.) were formed in 3–4 weeks. A plating efficiency of 55% was recorded when protoplasts were plated at a density of 1×10^5 protoplasts/ml in the liquid culture medium. Prior to developing the medium which sustained division of *P. glaucum* protoplast-derived cells, several modifications of the *MS* culture medium were tested. The *MS* medium (from Table 1) with the same level of mannitol, maple water (MW), only resulted in budding when plated at 1×10^5/ml. First division was never observed. It has been proposed that incomplete cell wall resynthesis, termed protoplast budding, occurs when pectin is not incorporated into the new cell wall (Hinke and Wershoe, 1967) and is the result of weakened areas in the new cell wall (Hinke and Gamborg, 1970). In the same culture

Fig. 1. Division and formation of plants from protoplasts of P. alpicola: Freshly isolated protoplasts suspended in culture medium. x 400 (a), first division in cell regenerated from protoplast. x 400 (b), protoplast-derived cell following the second mitotic division with non-dividing cell in immediate vicinity. x 400 (c), multicellular colony. x 400 (d), macro-colonies upon further plating in soft agar (actual size) (e), differentiation of shoots on protoplast-derived callus (actual size) (f), and adventitious roots produced on regenerated shoots on MS medium with 1.0 mg/l IBA(actual size) (g).


a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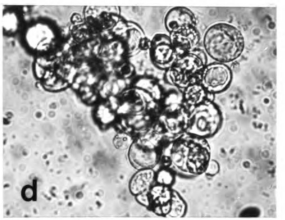
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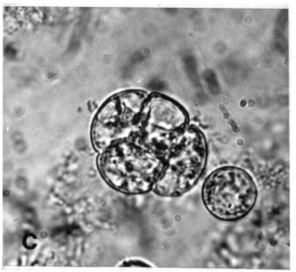
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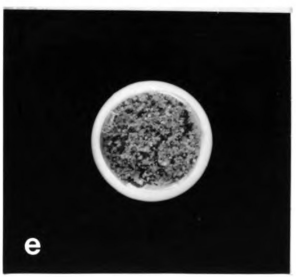
e


g

medium (Table 1) lacking only coconut water, protoplasts were observed dividing with a plating efficiency of 60%. Culture medium containing 20% coconut water, 2,4-D (1.0 mg/l), NAA (2.0 mg/l), and BAP (0.5 mg/l) increased the plating efficiency to 85%. The 25% increase might be due to the stimulating synergistic effect of coconut water and 2,4-D as seen by Steward and Caplin (1951) with the culture of potato tuber cells. Coconut water is generally believed to contain cytokinin-like substances as well as reduced nitrogen and possesses detoxifying properties, all of which may have value for certain tissue cultures (Pollard et al., 1961; Tulecke et al., 1961).

In initial experiments, the culture dishes were replenished every two weeks after plating with 0.5-ml aliquots of the appropriate culture medium containing reduced mannitol levels of 11, 9, 6, 3 and 0%. This procedure resulted in browning and eventually the death of all visible cell colonies.

In liquid culture medium there was limited growth after colonies reached the multicellular stage (Fig. 1c) unless transferred to the soft agar. Transferring cells at the multicellular stage to interfacing layers of semi-solid agar allowed further growth and development of the green, visible colonies (Fig. 1e), which resulted in the growth of cells in compact and discrete clusters. This method was a modification of the plating technique used by Nagata and Takebe (1971) for culturing isolated tobacco mesophyll protoplasts.

Calli were of sufficient size (3-4 mm) to be transferred to shoot regeneration medium (Fig. 1f) approximately 8-12 weeks after plating the protoplasts. At this stage the calli were moved to a higher light intensity of 38 $\mu Em^{-2}s^{-1}$ (G.E.F96-T12-CW) for 16 h at 28°C. Once a callus initiated shoot primordia it continued to produce nodulated callus and prolific shoots. Shoot-tips of 2 cm or longer were separated singly from the shoot regeneration cultures and transferred to rooting media, either MS with 0.01 mg/l NAA or 1.0 mg/l IBA (indolebutryic acid) (Fig. 1g). Root primordia generally emerged between the first and second week, although a few shoots had 1-3 mm roots after 6

protoplasts

Calli were of sufficient size (1–4 mm) to be transferred to shoot regeneration medium (Fig. 1f) approximately 8 weeks after plating the protoplasts. At this stage the calli were moved to a higher light intensity of 75 $\mu E\ m^{-2}\ s^{-1}$ (16 E l^{-1} d^{-1}; TL 20 CW) for 16 h at 25°C. Once a callus initiated shoot primordia it continued to produce modular shoots and produce shoots. Shoot tips of 2 cm or longer were separated singly from the shoot regeneration cultures and transferred to rooting media either (A) with 0.1 mg l^{-1} NAA (2.0 mg l^{-1} and IAA (2.0 mg l^{-1}) as the plating efficiency was 75%. The 2% increase might be due to the stimulating synergistic effect of coconut water and 2,4-D as seen by Sreenand and Chitra (1996) with the culture of pseudo-cereal. Coconut water is generally believed to contain cytokinin like substances as well as reduced nitrogen and possess growth promoting properties all of which may have value for certain tissue cultures (Hiremath et al. 1991; Tulecke et al. 1961).

In initial experiments the culture dishes were replenished every two weeks after plating with 0.5 ml aliquots of the appropriate culture medium containing reduced mannitol levels of 11.9, 8 and 0%. This procedure resulted in browning and eventually the death of all viable cell colonies

In liquid culture medium there was limited growth after colonies reached the multicellular stage (Fig. 1c) unless transferred to the soft agar. Transferring cells at the multicellular stage to interfacing layers of semi solid agar allowed further growth and development of the green visible colonies (Fig. 1e) which resulted in the growth of cells in compact and discrete clusters. This method was a modification of the plating technique used by Nagata and Takebe (1971) for culturing isolated tobacco mesophyll

days. Both auxins produced 100% rooting of shoots. In either medium, the regenerates were grown for four weeks to allow roots to develop. Shoot-tips were also transferred to an MS medium with auxins and cytokinins completely eliminated (MS0), but only few or no roots were initiated.

Establishment of the regenerates directly into soil or a soil-less planting medium was unsuccessful. A mixture of peat, perlite and vermiculite (V. S. P. - Bay Houston Towing Co.) was found to cause necrosis of the roots and all plants were highly susceptible to fungal attack. The in vitro rooted regenerates continued to grow when they were transferred to cell packs containing sterilized perlite. The regenerated plants, after growing in the highly protected, artificial culture environment were found to be very sensitive to moisture stress and susceptible to pathogen attack due to the water retention capacity of the initial planting medium. The gradual opening of polyethylene bags, used to provide a high humidity, was attempted to acclimate plantlets but dehydration repeatedly occurred. To date, all efforts to successfully acclimate the regenerated plants to the outside environment have failed. Sensitivity to stress during acclimation is apparently due in part to lack of cuticle on the leaves (Grout and Ashton, 1977; Sutter and Langhans, 1979). In addition, plantlets were highly sensitive to dehydration because their stomates may not have been functioning effectively (Brainerd and Fuchigami, 1981).

This study indicated that plants can be regenerated from protoplasts of P. alpicola and provides an experimental basis for future work in somatic cell genetics with this species.

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they were transferred to soil pots containing sterilized perlite. The regenerated

susceptible to fungal attack. The in vitro protected regenerants continued to grow when

was unsuccessful. A mixture of peat, perlite and vermiculite (1:1:1 by volume)

Potting Soil was found to cause necrosis of the roots and all plants were brittle

Transfer of the regenerates directly into soil or a soil less planting medium

only few or no roots were initiated

formed in MS medium with agitation and cytokinins completely eliminated (Abdul Kadir

were grown for four weeks to allow root development. Shoot tips were also trans-

ferred to MS medium with agitation and cytokinins completely eliminated (Abdul Kadir

References

Binding, H. and G. Krumbegel-Schroeren: Protoplast regeneration. In: Petunia (K. C. Sink, ed.) pp. 123-132. Monographs on Theoretical and Applied Genetics 9. Springer-Verlag, Berlin Heidelberg (1984).

Brainerd, K. E. and L. J. Fuchigami: Acclimatization of aseptically cultured apple plants to low relative humidity. J. Am. Soc. Hort. Sci. 106, 515-518 (1981).

Ferguson, M. C. and E. B. Coolidge: A cytological and a genetical study of Petunia. IV. Pollen grains and the method of studying them. Amer. J. Bot. 19, 644-659 (1932).

Fowke, L. C. and O. L. Gamborg: Applications of protoplasts to the study of plant cells. Int. Rev. Cytol. 68, 9-51 (1980).

Frearson, E. M., J. B. Power and E. C. Cocking: The isolation, culture and regeneration of Petunia leaf protoplasts. Dev. Biol. 33, 130-137 (1973).

Grout, B. W. W. and M. J. Ashton: Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res. 17, 1-7 (1977).

Hanke, D. E. and D. H. Northcote: Cell wall formation by soybean callus protoplasts. J. Cell Sci. 14, 29-50 (1974).

Izhar, S. and J. B. Power: Genetical studies with Petunia leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci. Lett. 8, 375-383 (1977).

Kao, K. N., W. A. Keller and R. A. Miller: Cell division in newly formed cells from protoplasts of soybean. Exp. Cell Res. 62, 338-340 (1970).

Murashige, T. and F. Skoog: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497 (1962).

Nagata, T. and I. Takebe: Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99, 12-20 (1971).

Pollard, J. K., E. M. Shantz and F. C. Steward: Hexitols in coconut milk: Their role in nuture of dividing cells. Plant Physiol. 36, 492-501 (1961).

Power, J. B., E. M. Frearson, D. George, P. K. Evans, S. F. Berry, C. Hayward and E. C. Cocking: The isolation, culture and regeneration of leaf protoplasts in the genus Petunia. Plant Sci. Lett. 7, 51-55 (1976).

Sink, K. C. and J. B. Power: Incongruity of interspecific and intergeneric crosses involving Nicotiana and Petunia species that exhibit potential for somatic hybridization. Euphytica 27, 725-730 (1978).

Steward, F. C. and S. M. Caplin: A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. Science 113, 518-520 (1951).

Sutter, E. and R. W. Langhans: Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Am. Soc. Hort Sci. 104, 493-496 (1979).

Tulecke, W., L. H. Weinstein, A. Rutner and H. J. Laurencot, Jr.: The biochemical composition of coconut water (coconut milk) as related to its use in plant tissue culture. Contribs. Boyce Thompson Inst. 21, 115-128 (1961).

Vasil, I. K.: The progress, problems, and prospects of plant protoplast research. Adv. Agron. 28, 119-160 (1976).

Widholm, J. M.: The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Tech. 47, 189-194 (1972).

SUMMARY AND CONCLUSIONS

Petunia alpicola was found to be self-incompatible caused by inability of the pollen tubes to grow beyond the stigmatic region of the style. This species also failed to produce hybrids when reciprocally crossed with P. parviflora and P. hybrida. Pre- and/or post-zygotic incompatibility was indicated as the reason for the failure to produce interspecific hybrids between these Petunia species. As one emphasis of breeding in the genus Petunia is to introduce desirable wild species traits into the cultivated species, P. hybrida Hort., incompatibility serves as a barrier in using P. alpicola to further breeding endeavors. Therefore, somatic hybridization appears to be a viable alternative to integrate desirable genes into P. hybrida. The isolation, culture, and regeneration to plants of protoplasts isolated from callus and suspension cultures of this species was achieved as a prerequisite to cell fusion. Further studies to successfully acclimate P. alpicola to the outside environment will aid its incorporation into somatic cell genetic research on Petunia, as this species is very amenable to in vitro culture and may serve as a valuable germplasm resource.

LIST OF REFERENCES

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Ahkong, Q.F., D. Fisher, W. Tampion and J.A. Lucy. 1975. Mechanism of cell fusion. Nature 253:194-195.

Aist, J.R. 1976. Papillae and related wound plugs of plant cells. Ann. Rev. Phytopathol. 14:145-163.

Anvari, S.F. and R. Stösser. 1978. Eine neue fluoreszenzmikroskopische Methode zur Beurteilung der Befruchtungs-Fahigkeit der Samenanlagen bei Prunus. Z. Pflanzenzucht 81:333-336.

Ascher, P.D. 1976. Self-incompatibility systems in floriculture crops. Acta Hort. 63: 205-215.

Ascher, P.D. 1984. Self-incompatibility. In: Petunia (K.C. Sink, ed.) pp. 92-110. Monographs on Theoretical and Applied Genetics 9. Springer-Verlag, Berlin Heidelberg.

Bidney, D.L. and J.F. Shepard. 1980. Colony development from sweet potato petiole protoplasts and mesophyll cells. Plant Sci. Lett. 18:335-342.

Binding, H. 1974. Cell cluster formation by leaf protoplasts from axenic cultures of haploid Petunia hybrida L. Plant Sci. Lett. 2:185-188.

Binding, H. and G. Krumbegel-Schroeren. 1984. Protoplast regeneration. In: Petunia (K.C. Sink, ed.) pp. 123-132. Monographs on Theoretical and Applied Genetics 9. Springer-Verlag, Berlin Heidelberg.

Bourgin, J.P., Y. Chupea and C. Missonier. 1979. Plant regeneration from mesophyll protoplasts of several Nicotiana species. Physiol. Plant. 45:288-292.

Bracha, M. and N. Sher. 1981. Fusion of enucleated protoplasts with nucleated miniprotoplasts in onion (Allium cepa L.). Plant Sci. Lett. 23:95-101.

Bradley, P.M. 1983. The production of higher plant subprotoplasts. Plant Mol. Biol. Rep. 1:117-123.

Brainerd, K.E. and L.J. Fuchigami. 1981. Acclimatization of aseptically cultured apple plants to low relative humidity. J. Am. Soc. Hort. Sci. 106:515-518.

Brewbaker, J.L. 1957. Pollen cytology and self-incompatibility systems in plants. J. Hered. 48:271-277.

Brewbaker, J.L. and S.K. Majumder. 1961. Cultural studies on the pollen population effect and the self-incompatibility inhibition. Amer. J. Bot. 48:457-464.

Britton, N.L., E.A. Sterns and J. Poggenburg. 1888. Preliminary catalogue of Anthophyta and Pteridophyta, p. 38. New York.

Burgess, J. and E.N. Fleming. 1974. Ultrastructural studies of the aggregation and fusion of plant protoplasts. Planta 118:183-193.

Caboche, M. 1980. Nutritional requirements of protoplast-derived, haploid tobacco cells grown at low cell densities in liquid medium. Planta 149:7-18.

Caplan, A., L. Herrera-Estrella, D. Inzé, E. Van Haute, M. Van Montagu, J. Schell and P. Zambryski. 1983. Introduction of genetic material into plant cells. Science 222:815-821.

Cella, R. and E. Galun. 1980. Utilization of irradiated carrot cell suspensions as feeder layer for cultured Nicotiana cells and protoplasts. Plant Sci. Lett. 19:243-252.

Chellapan, K.P., S. Seeni and A. Gnanam. 1980. The isolation, culture, and organ differentiation from mesophyll protoplasts of Mollugo nudicaulis Lam., a C_4 species. Experientia 36:60-61.

Chilton, M.-D., R.K. Saiki, N. Yadav, M.P. Gordon and F. Quétier. 1980. T-DNA from Agrobacterium Ti plasmid is in the nuclear DNA fraction of crown gall tumor cells. Proc. Natl. Acad. Sci. U.S.A. 77:4060-4064.

Chin, J.C. and K.C. Scott. 1979. A large-scale isolation procedure for cereal mesophyll protoplasts. Ann. Bot. 43:23-32.

Christen, A. and P. Lurquin. 1983. Infection of cowpea mesophyll protoplasts with cowpea chlorotic mottle virus (CCMV) RNA encapsulated in large liposomes. Plant Cell Rep. 2:43-46.

Clarke, A.E. and B.A. Stone. 1963. Chemistry and biochemistry of β-1,3 glucans. Rev. Pure Appl. Chem. 13:134-233.

Cocking, E.C. 1960. A method for the isolation of plant protoplasts and vacuoles. Nature 187:962-963.

Constabel, F. and K.N. Kao. 1974. Agglutination and fusion of plant protoplasts by polyethylene glycol. Can. J. Bot. 52:1603-1606.

Currier, H.B. 1957. Callose substance in plant cells. Amer. J. Bot. 44:478-482.

Davey, M.R., E.C. Cocking, H. Freeman, N. Pearce and I. Tudor. 1980. Transformation of Petunia protoplasts by isolated Agrobacterium plasmids. Plant Sci. Lett. 18:307-313.

De La Roche, A.I., W.A. Keller, J. Singh and D. Siminovitch. 1977. Isolation of protoplasts from unhardened and hardened tissues of winter rye and wheat. Can. J. Bot. 55:1181-1185.

Dermen, H. 1931. Polyploidy in Petunia. Amer. J. Bot. 18:250-261.

Dickinson, H.G. and D. Lewis. 1973. Cytochemical and ultrastructural differences between intraspecific compatible and incompatible pollinations in Raphanus. Proc. R. Soc. London, Ser. B. 183:21-38.

Doohan, M.E. and B.A. Palevitz. 1980. Microtubules and coated vesicles in guard-cell protoplasts of Allium cepa L. Planta 149:389.

Dos Santos, A.V.P., D.E. Outka, E.C. Cocking and M.R. Davey. 1980. Organogenesis and somatic embryogenesis in tissues derived from leaf protoplasts and leaf explants of Medicago sativa. Z. Pflanzenphysiol. 99:261-270.

Draper, J., M.R. Davey, J.P. Freeman and E.C. Cocking and B.J. Cox. 1982. Ti-plasmid homologous sequences present in tissues from Agrobacterium plasmid-transformed Petunia protoplasts. Plant Cell Physiol. 23:451-458.

Dudits, D., K.N. Kao, F. Constabel and O.L. Gamborg. 1976. Embryogenesis and formation of tetraploid and hexaploid plants from carrot protoplasts. Can. J. Bot. 54: 1063-1067.

Dumas, C. and R.B. Knox. 1983. Callose and determination of pistil viability and incompatibility. Theor. Appl. Genet. 67:1-10.

Durand, J. 1979. High and reproducible plating efficiencies of protoplasts isolated from in vitro grown haploid Nicotiana sylvestris Spegaz et Comes. Pflanzenphysiol. 93:283-295.

Durand, J., I. Potrykus and G. Donn. 1973. Plantes issues de protoplasts de Petunia. Z. Pflanzenphysiol. 69:26-33.

East, E.M. 1923. Genetical aspects of self- and cross-sterility. Amer. J. Bot. 10:468-473.

East, E.M. 1928. The genetics of the genus Nicotiana. Bibliogr. Genetica 4:243-320.

East, E.M. 1940. The distribution of self-sterility in the flowering plants. Proc. Amer. Phil. Soc. 82:449-518.

East, E.M. and A.J. Mangelsdorf. 1925. A new interpretation of the hereditary behavior of self-sterile plants. Proc. Nat. Acad. Sci. U.S.A. 11:166-171.

East, E.M. and A.J. Mangelsdorf. 1926. Studies on self-sterility. VII. Heredity and selective pollen tube growth. Genetics 11:466-481.

Edwards, G.E., S.P. Robinson, N.J.C. Tyler and D.A. Walker. 1978. Photosynthesis by isolated protoplasts, protoplast extracts, and chloroplasts of wheat. Plant Physiol. 62:313-317.

Eriksson, T. 1971. Isolation and fusion of plant protoplasts. Colloq. Int. Cent. Nat. Rech. Sci. 193:297-302.

Eriksson, T. 1977. Technical advances in protoplast isolation and cultivation. In: Plant Tissue Culture and Its Biotechnical Application (W. Barz, E. Reinhard and M.H. Zenk, eds.) pp. 313-322. Springer-Verlag, Berlin.

Evans, D.A. 1979. Chromosome stability of plants regenerated from mesophyll protoplasts of Nicotiana species. Z. Pflanzenphysiol. 95:459-463.

Ferguson, M.C. and E.B. Coolidge. 1932. A cytological and a genetical study of Petunia. IV. Pollen grains and the method of studying them. Amer. J. Bot. 19:644-659.

Ferguson, M.C. and A.M. Ottley. 1932. Studies in Petunia. III. A redescription and additional discussion of certain species of Petunia. Amer. J. Bot. 19:385-403.

Flaschenriem, D.R. and P.D. Ascher. 1979. Pollen tube expression on pseudo-self-compatibility (PSC) in Petunia hybrida. Theor. Appl. Genet. 54:97-101.

Fowke, L.C. and O.L. Gamborg. 1980. Applications of protoplasts to the study of plant cells. Int. Rev. Cytol. 68:9-51.

Fowke, L.C., L.R. Griffing, B.G. Massey and P. Van der Valk. 1983. Protoplasts for studies of the plasma membrane and associated cell organelles. In: Protoplasts 1983, Lecture Proceedings (I. Potrykus, C.T. Harms, A. Hinnen, R. Hütter, P.J. King and R.D. Shillito, eds.) p. 101. Birkauser Verlag, Basel.

Fraley, R. 1983. Liposome-mediated delivery of tobacco mosaic virus RNA into petunia protoplasts. Improved conditions for liposome-protoplast incubations. Plant Mol. Biol. 2:5-11.

Fraley, R., S. Dellaporta and D. Papahadjopoulos. 1982. Liposome mediated delivery of tobacco mosaic virus RNA into tobacco protoplasts: A sensitive assay for monitoring liposome-protoplast interactions. Proc. Natl. Acad. Sci. U.S.A. 79:1859-1863.

Fraley, R.T. and D. Papahadjopoulos. 1982. Liposomes: The development of a new carrier system for introducing nucleic acids into plant and animal cells. Curr. Top. Microbiol. Immunol. 96:171-191.

Frearson, E.M., J.B. Power and E.C. Cocking. 1973. The isolation, culture and regeneration of Petunia leaf protoplasts. Dev. Biol. 33:130-137.

Fries, R.E. 1911. Die Arten der Gattung Petunia. K. Sy. Vet. Akad. Handl. 46:25-40.

Fryxell, P.A. 1957. Mode of reproduction of higher plants. Bot. Rev. 23:135-233.

Galston, A.W., A. Altman and R. Kaur-Sawhney. 1978. Polyamines, ribonuclease and the improvement of oat leaf protoplasts. Plant Sci. Lett. 11:69-79.

Galun, E. 1981. Plant protoplasts as physiological tools. Ann. Rev. Plant Physiol. 32:237.

Gamborg, O.L. 1976. Plant protoplast isolation, culture, and fusion. In: Cell Genetics in Higher Plants (D. Dudits, G.L. Farkas, P. Maliga, eds.) pp. 107-128. Akademiai Kiado, Budapest.

Gamborg, O.L. 1977. Culture media for plant protoplasts. In: Handbook of Nutrition and Food (M. Rechcigl, ed.) pp. 415-422. CRC Press, Cleveland.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

Gamborg, O.L., J. Shyluk and K.K. Kartha. 1975. Factors affecting the isolation and callus formation in protoplasts from the shoot apices of Pisum sativum L. Plant Sci. Lett. 4:285-292.

Gatenby, A.A. and E.C. Cocking. 1977a. Callus formation from protoplasts of marrow stem kale. Plant Sci. Lett. 8:275-280.

Gatenby, A.A. and E.C. Cocking. 1977b. Polypeptide composition of Fraction I protein subunits in the genus Petunia. Plant Sci. Lett. 10:97-101.

Gerstel, D.U. 1950. Self-incompatibility studies in guayule. II. Inheritance. Genetics 35:482-506.

Gill, R., A. Rashid and S.C. Maheshwari. 1979. Isolation of mesophyll protoplasts of Nicotiana rustica and their regeneration into plants flowering in vitro. Physiol. Plant. 47:7-10.

Gill, R., A. Rashid and S.C. Maheshwari. 1981. Dark requirement of cell regeneration and colony formation by mesophyll protoplasts of Nicotiana plumbaginifolia Viv. Protoplasma 106:351-354.

Gleason, H.A. and A. Cronquist. 1963. Manual of vascular plants of northeastern United States and adjacent Canada. Van Nostrand, Princeton.

Gleba, Y.Y. 1978. Microdroplet culture. Tobacco plants from single mesophyll protoplasts. Naturwissenschaften 65:158.

Glimelius, K., A. Wallin and T. Eriksson. 1974. Agglutinating effects of Concanavalin A on isolated protoplasts of Daucus carota. Physiol. Plant. 3:225-230.

Gresshoff, P.M. 1980. In vitro culture of white clover: callus, suspension, protoplast culture, and plant regeneration. Bot. Gaz. 141:157-164.

Griesbach, R.J., R.L. Malmberg and P.S. Carlson. 1982. An improved technique for the isolation of higher plant chromosomes. Plant Sci. Lett. 24:55-60.

Grout, B.W.W. and M.J. Ashton. 1977. Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res. 17:1-7.

Grout, B.W.W. and R.H.A. Coutts. 1974. Additives for the enhancement of fusion and endocytosis in higher plant protoplasts: An electrophoretic study. Plant Sci. Lett. 2:397-403.

Gunning, B.E.S. and A.R. Hardham. 1982. Microtubules. Ann. Rev. Plant Physiol. 33:651.

Gutierrez, M., S.C. Huber, S.B. Ku and G.E. Edwards. 1975. Intracellular carbon metabolism in mesophyll cells of C^4 plants. Proc. Int. Congr. Photosynth. Res., 3rd, 1974. pp. 1219-1230.

Hadlaczky, G., G. Bisztray, T. Praznovszky and D. Dudits. 1983. Mass isolation of plant chromosomes and nuclei. Planta 157:278-285.

Hadlaczky, G., T. Praznovszky and G. Bisztray. 1982. Structure of isolated protein-depleted chromosomes of plants. Chromosoma 86:643-659.

Hanke, D.E. and D.H. Northcote. 1974. Cell wall formation by soybean callus protoplasts. J. Cell Sci. 14:29-50.

Harms, C.T., H. Lörz and I. Potrykus. 1979. Multiple-drop-array (MDA) technique for the large scale testing of culture media variations in hanging microdrop cultures of single cell systems. II. Determination of phytohormone combinations for optimal response in Nicotiana tabacum protoplast cultures. Plant Sci. Lett. 14: 237-244.

Harms, C.T. and I. Potrykus. 1978. Fractionation of plant protoplast types by iso-osmotic density gradient centrifugation. Theor. Appl. Genet. 53:57-63.

Hartmann, J.X., K.N. Kao, O.L. Gamborg and R.A. Miller. 1973. Immunological methods for the agglutination of protoplasts from cell suspension cultures from different genera. Planta 112:45-56.

Hasezawa, S., T. Nagata and K. Syōno. 1981. Transformation of Vinca protoplasts mediated by Agrobacterium tumefaciens spheroplasts. Mol. Gen. Genet. 182:206-210.

Hayward, C. and J.B. Power. 1975. Plant production from leaf protoplasts of Petunia parodii. Plant Sci. Lett. 4:407-410.

Herth, W., W.W. Franke, H. Bittiger, A. Kuppel and G. Keilich. 1974. Alkali-resistant fibrils of β-1,3 and β-1,4 glucans: structural polysaccharides in the pollen tube wall of Lilium longiflorum. Cytobiologie 9:344-367.

Heslop-Harrison, J. 1975. Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Physiol. 26:403-425.

Heslop-Harrison, J. 1978. Recognition and response in the pollen-stigma interaction. Symp. Soc. Exp. Biol. 32:121-138.

Heslop-Harrison, J., R.B. Knox and Y. Heslop-Harrison. 1974. Pollen-wall proteins: exine-held fractions associated with the incompatibility in Cruciferae and Compositae. Incompatibility Newsl. 3:73-76.

Heslop-Harrison, J., R.B. Knox, Y. Heslop-Harrison and O. Mattsson. 1975. Pollen-wall proteins: emission and role in incompatibility responses. In: The biology of the male gamete (J.G. Duckett and P.A. Racey, eds.) pp. 189-204. Academic Press, London.

Hess, D. and I. Potrykus. 1972. Teilung isolierter protoplasten von Petunia hybrida. Die Naturwissenschaften 6:273-274.

Huber, S.C. and G.E. Edwards. 1975. An evaluation of some parameters required for the enzymatic isolation of cells and protoplasts with CO_2 fixation capacity from C_3 and C_4 grasses. Physiol. Plant. 35:203-209.

Hughes, B.G., F.G. White and M.A. Smith. 1978. Effect of plant growth, isolation and purification conditions on barley protoplast yield. Biochem. Physiol. Pflanzen 172:67-77.

Izhar, S. and J.B. Power. 1977. Genetical studies with Petunia leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci. Lett. 8:375-383.

Johnson, L.B., D.L. Stuteville, R.K. Higgins and D.Z. Skinner. 1981. Regeneration of alfalfa plants from protoplasts of selected regenerated S clones. Plant Sci. Lett. 20:297-304.

Jussieu, A.L. 1803. Sur le Petunia, genre nouveau de a famille des plantes solanees. Ann. Mus. Nat. Paris 2:214-216.

Kameya, T. 1973. The effects of gelatin on aggregation of protoplasts from higher plants. Planta 115:77-82.

Kameya, T. 1975. Induction of hybrids through somatic cell fusion with dextran sulfate and gelatin. Jap. J. Genet. 50:235-246.

Kameya, T. 1979. Studies on plant cell fusion: Effect of dextran and pronase E on fusion. Cytologia 44:449-456.

Kameya, T. 1982. The method for fusion with dextran. Proc. Int. Congr. Plant Tissue Cell Cult., Plant Tissue Cult., 1982 pp. 613-614.

Kameya, T., M.E. Horn and J. Widholm. 1981. Hybrid shoot formation from fused Daucus carota and D. capillifolius protoplasts. Z. Pflanzenphysiol. 104:459-466.

Kameya, T. and N. Takahashi. 1972. The effects of inorganic salts on fusion of protoplasts from roots and leaves of Brassica species. Jap. J. Genet. 47:215-217.

Kao, K.N., F. Constabel, M.R. Michayluk and O.L. Gamborg. 1974. Plant protoplast fusion and growth of intergeneric hybrid cells. Planta 120:215-227.

Kao, K.N., W.A. Keller and R.A. Miller. 1970. Cell division in newly formed cells from protoplasts of soybean. Exp. Cell Res. 62:338-340.

Kao, K.N. and M.R. Michayluk. 1974. A method for high-frequency intergeneric fusion of plant protoplasts. Planta 115:355-367.

Kao, K.N. and M.R. Michayluk. 1975. Nutritional requirements for growth of Vicia hajastana cells and protoplasts at a very low population density in liquid media. Planta 126:105-110.

Kao, K.N. and M.R. Michayluk. 1980. Plant regeneration from mesophyll protoplasts of alfalfa. Z. Pflanzenphysiol. 96:135-141.

Kartha, K.K., M.R. Michayluk, K.N. Kao, O.L. Gamborg and F. Constabel. 1974. Callus formation and plant regeneration from mesophyll protoplasts of rape plants (Brassica napus L. cv. Zephyr). Plant Sci. Lett. 3:265-271.

Keller, W.A. and G. Melchers. 1973. The effect of high pH and calcium on tobacco leaf protoplast fusion. Z. Naturforsch. 28c:737-741.

Keller, F. and B.A. Stone. 1978. Preparation of Lolium protoplasts and their purification using an anti-galactan-sepharose conjugate. Z. Pflanzenphysiol. 87:167-172.

Kerhoas, C., R.B. Knox and C. Dumas. 1983. Specificity of the callose response in stigmas of Brassica. Ann. Bot. 52:597-602.

Klercker, J.A.F. 1892. Eine Methode zur Isolierung lebender Protoplasten. Svenka. Vet-Akad. Fork. Stockholm 9:463-471.

Klobutcher, L.A. and F.H. Ruddle. 1981. Chromosome mediated gene transfer. Ann. Rev. Biochem. 50:533-554.

Kostoff, D. and J. Kendall. 1931. Studies on certain Petunia aberrants. J. Genet. 24: 165-179.

Krens, F.A., L. Molendijk, G.J. Wullems and R.A. Schilperoort. 1982. In vitro transformation of plant protoplasts with Ti plasmid DNA. Nature (Lond.) 298:72-74.

Küster, E. 1909. Uber die Verschmelzung nackter Protoplasten. Ber. Dtsch. Bot. Ges. 27:589-598.

Laemmlen, F.F. and K.C. Sink. 1978. Evaluation of petunia cultivars for botrytis resistance. Plant Dis. Reptr. 62:361-365.

Lamarck, J.B. 1793. Tableau encyclopedique et methodique botanique. 2:7.

Lamprecht, H. 1953. Petunia axillaris (Lam.) B.S.P. und ihre synonyme P. inflata R. Fries. Agric. Hortic. Genet. 11:83-108.

Larkin, P.J. 1976. Purification and viability determinations of plant protoplasts. Planta 128:213-216.

Lehman, E. 1926. The heredity of self-sterility in Veronica syriaca. Mem. Hort. Soc. N.Y. 3:313-320.

Lesney, M.S., P.C. Callow and K.C. Sink. 1983. A simplified method for bulk production of cytoplasts from suspension-culture derived protoplasts of Solanum nigrum (L). In: Protoplasts 1983 (I. Potrykus, C.T. Harms, A. Hinnen, R. Hütter, P.J. King and R.D. Shillito, eds.) pp. 116-117. Birkhäuser Verlag, Basel.

Lewis, D. 1944. Incompatibility in plants, its genetical and physiological synthesis. Nature 153:575-578.

Lewis, D. 1956. Incompatibility and plant breeding. Brookhaven Symp. Biol. 9: 89-100.

Lewis, D.H. 1980. Are there interrelations between the metabolic role of boron, synthesis of phenolic phytoalexins and the germination of pollen? New Phytol. 84:261-270.

Li, X.H., Q.S. Yan, M.J. Huang, Y.R. Sun and W.B. Li. 1980. Division of cells regenerated from mesophyll protoplasts of wheat. In: Advances in Protoplast Research (L. Ferenczy and G.L. Farkas, eds.) pp. 261-267. Pergamon Press, Oxford.

Linskens, H.F. 1958. Zur Frage der Entstehung der Abwehr-Korper bei der Inkompatibilitats - Reaktion von Petunia. Ber. Deutsch. Bot. Ges. 71:3-10.

Linskens, H.F. and K. Esser. 1957. Uber eine spezifische Anfarbung der Pollenschlauche im Griffel und die Zahl der Kallosepfropfen nach Selbstung und Fremdung. Naturwissenschaften 44:16.

Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.

Lloyd, C.W., A.R. Slabas, A.J. Powell and S.B. Lowe. 1980. Microtubules, protoplasts and plant cell shape. Planta 147:500.

Lörz, H., I. Paszkowsky, C. Dierks-Ventling and I. Potrykus. 1981. Isolation and characterization of cytoplasts and miniprotoplasts derived from protoplasts of cultured cells. Physiol. Plant. 53:385-391.

Lörz, H. and I. Potrykus. 1978. Investigation on the transfer of isolated nuclei into plant protoplasts. Theor. Appl. Genet. 53:251-256.

Lurquin, P. 1979. Entrapment of plasmid DNA by liposomes and their interactions with plant protoplasts. Nucleic Acids Res. 6:3773-3784.

Lurquin, P. and R. Sheehy. 1982. Binding of large liposomes to plant protoplasts and delivery of encapsulated DNA. Plant Sci. Lett. 25:133-146.

Maheshwari, P. 1949. The male gametophyte of Angiosperms. Bot. Rev. 15:1-75.

Maliga, P., H. Lörz, G. Lázár and F. Nagy. 1982. Cytoplast-protoplast fusion for interspecific chloroplast transfer in Nicotiana. Mol. Gen. Genet. 185:211-215.

Malinowski, E. 1928. Variegation and chromosomes in Petunia. J. Hered. 19:522-526.

Malmberg, R.L. and R.J. Griesbach. 1980. The isolation of mitotic and meiotic chromosomes from plant protoplasts. Plant Sci. Lett. 17:141-147.

Márton, L., G.J. Wullems, L. Molendijk and R.A. Schilperoort. 1979. In vitro transformation of cultured cells from Nicotiana tabacum by Agrobacterium tumefaciens. Nature (Lond.) 277:129-131.

Mather, K. 1943. Specific differences in Petunia. I. Incompatibility. J. Genet. 45: 215-235.

Mather, K. and P.M.J. Edwardes. 1943. Specific differences in Petunia. III. Flower colour and genetic isolation. J. Genet. 45:243-260.

Matthews, B., S. Dray, J. Widhold and M. Ostro. 1979. Liposome-mediated transfer of bacterial RNA into carrot protoplasts. Planta 145:37-44.

Meizel, S. and C.L. Markert. 1967. Malate dehydrogenase isozymes of the marine snail Ilyanassa obsoleta. Arch. Biochem. Biophys. 122:753-765.

Menczel, L., G. Lazar and P. Maliga. 1978. Isolation of somatic hybrids by cloning Nicotiana heterokaryons in nurse cultures. Planta 143:29-32.

Mersey, B.G., L.C. Fowke, F. Constabel and E.H. Newcomb. 1982. Preparation of a coated vesicle-enriched fraction from plant cells. Exp. Cell Res. 141:459.

Mersey, B.G., L.R. Griffing, P.J. Rennie and L.C. Fowke. 1983. Coated vesicles from plant protoplasts. In: Protoplasts 1983, Poster Proceedings (I. Potrykus, C.T. Harms, A. Hinnen, R. Hütter, P.J. King and R.D. Shillito, eds.) p. 216. Birkhäuser Verlag, Basel.

Mulcahy, D.L. 1975. Gamete competition in plants and animals. American Elsevier, New York.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nagata, T. and S. Ishii. 1979. A rapid method for isolation of mesophyll protoplasts. Can. J. Bot. 57:1820-1823.

Nagata, T., K. Okada, I. Takebe and C. Matsui. 1981. Delivery of tobacco mosaic virus RNA into carrot protoplasts. Planta 145:37-44.

Nagata, T. and I. Takebe. 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12-20.

Nasrallah, J.B., R.C. Doney and M.E. Nasrallah. 1985a. Biosynthesis of glycoproteins involved in the pollen-stigma interaction of incompatibility in developing flowers of Brassica oleracea L. Planta 165:100-107.

Nasrallah, J.B., T.-H. Kao, M.L. Goldberg and M.E. Nasrallah. 1985b. A cDNA clone encoding an S-locus-specific glycoprotein from Brassica oleracea. Nature 318:263-267.

Nasrallah, J.B. and M.E. Nasrallah. 1984. Electrophoretic heterogenity exhibited by the S-allele specific glycoproteins of Brassica. Experientia 40:279-281.

Nasrallah, M.E. 1974. Genetic control of quantitative variation in self-incompatibility proteins detected by immunodiffusion. Genetics 76:45-50.

Nasrallah, M.E., J.T. Barber and D.H. Wallace. 1970. Self-incompatibility proteins in plants: detection, genetics, and possible mode of action. Heredity 25:23-27.

Nasrallah, M.E. and D.H. Wallace. 1967. Immunogenetics of self-incompatibility in Brassica oleracea L. Heredity 22:519-527.

Nasrallah, M.E., D.H. Wallace and R.M. Savo. 1972. Genotype, protein, phenotype relationships in self-incompatibility of Brassica. Genet. Res. 20:151-160.

Natarella, N.J. and K.C. Sink, Jr. 1974. A chromatographic study of phenolics of species ancestral to Petunia hybrida Vilm. J. Hered. 65:85-90.

Natarella, N.J. and K.C. Sink, Jr. 1975. Electrophoretic analysis of proteins and peroxidases of selected Petunia species and cultivars. Bot. Gaz. 136:20-26.

Nettancourt, D. de. 1977. Incompatibility in angiosperms. Springer, New York.

Niedz, R.P., S.M. Rutter, L.W. Handley and K.C. Sink. 1985. Plant regeneration from leaf protoplasts of six tomato cultivars. Plant Science 39:199-204.

Nishio, T. and K. Hinata. 1977. Analysis of S-specific proteins in stigma of Brassica oleracea L. by isoelectric focusing. Heredity 38:391-396.

Oelck, M.M., P.S. Rao, P. Ozias-Akins and O. Schieder. 1983. Protoplast regeneration of some legume species. In: Protoplasts 1983, Poster Proceedings (I. Potrykus, C.T. Harms, A. Hinnen, R. Hütter, P.J. King and R.D. Shillito, eds.) p. 50. Birkhäuser Verlag, Basel.

Ohgawara, T., H. Uchimiya and H. Harada. 1983. Uptake of liposome-encapsulated plasmid DNA by plant protoplasts and molecular fate of foreign DNA. Protoplasma 116:145-148.

Ooms, G., A. Bakker, L. Molendijk, G.J. Wullems, M.P. Gordon, E.W. Nester and R.A. Schilperoort. 1982. T-DNA organization in homogeneous and heterogeneous octopine-type crown gall tissues of Nicotiana tabacum. Cell 30:589-597.

Othman, R.B. and K. Paranjothy. 1980. Isolation of Hevea protoplasts. J. Rubb. Res. Inst., Malaysia 28:61-66.

Pandey, K.K. 1957. Genetics of self-incompatibility in Physalis ixocarpa Brot. A new system. Amer. J. Bot. 44:879-887.

Pandey, K.K. 1958. Time of the S allele action. Nature 181:1220-1221.

Pandey, K.K. 1960. Evolution of gametophytic and sporophytic systems of self-incompatibility in angiosperms. Evolution 14:98-115.

Pandey, K.K. 1968. Compatibility relationships in flowering plants. Role of the S gene complex. Amer. Natur. 102:475-489.

Pandey, K.K. 1977. Origin of complementary incompatibility systems in flowering plants. Theor. Appl. Genet. 49:101-109.

Partanen, C.R. 1981. Filter paper as a support and carrier for plant protoplast cultures. In Vitro 17:77-80.

Passiatore, J.E. and K.C. Sink. 1981. Plant regeneration from leaf mesophyll protoplasts of selected ornamental Nicotiana species. J. Am. Soc. Hort. Sci. 106:799-803.

Patnaik, G., D. Wilson and E.C. Cocking. 1981. Importance of enzyme purification for increased plating efficiency and plant regeneration from single protoplasts of Petunia parodii. Z. Pflanzenphysiol. 102:199-205.

Pelcher, L.E., O.L. Gamborg and K.N. Kao. 1974. Bean mesophyll protoplasts: Production, culture and callus formation. Plant Sci. Lett. 4:107-111.

Piwowarczyk, W. 1979. Modified method of purification of plant protoplasts by centrifugation in a new discontinuous gradient. Biochem. Physiol. Pflanzen 174: 318-321.

Pollard, J.K., E.M. Shantz and F.C. Steward. 1961. Hexitols in coconut milk: Their role in nuture of dividing cells. Plant Physiol. 36:492-501.

Potrykus, I. 1971. Intra and interspecific fusion of protoplasts from petals of Torenia bailloni and Torenia fournieri. Nature New Biol. 231:57-58.

Potrykus, I. 1973. Transplantation of chloroplasts into protoplasts of Petunia. Z. Pflanzenphysiol. 70:364-366.

Potrykus, I. 1980. The old problem of protoplast culture: cereals. In: Advances in Protoplast Research (L. Ferenczy and G.L. Farkas, eds.) pp. 243-254. Pergamon Press, Oxford.

Potrykus, I. and J. Durand. 1972. Callus formation from single protoplasts of Petunia. Nature New Biol. 237:286-287.

Power, J.B., S.F. Berry, J.V. Chapman and E.C. Cocking. 1980. Somatic hybridization of sexually incompatible petunias: Petunia parodii, Petunia parviflora. Theor. Appl. Genet. 56:1-4.

Power, J.B., S.E. Cummins and E.C. Cocking. 1970. Fusion of isolated plant protoplasts. Nature 255:1016-1018.

Power, J.B., M.R. Davey, J.P. Freeman, B.J. Mulligan and E.C. Cocking. 1986. Fusion and transformation of plant protoplasts. In: Methods in Enzymology, Vol. 118 Plant Molecular Biology (A. Weissbach and H. Weissbach, eds.) pp. 578-594. Academic Press, Orlando.

Power, J.B., E.M. Frearson, D. George, P.K. Evans, S.F. Berry, C. Hayward and E.C. Cocking. 1976. The isolation, culture and regeneration of leaf protoplasts in the genus Petunia. Plant Sci. Lett. 7:51-55.

Prell, H. 1921. Das Problem der Unbefruchtbarkeit. Naturwiss. Wochenschr. N.F. 20:440-446.

Rangaswamy, N.S. 1977. Applications of in vitro pollination and in vitro fertilization. In: Applied and fundamental aspects of plant, cell, tissue, and organ culture (J. Reinert and Y.P.S. Bajaj, eds.) pp. 412-424. Springer-Verlag, Berlin.

Reynolds, J.D. and W.V. Dashek. 1976. Cytochemical analysis of callose localization in Lilium longiflorum pollen tubes. Ann. Bot. 40:409-416.

Rick, C.M. 1971. Some cytogenetic features of the genome in diploid plant species. Stadler Genet. Symp. 1-2:153-176.

Robinson, S.P., G.E. Edwards and D.A. Walker. 1979. Established methods for the isolation of intact chloroplasts. In: Plant Organelles (E. Reid, ed.) pp. 13-24. Wiley, New York.

Robinson, D.G. and H. Quader. 1982. The microtubule-microfibril syndrome. In: The Cytoskeleton in Plant Growth and Development (C.W. Lloyd, ed.) p. 109. Academic Press, London.

Rose, R.J. 1980. Factors that influence the yield, stability in culture and cell wall regeneration of spinach mesophyll protoplasts. Aust. J. Plant Physiol. 7:713-725.

Schenk, H.R. and F. Hoffmann. 1979. Callus and root regeneration from mesophyll protoplasts of basic Brassica species: B. campestris, B. oleracea and B. nigra. Z. Pflanzenuchtg. 82:354-360.

Schieder, O. 1977. Hybridization experiments with protoplasts from chlorophyll-deficient mutants of some Solanaceae species. Planta 137:253-258.

Schwenk, F.W., C.A. Pearson and M.R. Roth. 1981. Soybean mesophyll protoplasts. Plant Sci. Lett. 23:153-155.

Sedgley, M. 1977. Reduced pollen tube growth and the presence of callose in the pistil of the male floral stage of the avocado. Sci. Hortic. 7:27-36.

Shepard, J.F. and R.E. Totten. 1975. Isolation and regeneration of tobacco mesophyll cell protoplasts under low osmotic condition. Plant Physiol. 55:689-694.

Shepard, J.F. and R.E. Totten. 1977. Mesophyll cell protoplasts of potato: Isolation, proliferation, and plant regeneration. Plant Physiol. 60:313-316.

Sims, J. 1825. Petunia nyctaginiflora. Bot. Mag. 52:2552.

Sink Jr., K.C. 1975. Inheritance of three genes for morphological characters in Petunia hybrida in crosses with four Petunia species. Can. J. Genet. Cytol. 17: 67-74.

Sink, K.C. 1980. Plant protoplasts - a novel method for producing new hybrids of horticultural crops. Part I. Flor. Rev. 167 (4323):22; 57-58. Part II. Flor. Rev. 167 (4324):18; 62.

Sink, K.C. 1981. Vegetative and floral characters of Petunia species hybrids as related to the origin of Petunia hybrida. Can. J. Genet. Cytol. 23:557-564.

Sink, K.C. 1984. Taxonomy. In: Petunia (K.C. Sink, ed.) pp. 3-9. Monographs on Theoretical and Applied Genetics 9. Springer-Verlag, Berlin Heidelberg.

Sink, K.C. and J.B. Power. 1977. The isolation, culture and regeneration of leaf protoplasts of Petunia parviflora Juss. Plant Sci. Lett. 10:335-340.

Sink, K.C. and J.B. Power. 1978. Incongruity of interspecific and intergeneric crosses involving Nicotiana and Petunia species that exhibit potential for somatic hybridization. Euphytica 27:725-730.

Sink, K.C., J.B. Power and N.J. Natarella. 1978. The interspecific hybrid Petunia parodii x P. inflata and its relevance to somatic hybridization in the genus Petunia. Theor. Appl. Genet. 53:205-208.

Skalinska, M. and S. Cuchtma. 1927. Karyologische Analyse einer polymorphen Rasse von Petunia violacea Lindl. Bibl. Univ. Liberae Polonae 19:1-23.

Slabas, A.R., A.J. Powell and C.W. Lloyd. 1980. An improved procedure for the isolation and purification of protoplasts from carrot suspension culture. Planta 147: 283-286.

Smith, L.B. and R.J. Downs. 1966. Solanacea. In: Flora illustrada Catarinense (P. R. Reitz, ed.) p. 321. Itajai, S. Catarina, Brazil.

Sood, R., K. Prabha and S.C. Gupta. 1982. Is the 'rejection reaction' inducing ability in sporophytic self incompatibility systems restricted only to pollen and tapetum? Theor. Appl. Genet. 63:27-32.

Steere, W.C. 1930. Petunia parodii, a new species of the subgenus Pseudonicotiana from Argentina. Papers Mich. Acad. Sci. 13:213-215.

Steere, W.C. 1932. Chromosome behaviour in triploid Petunia hybrids. Amer. J. Bot. 19:340-357.

Steward, F.C. and S.M. Caplin. 1951. A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. Science 113:518-520.

Sullivan, T.D. 1947. Somatic chromosomes of pedigreed hybrid Petunia. Bull. Torr. Bot. Club 74:453-475.

Sutter, E. and R.W. Langhans. 1979. Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Am. Soc. Hort. Sci. 104:493-496.

Szabados, L., G. Hadlaczky and D. Dudits. 1981. Uptake of isolated chromosomes by plant protoplasts. Planta 151:141-145.

Takahashi, H. 1973. Genetical and physiological analysis of pseudo-self-compatibility in Petunia hybrida. Jap. J. Genet. 48:27-33.

Takebe, I., Y. Otsuki and S. Aoki. 1968. Isolation of tobacco mesophyll cells in intact and active state. Plant and Cell Physiol. 9:115-124.

Tanchak, M.A., L.R. Griffing, B.G. Mersey and L.C. Fowke. 1983. Functions of coated vesicles in plant protoplasts: Endocytosis of cationized ferritin and transport of peroxidase. J. Cell Biol. 97:177.

Tanchak, M.A., L.R. Griffing, B.G. Mersey and L.C. Fowke. 1984. Endocytosis of cationized ferritin by coated vesicles of soybean protoplasts. Planta 162:481.

Thomashow, M.F., R. Nutter, A.L. Montoya, M.P. Gordon and E.W. Nester. 1980. Integration and organization of Ti Plasmid sequences in crown gall tumors. Cell 19:729-739.

Tulecke, W., L.H. Weinstein, A. Rutner and H.J. Laurencot, Jr. 1961. The biochemical composition of coconut water (coconut milk) as related to its use in plant tissue culture. Contribs. Boyce Thompson Inst. 21:115-128.

Uchimiya, H. and H. Harada. 1981. Transfer of liposome-sequestering plasmid DNA into Daucus carota protoplasts. Plant Physiol. 68:1027-1030.

Uchimiya, H. and T. Murashige. 1976. Influence of the nutrient medium on the recovery of dividing cells from tobacco protoplasts. Plant Physiol. 57:424-429.

Van der Valk, P. and L.C. Fowke. 1981. Ultrastructural aspects of coated vesicles in tobacco protoplasts. Can J. Bot. 59:1307.

Vasil, I.K. 1976. The progress, problems, and prospects of plant protoplast research. Adv. Agron. 28:119-160.

Vasil, V. and I.K. Vasil. 1974. Regeneration of tobacco and petunia from protoplasts and culture of corn protoplasts. In Vitro 10:83-96.

Vasil, I.K. and V. Vasil. 1980. Isolation and culture of protoplasts. Int. Rev. Cytol. Suppl. 11B:1-19.

Vithanage, HIMV, P.A. Gleeson and A.E. Clarke. 1980. The nature of callose produced during self-pollination in Secale cereale. Planta 148:498-509.

Vithanage, HIMV and R.B. Knox. 1977. Development and cytochemistry of stigma surface and response to self and foreign pollination in Helianthus annus. Phytomorphology 27:168-179.

Von Arnold, S. and T. Eriksson. 1977. A revised medium for growth of pea mesophyll protoplasts. Physiol. Plant. 39:257-260.

Wallin, A., K. Glimelius and T. Eriksson. 1974. The induction of aggregation and fusion of Daucus carota protoplasts by polyethylene glycol. Z. Pflanzenphysiol. 74:64-84.

Wallin, A., K. Glimelius and T. Eriksson. 1978. Enucleation of plant protoplasts by cytochalasin B. Z. Pflanzenphysiol. 87:333-340.

Watanabe, Y., T. Ohno and Y. Okada. 1982. Virus multiplication in tobacco protoplasts inoculated with tobacco mosaic virus RNA encapsulated in large unilamellar vesicle liposomes. Virology 120:478-480.

Widholm, J.M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Tech. 47:189-194.

Wilson, H.M., D.J. Styler, P.L. Conrad, R.D. Durbin and J.P.Helgeson. 1980. Isolation of sterile protoplasts from unsterilized leaves. Plant Sci. Lett. 18:151-154.

Wullems, G.J., L. Molendijk, G. Ooms and R.A. Schilperoort. 1981. Differential expression of crown gall tumor markers in transformants obtained after in vitro Agrobacterium tumefaciens-induced transformation of cell wall regenerating protoplasts derived from Nicotiana tabacum. Proc. Natl. Acad. Sci. U.S.A. 78:4344-4348.

Yamaguchi, Y. and R. Nagai. 1981. Motile apparatus in Vallisneria leaf cells. I. Organization of microfilaments. J. Cell Sci. 48:193.

Zapata, F.J., P.K. Evans, J.B. Power and E.C. Cocking. 1977. The effect of temperature on the division of leaf protoplasts of Lycopersicon esculentum and Lycopersicon peruvianum. Plant Sci. Lett. 8:119-124.

Zapata, F.J. and K.C. Sink. 1981. Somatic embryogenesis from Lycopersicon peruvianum leaf mesophyll protoplasts. Theor. Appl. Genet. 59:265-268.

Zapata, F.J., K.C. Sink and E.C. Cocking. 1981. Callus formation from leaf mesophyll protoplast of three Lycopersicon species: L. esculentum, c.v. Walter, L. pimpinellifolium and L. hirsutum f. glabratum. Plant Sci. Lett. 23:41-46.

Zimmermann, U. 1982. Electric field-mediated related electrical phenomena. Biochim. Biophys. Acta. 694:227-277.

Zimmermann, U., G. Kuppers and N. Salhani. 1982. Electric field-induced release of chloroplasts from plant protoplasts. Naturwissenschaften 69:451-452.

Zimmermann, U. and P. Scheurich. 1981a. Fusion of Avena sativa mesophyll cell protoplasts by electrical breakdown. Biochim. Biophys. Acta. 641:160-165.

Zimmermann, U. and P. Scheurich. 1981b. High frequency fusion of plant protoplasts by electrical field. Planta 151:26-32.