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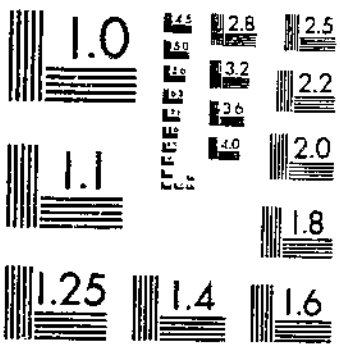

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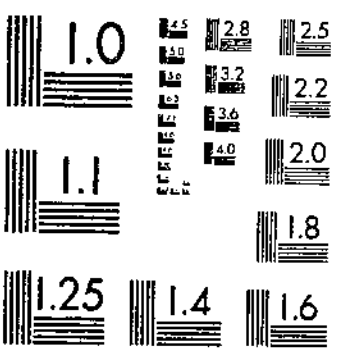

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NICOTIANA

PROCEDURES FOR EXPERIMENTAL USE

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Abstract

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Background information and procedures for using the genus *Nicotiana* as experimental subjects are presented. Each chapter first surveys a topic, then, in detail, presents the associated experimental techniques. The topics include plant propagating and hybridizing; cytogenetical techniques; organ, tissue, cell and protoplast culture; protoplast fusion and organelle transfer; selection of biochemical cell varients; virology; disease resistance; and bacterial hypersensitivity. In addition, a genetical overview is given of the genus.

Keywords: *Nicotiana,* tobacco, tissue culture, protoplast, disease resistance, haploids, protoplast and organelle fusion, organ culture, and bacterial hypersensitivity.

Foreword

Advances in botanical research have often depended upon the proper choice of an experimental plant. In this regard, the genus *Nicotiana* has been preeminently successful. A few such advances in which it has played a crucial role include photoperiodism, whose study has contributed much to our knowledge of flowering and other aspects of plant growth, and which was first elucidated in Maryland Mammoth tobacco by Garner and Allard (Journal Agricultural Research 18:553); the discovery of cytokinins as a class of plant growth substances depended upon tobacco tissue cultures; and quantitative virus assays were revolutionized by Holmes (Phytopathology 28:553) when he used leaves of *N. glutinosa* as a local lesion indicator for tobacco mosaic virus. Pioneering research in haploid plant production, quantitative genetics, incompatibility systems, and parasexual hybridization also have used tobacco as the experimental plant of choice.

Because of its many advantages, the prospect for the continued and even expanded employment of *Nicotiana* in theoretical biology seems assured. However, the necessary background information and procedures for taking full advantage of its attributes have never been brought together and, in some cases, are not published. Hence, the reason for this volume. Our hopes in presenting this information are that it will benefit experimenters in various disciplines and that they will continue to find *Nicotiana* useful.

Suppliers of the specialized materials mentioned (plants, chemicals and apparatus) are listed in the Appendix. The Index contains all references to *Nicotiana* spp. in the text, but it does not include those mentioned in figures or tables.

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THE GENUS AS A GENETIC RESOURCE

H. H. Smith[1]

Introduction

The genus *Nicotiana* has been used widely in genetic research, and in related botanical disciplines, largely because of the great variation and different stages of evolutionary divergence that species offer. Within a species, genetic studies are aided by the simplicity of controlled pollination and the abundant yield of seeds and progeny (ch. 3). Between species, hybridizing has revealed distinctive, cytogenetic relationships, and hereditary characetristics such as genetic tumors, differences in biochemical traits of alkaloids and isozymes, markers for chloroplast deoxyribonucleic acid (DNA), and certain somatic instabilities that are used to demonstrate the genetic control of gene expression. The applications that have been made in the past suggest that, as new techniques and concepts evolve, *Nicotiana* spp. will continue to find use in and to be applied to arising problems.

The favorableness of *Nicotiana* spp. for experimental research did not escape the notice of the pre-Mendelian hybridizers. Kolreuter, the first to undertake systematic, scientific hybridizing in plants, succeeded in obtaining the cross *N. rustica* × *N. paniculata*, describing it in 1761 more than 100 years before Mendel's results were published (*109, 151*).[2] Throughout the first half of the 20th century, *Nicotiana* spp. were used in many studies aimed at establishing the basis of classical genetics. These advances in genetics included forming the multiple factor hypothesis, producing new species through amphiploidy and postulating the origin of *N. tabacum*, establishing a series of monosomes, interspecific transferring of genes for disease resistance, and clarifying cytogenetic and phylogenetic relations among species. Reviews on the cytogenetics of the genus *Nicotiana* have been published by East (*42*), Kostoff (*76*), Goodspeed (*53*) and Smith (*133, 139*).

Throughout the 1950's and 1960's, parallel to the study of tobacco cytogenetics, a separate discipline of single cell culture was being developed, which made extensive use of *Nicotiana* spp. (ch. 7). Several investigators clearly demonstrated that whole tobacco plants could be differentiated from single somatic cells (*157*). In the late 1960's fungal cellulases, hemicellulases, and pectinases became commercially available from Japan. Techniques for their wide-scale use in isolating protoplasts rapidly advanced (ch. 8). In 1969-70 Takebe and his associates (*106, 156*) isolated protoplasts from tobacco leaves that underwent mitotic division and regenerated into whole plants (*152*).

With the consolidation of the fields of cell culture and plant genetics using such model systems as *Nicotiana* (*138*), the significance and potential of somatic cell plant genetics became recognized. Research with tobacco is in a better position than with any other crop plant to take advantage of the new developments in genetic engineering (*141*). Not only is this because the cells and tissues of tobacco are inherently relatively easy to culture, but the techniques themselves have been developed to a considerable extent by using species of the genus *Nicotiana*. Also, as noted above, the phylogenetic structure of the genus provides a rich reservoir of genetic resources for experiments.

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[2]Italic numbers in parentheses in each chapter refer to "References" listed at the end of each chapter.

The Species

A taxonomic monograph of the genus *Nicotiana* is included in Goodspeed's book (*53*). It is classified into 3 subgenera, 14 sections, and 60 species. Forty-five of the species are indigenous to North or South America and 15, comprising the Suaveolentes section, to Australia. In a subsequent revision, Burbidge (*18*) added five new species to the Australian group and changed *N. stenocarpa* to *N. rosulata*. A minor revision was suggested by Wells (*158*), who found a continuous intergradation of *N. palmeri* with *N. trigonophylla* and hence questioned the validity of assigning species rank to *N. palmeri*. The 64 presently recognized species of *Nicotiana* are listed in table 1-1 with the chromosome number of each. *N. sanderae* is not included in the table because it is a horticultural species that originated as a hybrid between *N. forgetiana* and *N. alata*. New collections of *N. cavicola* have been shown (*162*) to have $2n = 40$ instead of the originally reported $2n = 46$.

Two tentative new species of *Nicotiana* have been described in recent years. One, *N. africana* (*101*), was found on several isolated mountains in the middle of Namib (South-West Africa). Its taxonomic position is obscure, but the species might best be placed in subgenus Petunioides, although some characteristics are clearly reminiscent of the subgenera Rustica and Tabacum. Merxmüller and

TABLE 1-1.—*Classification of the genus* Nicotiana[1]

Subgenus	Section	Species	Authority	Somatic chromosome number
Rustica	Paniculatae	*glauca*	Graham	24
		paniculata	Linnaeus	24
		knightiana	Goodspeed	24
		solanifolia	Walpers	24
		benavidesii	Goodspeed	24
		cordifolia	Philippi	24
		raimondii	Macbride	24
	Thyrsiflorae	*thyrsiflora*	Bitter ex Goodspeed	24
	Rusticae	*rustica*	Linnaeus	48
Tabacum	Tomentosae	*tomentosa*	Ruiz and Pavon	24
		tomentosiformis	Goodspeed	24
		otophora	Grisebach	24
		setchellii	Goodspeed	24
		glutinosa	Linnaeus	24
	Genuinae	*tabacum*	Linnaeus	48
Petunioides	Undulatae	*undulata*	Ruiz and Pavon	24
		arentsii	Goodspeed	48
		wigandioides	Koch and Fintelman	24
	Trigonophyllae	*trigonophylla*	Donal	24
	Alatae	*sylvestris*	Spegazzini and Comes	24
		langsdorffii	Weinmann	18
		alata	Link and Otto	18
		forgetiana	Hort, ex Hemsley	18
		bonariensis	Lehmann	18
		longiflora	Cavanilles	20
		plumbaginifolia	Viviani	20
	Repandae	*repanda*	Willdenow ex Lehmann	48
		stocktonii	Brandegee	48
		nesophila	Johnston	48
	Noctiflorae	*noctiflora*	Hooker	24
		petunioides	(Grisebach) Millán	24
		acaulis	Spegazzini	24
		ameghinoi	Spegazzini	?
Petunioides Continued	Acuminatae	*acuminata*	(Graham) Hooker	24
		pauciflora	Remy	24
		attenuata	Torrey ex Watson	24
		longibracteata	Philippi	?
		miersii	Remy	24
		corymbosa	Remy	24
		linearis	Philippi	24
		spegazzinii	Millán	24
	Bigelovianae	*bigelovii*	(Torrey) Watson	48
		clevelandii	Gray	48
	Nudicaules	*nudicaulis*	Watson	48
	Suaveolentes	*benthamiana*	Domin	38
		umbratica	Burbidge	46
		cavicola	Burbidge	40
		debneyi	Domin	48
		gossei	Domin	36
		amplexicaulis	Burbidge	36
		maritima	Wheeler	32
		velutina	Wheeler	32
		hesperis	Burbidge	42
		occidentalis	Wheeler	42
		simulans	Burbidge	40
		megalosiphon	Heurck and Mueller	40
		rotundifolia	Lindley	44
		excelsior	J. M. Black	38
		suaveolens	Lehmann	32
		ingulba	J. M. Black	40
		exigua	Wheeler	32
		goodspeedii	Wheeler	40
		rosulata	(S. Moore) Domin	40
		fragrans	Hooker	48

[1]From Smith (*133*), after Goodspeed (*53*).

Buttler, state that, "It probably, therefore, can be considered as an endemic relict of considerable age. It may form a parallel to the section Suaveolentes, endemic to Australia and the South Pacific, with which it agrees in being dysploid (2n = 46)." *N. africana* is unique—it is the only member of the genus known from Africa.

The second recently described species has been named *N. kawakamii* (*108*). This species was found by Japanese explorers in the Andes of South America; it appears to fit in the Tomentosae section though it has some unique cytological features and is day neutral.

The origins and evolution of species of the genus *Nicotiana* have been presented in detail by Goodspeed (*53*) and Goodspeed and Thompson (*57*). Goodspeed (*53*) has summarized these relations diagrammatically in the form of three phylogenetic arcs. In the first two arcs, the genus is envisaged as derived from a pregeneric reservoir of related forms with six pairs of chromosomes that evolved into three complexes at the 12-paired level. These are hypothetical precursors of the three modern subgenera. The third arc contains the present-day species, with their various degrees of genetic interconnection, at the 12- and 24-paired chromosome level. The evidence is consistent with the conclusion that interspecific hybridization with subsequent amphiploidy, as well as genetic recombination, has played an important role in the evolution of the genus *Nicotiana*.

Polyploidy, Aneuploidy and the Origin of *Nicotiana tabacum*

Eleven of the known species of *Nicotiana* have 24 pairs of chromosomes and hence are of probable amphiploid origin. In addition, a large number of amphiploids have been produced, either spontaneously following interspecific hybridization or by artificial means, principally with colchicine.

The main tobacco species of commerce, *N. tabacum*, is of amphiploid origin (n = 24), and much interest and research has centered on its derivation from putative wild progenitors. The original evidence of Goodspeed and Clausen (*56*) was interpreted to indicate that *N. tabacum* arose from chromosome doubling following hybridization between a progenitor of *N. sylvestris* (S′ genome, n = 12) and a member of the Tomentosae section, either *N. otophora*, *N. tomentosiformis* or, more likely, an ancestral type similar to, but not identical with, either of these present-day species (T′ genome, n = 12).

To determine genetically whether the chromosomes of *N. tomentosiformis* or those of *N. otophora* are more nearly homologous with the T genome of *N. tabacum*, Gerstel (*45*) examined segregation frequencies, which indicated that *N. tomentosiformis* is the more closely related. This is supported by observations on flower morphology (*47*), isozyme patterns (*123*), and analysis of Fraction I proteins (*58*). Gerstel (*47*), further notes, "Chromosome pairing in undoubled F_1 hybrids between *N. sylvestris* and Tomentosae species is very low and multivalent formation in their amphiploids is near zero. Therefore, a mechanism suppressing homeologous associations like the one found in chromosome 5B in the polyploid wheats is not required in *N. tabacum;* differential affinity assures regularity of meiosis here" (*46*). The germplasm of progenitor species may still contain genetic material that can find use in further improvement of the cultivated tobacco (*160*).

The monosomics of *N. tabacum* are aneuploid types of particular interest because they provide material for a rapid method of locating genes on specific chromosomes (ch. 2). These monosomics have arisen spontaneously (*32*) as derivatives from hybridizing *N. tabacum* and *N. sylvestris* (*33*) and by using a genetically controlled, asynaptic condition (*31*). The 24 monosomic lines have been characterized on the basis of their most readily identifiable features by Cameron (*22*). They are listed in table 1-2 and described according to their appearance on a common genetic background, the so-called Red Russian tobacco.

All primary trisomic types have been identified only in *N. sylvestris* (n = 12) (*54, 55*). Eight of the nine possible trisomics have been identified in *N. langsdorffii* (*1, 81, 126*). In both of these species the trisomic types are readily distinguishable from the diploid types in morphological features of plant, leaf and flower.

Aneuploidy, as well as amphiploidy, has played a part in the evolution of the genus *Nicotiana*, as shown by the occurrence of 9- and 10-paired species in the Alatae section and 16- and 23-paired species in the Suaveolentes section (table 1-1). The former are considered to have resulted from chromosomal loss at the 12-paired level and the latter from loss at the 24-paired level.

In an effort to explore the limits and consequences of multiple allopolyploidy, a hybrid was produced (*72*) that combined the genomes of three distantly related amphiploid species: *N. bigelovii* (n = 24, North America), *N. debneyi* (n = 24, Australia) and *N. tabacum* (n = 24, South America). In an individual that contained the doubled (by colchicine) complement of 144 chromosomes, only bivalents were formed; laggards were observed at metaphase and anaphase stages of meiosis. Inbreeding and selection were practiced for 10 generations, which established three morphologically distinct races, each involving a loss of different chromosomes from the original 144 to give 108 ± 6 (*148*). The phenomenon observed in this multiple allopolyploid would seem to offer opportunities for exploitation in the natural evolution of some plant groups because of the wide variability in early generations without the serious loss of fertility usually associated with species hybridization at the diploid level.

TABLE 1-2.—*The monosomic types of* Nicotiana tabacum[1]

Designation	Distinguishing characteristics					Associated genes
	Plant height	Leaves	Flowers,[2] length in mm and characteristics	Pollen	Monosome	
Haplo-A	Somewhat below normal	Smaller; basal constriction more pronounced	48.6–43.1; somewhat paler in color, fading earlier	Essentially normal	Medium small	*hf*$_1$, hairy filaments; *pa*, asynaptic; *au'*, auren
Haplo-B	Subnormal; sparsely branched	Smaller; narrow; basal constriction less abrupt; auricles strongly reduced	53.6–41.0; more strongly bent; color darker	Essentially normal	Very small	*Ml*, many leaves; *Pp*, purple plant; *yb*$_1$, yellow burley; *Pb*, purple buds, *N. otophora*[3]
Haplo-C	Often taller than normal; longer internodes	Narrow; basal constriction less abrupt	58.6–43.7; longer and broader; color paler in tube and throat	Marked abortion	Medium small	*cd*, crinkled dwarf; *lf*, light filaments; *wh*, white flower; *bf*, bent flower; *Wh-P*, pale; *wc*, white center, *N. otophora*[3]
Haplo-D	Normal but maturity delayed	Brighter green in young plants; leaf base semibroad	50.6–39.7; slightly reduced in size	Essentially normal	Incorporated in a trivalent in about 50 percent PMC	*fs*$_1$, fasciated
Haplo-E	Subnormal	Smaller; constriction less abrupt	51.3–41.1; calyx inflated	Essentially normal	Very small	——
Haplo-F	Subnormal; shorter internodes	Small; more erect	44.5–37.5, distinctly shorter limb, fluted	Moderate abortion	Large with characteristic median construction	*co*, coral flower; *mm*$_1$, mammoth; *sn*$_1$, spontaneous necrosis
Haplo-G	Subnormal; meager inflorescence; maturity delayed	Small with rounded tips; basal constriction pronounced	55.6–42.3, tapering gradually to limb; style short; capsules small and poorly filled	Variable as to cytoplasmic content but few grains completely aborted	Large	*tg*, tinged; *vb*, vein-banding; *ws*$_1$, white seedling; *vp'*, variegiated plant
Haplo-H	Normal but stems and branches slender; reduced branching	Small; narrow; basal constriction less pronounced	51.8–38.3; narrow tube; limb reduced; calyx lobes pointed	High abortion but variable as to contents	Medium large	*Nc*, necrotic, *N. glutinosa*,[3] *td*, toadskin
Haplo-I	Normal; slender branches; delayed maturity	Small; more sharply pointed	53.1–41.2, corolla lobes pointed; capsules long, narrow, poorly filled; calyx inflated	Low abortion but dimorphic	Very small	*cc*$_1$, catacorolla,[4] *rd*, red modifier
Haplo-J	Subnormal; maturity delayed; leaves small, narrow	Small and narrow	52.3–42.6, limb characteristically wavy at maturity; color less intense; capsules small, poorly filled	High abortion, sharp distinction between stainable and aborted grains	Medium	*cy*, calycine,[4] *lc*, lacerate,[4] *vi A'*, virescent
Haplo-K	Subnormal; maturity delayed	Semibroad at base	48.3–36.7; tube short; infundibulum proportionately longer; anthers small with delayed dehiscence	Low abortion; dimorphic	Very large, medianly constricted (cf. *31*)	——
Haplo-L	Above normal; stem heavy; maturity somewhat delayed	——	48.6–37.1; tube shorter and broader; color distinctly paler	High abortion; variable in size	Large with prominent constriction	*al*, Ambalema tall; *gb*, green buds; *Tr*, tube retarder, *N. setchellii*[3]

See footnotes at end of table.

TABLE 1-2.—*The monosomic types of* Nicotiana tabacum[1]

Designation	Distinguishing characteristics					Associated genes
	Plant height	Leaves	Flowers,[2] length in mm and characteristics	Pollen	Monosome	
Haplo-M	Subnormal; branching at the base	Large; basal constriction less pronounced	53.9–40.7; color fades to a purplish hue at maturity; calyx conspicuously longer	High abortion; variable in content	Medium large; characteristically ovoid	*Ap*, apetalous; *Rf*, ruffled; *pvb*, progressive vein banding
Haplo-N	Distinctly subnormal; short internodes; compact inflorescence	Small; erect	43.9–34.0; visibly smaller; color darker red	Low abortion; dimorphic	Large with median constriction (cf. Haplo-F)	mm_2, mammoth; sn_2, spontaneous necrosis
Haplo-O	Close to normal	Slightly smaller; basal constriction more pronounced	49.2–39.5; size reduced; paler in color; stamens and pistils slightly exserted; pollen shedding delayed; capsule small and poorly filled	Low abortion	Medium large	hf_2, hairy filaments; yb_2, yellow burley; *au''*, aurea
Haplo-P	Normal; maturity delayed	Small; tips rounded; semibroad at base	49.3–37.5; limb narrow; corolla lobes less pronounced; capsules small and poorly filled	Marked abortion; subnormal grains variable in size	Medium large with characteristic subterminal constriction	*Br*, broad; Fs_2, fasciated; *pk*, pink flower; *sg*, stigmatoid; *vi A''*, virescent; *yc*, yellow crittenden
Haplo-Q	Reduced; little branching; maturity delayed	Narrow; basal constriction pronounced; auricles strongly reduced; ruffled	54.8–38.8; tube longer; limb spread reduced; capsules pointed, small, and poorly filled	Very high abortion; sharply divided into two classes	Medium	—
Haplo-R	Subnormal; thick stems; profusely branched	Small; darker green; auricles reduced	49.2–41.9; enlarged infundibulum, wide throat; color paler	High abortion, but completely empty grains rare	Very large	mi_2, mosaic tolerant; *Pd*, petioloid
Haplo-S	Normal; maturity usually retarded	Lighter green; surface smooth	47.3–41.7; color more vivid; stamens and pistils exserted; pollen shedding delayed	Low abortion; grains variable in size	Large, frequently associated with a bivalent	*ch*, chimeral; *yg*, yellowish green; *su*, sulfur
Haplo-T	Subnormal; maturity delayed	Small; darker green; basal constriction elongated	56.0–41.7; tube longer, merging gradually into the infundibulum; stamens and pistils relatively short; capsules small, poorly filled	High abortion	Large, usually with a well-defined constriction	ws_2, white seedling; *vp''*, variegated plant
Haplo-U	Subnormal; bushy	Large; frequently with a pronounced petiole	48.5–40.9; corolla lobes acutely pointed; tube pale; limb and throat strongly colored	High abortion; aborted grains variable in size	Medium large	—
Haplo-V	Subnormal	Small; basal constriction less abrupt; auricles reduced	47.0–39.8; tube stout	High abortion, visibly so in freshly opened flowers	Medium large	—
Haplo-W	Subnormal; elongated internodes; sparsely branched; maturity delayed	Long; narrow; sharply pointed; auricles reduced	52.8–40.8; color lighter; pollen scanty, sometimes lacking in early flowers	High abortion; aborted grains small	Large, but PMC frequently unobtainable during early flowering	—

TABLE 1-2.—*The monosomic types of* Nicotiana tabacum[1]—Continued

Designation	Distinguishing characteristics					Associated genes
	Plant height	Leaves	Flowers,[2] length in mm and characteristics	Pollen	Monosome	
Haplo-Z	Normal; maturity conspicuously delayed	Small; basal constriction less pronounced; auricles less ruffled	52.9–40.3; style tends to be curved; limb frequently fails to open fully	Abortion very high; sharp distinction between normal and aborted grains	Large	——

[1]From Smith (*139*), after Cameron (*22*).

[2]The flower measurements are averages of 10 representative flowers and show tube length-limb spread (mm). These are to be compared with normal values of about 53–43.

[3]Transfers to *N. tabacum* from other species.

[4]Not clear-cut distinct characters.

Biometrical Studies

The dimensions of flower parts were used in early experiments on the inheritance of quantitative characters in crosses between *Nicotiana* spp. (*41*). This material was especially favorable for such studies because of the conspicuous differences between species in corolla size, the relative independence of this characteristic from environmental influences, and the partial fertility of some interspecific hybrids. East was able to verify the main tenants of the multiple factor hypothesis for explaining the inheritance of continuous variation by noting that experimental results fitted the main postulates of Mendelism restated in quantitative terms. Linkage between qualitative and quantitative characters was later demonstrated in progeny of a cross between *N. langsdorffii* and *N. sanderae* (*125*). Additional advantages of this material for such studies were the large number of interspecific differences in genes for flower color (*124*) and the few linkage groups because each of the two species has only nine pairs of chromosomes.

These early genetic studies showed that, with simple biometrical techniques, quantitative character inheritance is explainable on the basis of the accumulative action of many genes, each with similar expression and with small effects compared to environmental influences. Because of the prevalence of these multiple gene systems on the one hand and the preponderance of repeated sequences of DNA in eukaryotic genomes on the other, repetitive DNA may indicate the physical basis of polygenic systems (*135*).

More advanced biometrical methods were initiated in the late 1940's. For example, an analysis of means and variance components applied to data from a series of generations following prescribed breeding programs made possible interpretations about kinds of gene action, that is, additive, dominance, and various nonallelic interactions. The total phenotypic variance for quantitative characters can be partitioned by appropriate methods into genetic and environmental components, and the genetic component further partitioned into the proportionate contribution by additive, dominance, and various epistatic gene effects. Information gained from such genetical analyses can be used in designing breeding methods to give maximum expectations for achieving desired practical goals.

Studies on plant height and other quantitative characters in *N. rustica* and *N. tabacum* (*89, 90, 112, 128*) showed that, in general, additive gene effects contributed more to the total variance than dominant effects (allelic interaction). Consequently, heritability was high and the expected efficacy of selection was great. For most quantitative characters in crosses among varieties of *N. tabacum*, most workers reported a preponderance of additive gene effects and high heritability (*91, 92*); however, large genotype × year interactions may occur (*93*).

Biochemical Genetics

Alkaloids

A characteristic feature of *Nicotiana* spp. is the presence of nicotine or other alkaloids, or both. At least 12 different alkaloids have been obtained and identified from *N. tabacum* (*88*). However, little is known about their inheritance. The alkaloid composition of at least 52 other species also has been determined, usually by paper partition chromatography (*64, 142*). Most of these species contain predominantly one of the three identified alkaloids: nicotine, nornicotine and anabasine; other alkaloids, separable by chromatography, have been found but not yet identified chemically.

Numerous studies on the site(s) of alkaloid formation (*38, 104, 155*) indicate that nicotine is formed in the root and translocated apically through the xylem; nornicotine, a demethylation product of nicotine, is formed in the shoot;

and anabasine is formed in both root and shoot. Because all species contain one or more alkaloids, their presence may have had an adaptive significance early in the evolution of the genus and the biochemistry of their formation been fixed with a significant role in metabolism at the cellular level in present day *Nicotiana* plants.

When a predominantly "anabasine species" is crossed with an anabasine, nicotine, or nornicotine species, the main alkaloid in the hybrid is most frequently anabasine (*142, 145*). The biosynthesis of this alkaloid is an essentially dominant, genetic characteristic. The main alkaloid produced in crosses between predominantly nicotine and nornicotine species is most frequently nornicotine. The genetic factors controlling nornicotine formation are usually partly dominant over those producing nicotine, but the relationship is not simple (*19, 85, 131*).

Most cultivars of *N. tabacum* produce and retain nicotine as their primary alkaloid. However, related species, including the modern descendents of the probable progenitors of *N. tabacum* as well as certain cultivars of tobacco, produce and convert nicotine to nornicotine (*51, 86*). There are two dominant loci for nicotine conversion, C_1 and C_2 (*87*). Most tobacco types are c_1c_1 and, therefore, are nonconverters and produce mainly nicotine, but this recessive locus apparently may mutate to the nicotine-converting allele at a high frequency (*159*). The inheritance of the total alkaloid content in certain strains of burley tobacco has been reported to be governed by two independent pairs of genes (*36, 82, 83*). Many successful efforts have been made to select high and low nicotine strains of commercial tobacco.

Isozymes

Electrophoretic separation of proteins on starch or polyacrylamide gels together with visualization of different enzymes has provided biochemical markers for genetic and phylogenetic studies of *Nicotiana* spp. (*111, 122, 149*). At least 61 species have been so analyzed. Leaf, root, or seed extracts have been used, and the preparations have been most commonly stained for peroxidases and esterases, though other enzymes have also been investigated. With few possible exceptions, each species has an isozyme band pattern that is different from all other species. Some variation in pattern (one or two bands) has been found among collections within a diploid species (*111*).

Because most of the species analyzed have been represented by a single inbred type, caution should be exercised in phylogenetic interpretation. No intraspecific variation has been found among polyploid species (*59*). No single isozyme band is characteristic of all species. One broad phylogenetic relationship that became evident from comparing species zymograms was a significantly more frequent matching of isozyme band positions among species within a taxonomic section than among those from different sections. Thus, there was general agreement with relationships established on the basis of more conventional methods of systematics.

On the assumption that matching band mobility indicates genetic equivalency, the isozyme pattern of interspecific hybrids compared to the parental species can yield some genetic information. A comparison of 17 amphiploid patterns with those of the parents showed that most of the bands were found in one parent or the other and therefore exhibited a dominant or codominant gene action. In view of this result from synthesized amphiploids, the matching of band positions was used to assess the probable diploid progenitor species among putative parents of an established species of presumed, amphiploid origin. Using polyacrylamide gel electrophoresis of leaf extracts and staining for eight enzyme systems, Sheen (*123*) found that the band positions of *N. sylvestris* × *N. tomentosiformis* were more similar to *N. tabacum* than were those of *N. sylvestris* × *N. otophora*. This result supports the hypothesis that ancestors of *N. sylvestris* and *N. tomentosiformis* are the more likely progenitors of *N. tabacum*.

Genetic studies are essential to establish the hereditary basis of a particular isozyme. Segregation and linkage data have been obtained on the inheritance of two peroxidase variants, Px_1-I and Px_1-II, in crosses between *N. langsdorffii* and *N. sanderae*. The variants behaved as codominant alleles in the F_1. In the F_2 generation they gave a 1:2:1 ratio and provided evidence for linkage of the Px_1 locus with two, simply inherited genes for flower color (*61*).

Electrophoretic techniques have also been useful in demonstrating induced biochemical mutations (*134*), differences between tumor and normal plant tissues (*5, 17*), biochemical effects of irradiation (*30, 37*) and the influence of alien chromosomes on isozyme patterns (*135*).

Genetic Tumors

Evidence for Genetic Control

The occurrence of genetic tumors is not unique to interspecific hybrids of *Nicotiana* spp., but the phenomenon is found more frequently and has been studied more thoroughly in this genus than in any other plant (*8, 15, 136*). Genetic tumors are neoplastic growths that arise without any apparent external cause in organisms of certain genotypes. They are found within at least seven plant species and in hybrids between species in at least seven different genera (*136*). In *Nicotiana* they were first observed by Kostoff (*74*). The genetic constitution of the hybrid determines the potential of the constituent cells to undergo spontaneous change from normal growth to an abnormal, relatively undifferentiated proliferation.

The evidence for genetic control of tumor formation is extensive and can be summarized as follows:

(a) Among more than 300 different interspecific hybrids of *Nicotiana*, about 30 produce spontaneous tumors (*72*). The restriction of tumor occurrence to only certain genotypic combinations (*105*) can be considered as general evidence for their genetic basis.

(b) No causative agent of external origin has been isolated from *Nicotiana* hybrid tumors (*71*) nor transmitted across a graft union (*150*). No major differences at the ultrastructure level have been observed between parenchymal cells of genetic tumors and their normal counterparts from stems devoid of tumors (*6*). Tumors are produced only from cells that are genetically constituted to form them.

(c) Tumor formation is the same in hybrids from reciprocal crosses, thus indicating that the tumors are caused by nuclear elements contributed from each parent.

(d) Genetic manipulation of the ratio of genomes of *N. glauca* and *N. langsdorffii* does not alter qualitatively the potential of hybrid combinations to form tumors so long as an appropriate contribution of chromosomes from each parent is present (*73*).

(e) Location of the tumor-governing factors on certain chromosomes is evidenced by the appearance of tumors in plants with a few *N. glauca* chromosomes added to diploid *N. langsdorffii* or with a single *N. longiflora* chromosome added to the amphiploid *N. debneyi-tabacum* (*2, 3, 4, 73*). With plants of *N. langsdorffii* having one or two selected, extra chromosomes of *N. glauca*, tumor expression is much reduced, and the tissue culture requirements differ from those of the F_1 or amphiploid (Cheng and Smith, unpublished). This situation appears to present an opportunity to "genetically disect" the causative components of genetic tumors in *Nicotiana* spp.

(f) The factors that affect tumor formation exhibit features that characterize gene control, namely segregation, linkage (*146*), recombination (*130*) and mutation (*62*).

Physiological Characteristics

The genetically tumor-prone *Nicotiana* hybrids show differences in growth substance levels in the plants and differences in phytohormone requirements for tissue culture from nontumorous genotypes. Abnormal phytohormone relationships have been associated with tumor formation in *Nicotiana* (*71*). The tumor-forming amphiploid *N. glauca-langsdorffii* is higher in free indoleacetic acid (IAA), is more effective in converting tryptophan to IAA, and has a higher free tryptophan content than either parent (*73*). Bayer (*12, 13*) and Bayer and Ahuja (*16*) also have shown that tumorous hybrid genotypes have a higher level of IAA than plants of their nontumor-forming parental species. Hybrid tissues show an increased uptake and a lower transport capacity for IAA than do the parents (*14*). These elevated levels of IAA are considered to be physiologically responsible for tumor formation.

Recent findings that shed light on the physiology of spontaneous tumors are:

(a) Cheng (*28*) provided evidence that IAA induces its own synthesis in IAA-deprived cells of the tumor prone-hybrid *N. glauca* × *N. langsdorffii* but not in nontumorous parent cells;

(b) A reduction in the endongenous level of IAA has been proposed as the trigger for tumor induction in tumor-prone *Nicotiana* hybrids, and this has been substantiated by extensive experimental evidence (*7, 9, 10*);

(c) Liu and others (*84*) showed that the rate of synthesis of IAA conjugates was higher in tumorous hybrids than in corresponding, nontumorous types.

They postulated from these and other findings that IAA conjugates play an important role in tumorigenesis in *Nicotiana* spp.

Tissue culture of genetic plant tumors has clearly demonstrated that cells of the genetically tumor-prone *Nicotiana*, in contrast to genotypes governing normal differentiation, are capable of synthesizing or accumulating sufficient amounts of growth-promoting substances—auxin and cytokinins—so they can undergo rapid autonomous growth on a culture medium consisting solely of inorganic salts and sugar (*119*).

The two most significant results that have emerged from experiments on these spontaneous plant tumors are that their genetic basis is firmly established and that they can contain a higher than normal level of phytohormones (*136*). An explanation of the phenomenon in harmony with the experimental results to date is that the switch to tumorous growth is due to the activation of genes, normally repressed in differentiated tissues, that synthesize and accumulate products (mainly IAA) essential for continued or renewed cell divisions. This results in relatively undifferentiated, proliferative growth.

In short, the phenomenon appears to be basically a consequence of abnormal gene regulation (*130, 132, 136, 139*). This interpretation is substantiated by the following observations:

(a) Tumor cells can be reverted to normal differentiated tissue. For example, single hybrid cells produced by fusion of protoplasts of *N. glauca* and *N. langsdorffii* can be grown into a tumorous callus, then, by altering culture conditions, can be differentiated into a hybrid plant (*147*).

(b) Tumor acceleration and enhancement can be induced by such nonmutagenic stress conditions as merely crowding during seedling growth.

(c) Localized initiation of tumors at nodes or leaf scars is more amendable to interpretation as a result of local concentrations of metabolites affecting gene action than to explanation based on somatic mutation.

Some Unique Advantages of *Nicotiana* for Future Genetic Research

Built-in Selective System at the Cell Level

To produce parasexual hybrids by fusion of somatic cell protoplasts, an effective selective system is essential to pick up heterofusion products out of a mixed population containing parental protoplasts (ch. 10). The success of protoplast fusion and subsequent production of interspecific hybrids in *Nicotiana* (*24, 147*) is largely due to selecting hybrid cells on the basis of their less stringent tissue-culture requirements. This "built-in" system was known earlier because of previous work on tumorous hybrids, and in principle, it may have more general application (*63, 110*), particularly with heterotic hybrids (*143*).

This selection system is presently being exploited in an effort to extend the range of interspecific hybridization in *Nicotiana* spp. The scheme is based on the prediction that a hybrid, which has not previously been obtainable by cross pollination, would be expected to be tumorous and consequently have less stringent tissue culture requirements if it could be made by protoplast fusion (*141*). The basis for the prediction is the observation of Näf (*105*) that *Nicotiana* spp. can be divided into two groups (arbitrarily called plus and minus) so intragroup hybrids develop normally, but intergroup hybrids form tumors and their tissues can be cultured on media lacking phytohormones. Based on a survey of literature on hybrids of *Nicotiana* (*43, 53, 72, 76*), at least four, different, interspecific combinations exist that meet the two criteria of predictable tumor proneness and failure of cross pollination (*143*). These offer favorable material for further experimental use of *Nicotiana* in parasexual hybridization.

Another type of selection system in *N. tabacum* makes use of two genes that govern chlorophyll deficiency (ch. 3) and sensitivity to high temperature; they complement each other to produce a fully green hybrid callus and normal plants. Parasexual hybrids have been obtained by this method (*98, 99*) and by using a semidominant chlorophyll deficient mutant to mark the hybrid genome and simultaneously by another mutant (which gives variegated leaves in hybrids) to mark the chloroplasts (*52*). These methods may find general application because of the prevalence of recessive genes that control heritable chlorophyll deficiencies in plants (*35*). A possible shortcoming is that such genes may not be complementary in wider combinations between distant species, genera, or families.

Fraction I Protein: a Molecular Genetic Marker

Fraction I protein, ribulose-1,5-diphosphate carboxylase-oxygenase (*67*), which is involved in both photosynthesis and photorespiration (*77*), is the most abundant protein in nature and is found in all organisms that contain chlorophyll a. Fraction I protein can be dissociated into large and small subunits, which can be resolved into distinct polypeptide patterns by isoelectric focusing in polyacrylamide gels. Electrofocusing of carboxymethylated Fraction I protein of *N. tabacum* showed the large subunit to be composed of three peptide bands and the small subunit of two (*79*). Examination of Fraction I protein from 63 *Nicotiana* spp. revealed that all large subunits consisted of three polypeptides whereas small subunits varied from one to four polypeptides (*161*).

Chloroplast DNA contains the genetic coding information for the large subunit (*25*) as shown by an analysis of the following reciprocal, interspecific hybrids where the inheritance of the large subunit polypeptides is strictly maternal: *N. tabacum* × *N. glauca*, *N. tabacum* × *N. glutinosa*, *N. tabacum* × *N. sylvestris*, *N. tabacum* × *N. gossei*, and *N. glauca* × *N. langsdorffii* (*77*). The small subunits, on the other hand, are coded by nuclear DNA (*68*). Kung (*78*) suggested that after the small subunits are formed on cytoplasmic ribosomes they are transported across the chloroplast membrane where they link up with the large subunits, synthesized on chloroplast ribosomes, to form the native protein. The dual and localized genetic control of subunit synthesis provides a unique and valuable molecular marker that can be applied to various biological problems.

The Fraction I polypeptides are useful as a measure of evolutionary differences among species at the molecular level. Although the large subunit in all *Nicotiana* spp. consists of three polypeptides, their mobilities on a gel differ. Australian species all have the same pattern and differ as a group from Western Hemisphere species, which in turn can be grouped into three different mobility types (*161*). From these results and from an analysis of tryptic peptides (*69*), it was concluded that only small changes have occurred over the past 150 to 250 million years in the chemical composition of the chloroplast DNA genes that code for the large subunit.

The number of peptides comprising the small subunit ranges from one to four, and this is correlated with phylogenetic relationships. Species with 24 pairs of chromosomes and others of the Australian group, which are considered to be of amphiploid origin, all have two to four small subunit polypeptides. Most 12-paired species contain a single polypeptide. An analysis of the cross *N. glutinosa* × *N. tabacum* to form the amphiploid "species" *N. digluta* showed that the small subunit polypeptides, which are coded by nuclear DNA, were a composite of adding the small subunit polypeptides from both *N. glutinosa* and *N. tabacum;* whereas the large subunit polypeptides were identical to those from *N. glutinosa*, the maternal parent of the original hybrid (*80*).

This same approach can be used to obtain evidence at the molecular level on the origin of *N. tabacum*. As previously discussed, *N. tabacum* ($n = 24$) is considered to have arisen by chromosome doubling after hybridization

of an *N. sylvestris* predecessor with a member of the Tomentosae section (*N. tomentosiformis* or *N. otophora*). Comparison of the polypeptide compositions of Fraction I protein indicates that *N. sylvestris* contributed the large subunit polypeptides and, therefore, was the maternal parent of the original hybrid. *N. sylvestris* also contributed one of the two small subunit polypeptides of *N. tabacum*, the other being contributed by *N. tomentosiformis* (*58*). This analysis indicated, therefore, that the original hybrid was *N. sylvestris* (female) × *N. tomentosiformis* (male) and that *N. otophora* was not involved. This conclusion was verified by a comparison of chymotryptic peptides (*70*) and is in agreement with cytogenetic (*46*), segregational (*45*), morphological (*47*), and isozyme (*123*) evidence. Other molecular level markers that may find use in evolutionary studies in *Nicotiana* are ferredoxin (*161*), tentoxin sensitivity (*40*), and possibly chromosome pairing in hybrids (*140*).

Fraction I protein has been used recently to verify the hybridity of plants regenerated from fusing protoplasts of *N. glauca* and *N. langsdorffii* (*147*) and to determine chloroplast DNA distribution in these parasexual hybrids (*27*). Hybridity was confirmed by showing that all the plants selected as fusion products displayed the nuclear coded small subunit polypeptides of both *N. glauca* and *N. langsdorffii*. Contrary to expectation, the chloroplast-coded, large subunit polypeptides in the parasexual hybrids were not a mixture of parental types, but with a single exception, were either all of *N. glauca* or all of *N. langsdorffii*. The reasons for this rapid indiscriminate sorting out to a monotypic population of chloroplasts are unclear but may involve incompatability of mixed chloroplasts and random fixation of their reduced number.

Consequences of Interspecific Gene Transfer

Gene transfer is currently an active area of genetic research, particularly with the development of new techniques from molecular biology. Of major impetus has been the development of a capability for cleaving DNA with restriction enzymes, splicing it into a carrier molecule of a bacterial plasmid, transferring the recombinant DNA back into a bacterial cell, then cloning the "genetically engineered" genotype. Neither this method for gene transfer nor transformation by direct incorporation of purified donor DNA has yet been demonstrated unequivocally for higher plants. One of the more promising vehicles for gene transfer between plants in the future is the so called Ti-plasmid that is responsible for the oncogenic properties of *Agrobacterium tumefaciens* in most dicotyledonous plants including tobacco (*29, 39, 120, 121*). Several kinds of evidence suggest that *A. tumefaciens* in transforming normal to tumorous plant cells, confers new heritable traits by DNA transfer. The general strategy for future research in applying Ti-plasmids would ideally be to incorporate only useful genes into the plasmid, then to introduce the recombinant DNA into protoplasts or cells of tobacco. The ultimate goal would then be to stabilize expression through either synchronized replication of an extra chromosomal element or incorporation into the host eukaryotic DNA.

Other newly available techniques for gene transfer, which might be termed *macro* in contrast to more molecular methods, are chromosome-mediated and genome fusion followed by uniparental chromosome elimination. The latter, an important process in animal somatic cell hybrids, has been used with outstanding success to map chromosomes and establish linkage relationship in man (*97*). In higher plants, the phenomenon has recently been reported following fusion of somatic cell protoplasts of soybean (*Glycine max*) with *N. glauca* (*66*). The two genomes did not divide synchronously during the early cell generations, and the *N. glauca* chromosomes underwent mitotic disturbances that gradually reduced their size and, in time, their numbers. However, some of the reconstructed *N. glauca* chromosomes were still retained in later generations and became synchronized with the full soybean complement. In this way, a few gene blocks of *N. glauca* were introgressed into soybean cells during several months of culture. This phenomenon might be developed into a more general technique for introgressing desirable donor genes, gene blocks, or chromosomes from distant taxa into a host plant. The possibility may even exist for gene transfer between tobacco and man (*65, 163*).

Metaphase chromosomes can be taken up *in vitro* by mammalian cells (*94*). This probably occurs by phagocytosis, and most of the foreign DNA is rapidly degraded to small fragments by lysosomal enzymes. Only a small functional chromosome fragment is stably transferred. This "transgenome" is evidently integrated into the DNA of the recipient cell. No comparable experiments have yet been reported with plants. However, the same type of result can be anticipated because the pattern of DNA organization, that is, the interspersion of short repetitive sequences, long repetitive sequences, and single copy sequences in the genome of *N. tabacum*, is similar to that found in animals (*164*).

Although the application of these newer, more rapid methods for transferring genes between species has not yet been used in *Nicotiana*, the consequences of gene introgression by classical methods of cross pollination and recurrent backcrossing are well known. The urgency to develop disease-resistant commercial tobacco by using resistance found mainly or exclusively in wild species of the genus has been an impetus to studies on gene transfer (ch. 12). For the most part, the desirable characteristic from the wild species, when introgressed into a cultivar has exhibited its original phenotypic expression. These familiar experiences with gene transfer provide a foundation for predicting the outcome of applying unaccustomed tech-

niques in the future. However there are some surprises, even with old techniques, in the realm of interspecific gene transfer (*137*). Three examples will be discussed of novel unexpected genetic consequences of interspecific gene transfer.

The first involves an altered, flowering response in derivatives of the cross *N. rustica* × *N. tabacum* mammoth (*127, 137*). The mammoth gene causes the otherwise, day-neutral tobacco plant to flower under short photoperiods, 24 C or higher, thereby increasing the number of leaves produced in the regular growing season. An effort was made to transfer the mammoth gene to *N. rustica* by repeated backcrossing accompanied by selection for mammoth segregants. In the third backcross generation, the *rustica*-like mammoths failed to flower under short-day conditions but could be made to flower under low temperatures (7 to 10 C night). Thus, the mammoth gene no longer behaved as a day-length, flowering preceptor but as a temperature, flowering preceptor.

In succeeding generations, following the fourth backcross, *rustica* mammoths failed to flower even under low temperatures. Nonflowering plants of the seventh backcross generation were brought into flower (*60*) only under prolonged treatment with gibberellic acid combined with low temperatures (11 to 16 C). The effect was much greater with short days (8 hr) than with long. Beginning in the sixth backcross generation, a further anomaly in flowering appeared. It was characterized by an early brief occurrance of abnormal flowers followed by reversion to a typical nonflowering mammoth. The genetic factor controlling this characteristic was linked to mammoth and may have been activated by a further reduction in *tabacum* genes associated with the selected block containing the mammoth locus.

A second example of unexpected consequences from interspecific gene transfer was afforded by combining the nuclear genome of *N. tabacum* with the cytoplasm (presumably organelles) of the Australian species *N. debneyi*. In the first backcross generation of the amphiploid *N. debneyi-tabacum* as female to *N. tabacum* as the pollen parent, Clayton (*34*) observed male sterility and abnormal flowers with split corolla and aberrant anthers. In a succession of subsequent backcrosses (to BC_{10}) using *N. tabacum* as the male, the male sterility remained complete and the most extreme type of split blossom became fixed. This occurred only when *N. debneyi* contributed the cytoplasm in recurrent backcrosses toward a residual *N. tabacum* genome.

In a genetic analysis of the phenomenon (*114*), the progeny were classified into four main categories of corolla types (*116*) and seven aberrant stamen conditions. The more extreme type of each appears to be strictly cytoplasmic in inheritance, that is, due to *debneyi* cytoplasm combined with a *tabacum* genome. The progressively more normal types appear to result from interaction of one or more *debneyi* chromosomes, retained by selection, which contain genes that restore anther development to a more normal condition (*153, 154*). The Australian cytoplasm of the female parent can be recognized at a molecular level because the polypeptide pattern of the Fraction I protein large subunit coded by chloroplast DNA is different from that of *N. tabacum* (*27*).

These examples provide clear evidence of the interaction of cytoplasmic (organelle) and chromosomal elements from different species effecting a marked, unanticipated alteration in differentiation. Such interactions may be common among interspecific hybrids in the genus *Nicotiana* (*26*).

A third category of unpredictable phenomena that occur in hybrids among *Nicotiana* spp. are various manifestations of cytogenetic instability. These include variations in pigment of flower or leaf, variations in morphology and habit during individual plant development, and variability in growth among hybrid plants (*44, 72, 75, 76, 96*). All hybrid combinations of *N. tabacum* with *N. plumbaginifolia* that were studied by Ar-Rushidi (*11*) Cameron and Moav (*23*), Moav (*102*), and Moav and Cameron (*103*) showed somatic variegation of the dominant characters carried on the *plumbaginifolia* genome. Mitotic bridge-like structures in the hybrids have been observed; however, the exact cytological nature of the chromosomal elimination that causes variegation is still to be found.

Genetic instability also has appeared repeatedly in hybrids and hybrid derivatives between *N. tabacum* and diploid species that are representatives of putative ancestral forms. In hexaploids synthesized from *N. tabacum* × *N. otophora*, abnormal segregation ratios, variegation, and chromosomal aberrations were encountered (*45, 49*). Chromosomes of extraordinary size, called megachromosomes (*48*), were found in scattered cells of derivatives from *N. tabacum* × *N. otophora* showing variegation. The patterns of distribution of heterochromatin in *N. tabacum* and *N. otophora* are greatly different and the total amount of heterochromatin is much larger in *N. otophora* (chs. 2, 9) (*20*). The heterochromatic blocks from *N. otophora*, and also from *N. tomentosiformis* (*21*), underwent spontaneous breakage and great enlargement when transferred to *N. tabacum* (*50*).

A recent survey of the genus for locations of heterochromatin (*100*) showed that all *Nicotiana* spp. studied have small heterochromatic knobs, but only a few species scattered throughout the three subgenera, possess, in addition, large blocks of heterochromatin. These include one of the ancestral species of each of the cultivated amphiploid species, *N. tabacum* and *N. rustica*, which in turn have only small blocks and thereby pose a puzzling evolutionary phenomenon (*47*).

Another example of hybrid instability is the sporadic ap-

pearance of variegated, anthocyanin pigmentation in the flower of rare segregants from the cross *N. langsdorffii* × *N. sanderae* (*144*). One of these types (variegated-1) has been analyzed in detail and found to be governed by a mutable locus, *v*. Two alleles at this locus are necessary but sufficient to account genetically for the different model breeding behavior of the three major variegated phenotypes: speckled ($v_s v_s$), sectorial ($v_s v_s$) and rare sectorial ($v_S v_S$). Both alleles are unstable, and somatic mutations occur in both directions so a chromosomal loss is apparently not involved. Differences in frequency and developmental timing of these reversible mutations have both heritable and environmental components (*113*). The unstable gene, *v*, is about 10 times more sensitive to gamma radiation than stable gene, *R*, at low dose levels below 12 rad/day (*117, 118*).

Recently an ingenious genetic analysis was made by Sand (*115*) of a related, unstable type termed variegated-3 with a distinctive variegation pattern. This includes a unique and independent segregating regulator component, termed Fleck-timer (*Flt (3)*), in addition to the *v* locus element held in common with variegated-1. Presence of *Flt (3)* modifies the timing of somatic sectoring events and is correlated with the capability of producing frequent changes at the *v* locus to a stable nonvariegating dominant allelic form, *V*. Individuals containing the newly-arisen *V* alleles fail to reveal the continued presence of *Flt (3)*. The mechanism generating *V* from *v* appears to be correlated invariably with loss, inactivation, or transposition of a controlling element that is located initially on a different chromosome from that bearing *v*. Thus, the consequences of interspecific hybridization have led to evidence for a two-element control system in *Nicotiana* spp. similar to that first shown by McClintock (*95*) in maize and to transposons, or insertion sequences, found in prokaryotes (*107*).

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2

CYTOGENETIC TECHNIQUES

G. B. Collins[1]

Introduction

The genus *Nicotiana* has been extensively investigated cytogenetically during the past 50 years. At least two major characteristics of the genus have stimulated these investigations. First, the widely cultivated species *N. tabacum* is of allopolyploid hybrid origin, and considerable effort has been devoted to elucidating its phylogenetic relationships to that of its progenitor species (ch. 1). Also, an understanding of the phylogenetic relationships of the other species and species groups has been based largely on the determination of chromosome numbers, karyotype analyses, and chromosome pairing at meiosis in the appropriate hybrid combinations.

The second major impetus to cytogenetic study has come from the numerous efforts to transfer desirable disease and insect resistance and biochemical traits from *Nicotiana* spp. to *N. tabacum*. Prediction and execution of successful interspecific transfers and the subsequent incorporation of desirable genetic factors have required cytogenetic information from donor and recipient species.

Cytogenetic studies involving *Nicotiana* also have been stimulated by the availability of the complete monosomic series, the occurrence of interspecific hybrid instability and megachromosomes, cytoplasmic male sterility, androgenetically derived haploids, and chromosomal instabilities associated with *in vitro* cultures of *Nicotiana*. Reviews on the cytogenetics of *Nicotiana* have been provided by East (*14*), Kostoff (*20*), Goodspeed (*18*) and Smith (*35, 36*).

The recent availability of haploid plants (ch. 5), tissue cultures (ch. 7), and protoplasts (ch. 8) obtained through anther or pollen cultures has stimulated exciting research including somatic cell hybridization (ch. 9), selection at the cellular level (ch. 10), and the direct transfer of genetic material in *Nicotiana*. Cytogenetic evaluation of somatic hybrids, plants regenerated from selected cells or tissues, and plants obtained following the direct transfer of genetic material is required to establish the correct chromosome number of such derived materials.

Mitosis

Numerous types of studies with *Nicotiana* require the determination of the somatic chromosome number. Such somatic chromosome counts are readily obtained from squash preparations of tissues in which the metaphase stage of mitosis is observed under the light microscope. Chromosome numbers range from $2n = 18$ to $2n = 48$ with a size range of about 2 μ to 5.5 μ (ch. 1) (*18*). The high chromosome number and small chromosome size typical of *Nicotiana* spp. require a high degree of refinement in cytological techniques to obtain accurate chromosome counts and to determine chromosome morphology. In general, the metaphase stage of mitosis is most useful for making accurate chromosome counts because at this stage the chromosomes reach their maximum degree of contraction and spread in the cell following the use of a pretreating agent in the preparation procedure.

Procedures

The most widely used squash procedures for mitotic cytology in *Nicotiana* spp. are the aceto-carmine and Feulgen techniques. Steps in these procedures are similar although the fixative solution and stain used are different. The steps to be followed can be divided into collection, pretreatment, fixation, hydrolysis, staining, mounting, and observation.

Collection. Efficient mitotic cytology requires active mer-

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istematic tissue in which a large number of somatic divisions are occurring. The most frequently used intact plant tissues for cytology are root tips and immature corolla tissue. Root tips produced from stem and bud cuttings or roots developed on regenerating plants grown under *in vitro* culture conditions also can be analyzed using the same techniques. In addition, some investigators use leaf tissue, microspores, or callus tissue. Somatic cell counts from leaf tissues are best accomplished by applying the methods to be outlined for corolla tissue.

Root tips and immature corolla tissues must be collected from vigorous, healthy plants growing under optimum environmental conditions conducive to rapid plant growth and development (ch. 4). Root tips are best obtained from potted plants or plants growing in peat balls or other containers in which newly formed roots are readily accessible. Corollas are easily obtained from vigorously growing plants in the greenhouse or field without contaminant dirt or foreign material, which is often a problem with root tips. Burns (*5*) has detailed the steps to be followed in the corolla technique and recommends the use of tissue from small buds in the 3 mm range.

Pretreatment. Collected root tips or dissected corollas are placed directly into a pretreatment solution of 3 m*M* 8-hydroxyquinoline. This pretreatment results in a disruption of the spindle with the arrest and collection of many cells at the metaphase stage, and a shortening and contracting of the chromosomes. Burns (*5*) has suggested the addition of two drops of saturated maltose solution to the pretreatment to minimize stickiness and clumping of chromosomes. Most schedules recommended a 4- to 5-h pretreatment at 16 to 18 C to obtain cells. Sharma and Sharma (*32*), in contrast, recommended a 2 m*M* solution of oxyquinoline and a 3- to 4-hour treatment time at 12 to 16 C.

In addition to its c-mitotic activity, 8-hydroxyquinoline maintains the relative arrangements of the metaphase chromosomes at the metaphase plate and causes a conspicuous display of the primary and secondary constrictions, along with an exaggerated satellite gap (*32*). These characteristics facilitate the study of heterochromatin distribution and morphological differentiation of the chromosomes, especially in late prophase and early metaphase cells. The individual investigator should determine the exact 8-hydroxyquinoline concentration, treatment time, and temperature required to achieve the desired pretreatment effect; other pretreating agents, such as colchicine, γ-dichlorobenzene, α-bromonapthalene, and cold water, should be evaluated if specific chromosomal effects are required.

Fixation. At the end of the pretreatment, place the root tip or corolla tissue into freshly prepared Farmer's fluid consisting of glacial acetic acid: 95 percent ethanol (1:3) or Carnoy's fixative consisting of glacial acetic acid: chloroform: 95 percent ethanol (1:3:6). The former fixative is recommended for tissues to be stained by the Feulgen procedure whereas Carnoy's fixative is more suitable for aceto-carmine stained root tips or corollas. Fixation should proceed for 12 to 24 h at room temperature. Chromosome spreading may be facilitated refrigerating the fixed tissues before further processing if storage time does not exceed 2 to 4 wk. For longer storage, transfer the fixed tissues to 70 percent ethanol and place them in a freezer.

Hydrolysis. This treatment is done before squashing and staining because it softens the tissue and breaks down the middle lamella so that cells easily separate. Hydrolysis also exposes the aldehyde groups in the nucleic acids so that the reaction between aldehydes and the leuco-basic fuchsin in the Feulgen procedure can occur. Standard procedure involves placing the tissues in 1 *N* HCl for 6 to 10 min at 60 C. Determine the exact hydrolysis time for each tissue. The more recent hydrolysis procedure of Fox (*16*) is being adopted by many cytologists. With this procedure, hydrolysis is carried out at room temperature in 5 *N* HCl for 5 to 10 min with trial runs required to establish the exact time needed. Immerse the tissue in water at the end of hydrolysis to stop hydrolytic activity in the tissue. Then thoroughly rinse the tissue and place it in water until used.

Staining. For the Feulgen procedure, immerse the tissue in leuco-basic fuchsin stain previously warmed to room temperature. Darlington and LaCour (*13*) give recipes and preparative procedures in their book for commonly used chromosome stains, including leuco-basic fuchsin and aceto-carmine stains. Staining in leuco-basic fuchsin requires 1 to 2 h for complete staining to occur, and three or more distilled water rinses are needed to remove the excess stain. When the cells are to be evaluated cytophotometrically, rinse in SO_2 water to completely remove the stain. Store the washed tissue in 45 percent acetic acid or in a drop of aceto-carmine stain.

To stain with aceto-carmine, place tissue to be stained with aceto-carmine in the stain in a watch glass for 10 to 30 min or mount directly onto a glass slide in a drop of the stain. Addition of a small amount of iron from a rusty dissecting needle or from a $FeCl_3$ crystal will intensify the staining.

Mounting. With root tips, transfer only the whitish colored meristematic tip to the microscope slide. In the case of corolla tissue, remove a tiny piece of tissue from the margin of a young corolla lobe and place it on the slide. Apply a single drop of stain over the tissue and gently lower a clean cover slip onto the stain and tissue so that air bubbles are excluded and the tissue remains under the center of the cover slip. Hold the cover slip securely at two corners with the index and middle fingers while gently tapping the area of the cover slip over the tissue with a blunt instrument to spread and flatten the cells. Blot excess stain from the preparation and heat the squash preparation over a water bath or low spirit flame. Heating evacuates stain and air and pulls the cover slip tight to the slide. For additional

flattening of cells for counting purposes, press between layers of filter or bibulous paper taking care to avoid movement of the cover slip.

Follow the same squash and mounting procedure for tissue stained with leuco-basic fuchsin, except mount the tissue in a drop of 45 percent acetic acid. An optional procedure involves the use of aceto-carmine or another stain as the mounting medium. Using a second stain for mounting is desirable when the chromosomes are only lightly stained with leuco-basic fuchsin or when light staining of the cytoplasm aids in the analysis of the preparations.

Observation. Preliminarily observe temporary squash preparations as just described under the microscope for the presence of cells at the desired stage, for cell flatness and separation, and for general acceptability of the preparation. Additional tapping, flattening, and heating may be required before applying a temporary or permanent seal around the edges of the cover slip. Nail polish or rubber cement are satisfactory temporary seals that prevent stain evaporating and drying. Make chromosome counts, drawings, and photomicrographs from temporarily sealed preparations. Sharma and Sharma (*32*) have presented detailed schedules on permanent mounting procedures involving the use of mounting media such as euparal.

Chromosome Numbers

Numerous cytogenetic investigations have been completed for plant tissue and cell cultures. Polyploidy, aneuploidy, and rearrangements involving individual chromosomes have been observed in several species (*12, 39, 43*) as chromosomal changes occur in culture. In *Nicotiana*, chromosome numbers of callus cultures vary widely. For example, Fox (*15*) reported chromosome numbers of 130 to over 220 in a pith-derived tobacco callus line designated KX-1. A second line, not requiring auxin or cytokinin for growth, designated 0-1 exhibited chromosome numbers between 70 and 100. A relationship between chromosome number and exogenous hormone supply was suggested by the author. The chromosome numbers observed by Fox (*15*) are both aneuploid and polyploid variation from the amphidiploid number of $2n = 48$ typical of *N. tabacum*.

Shimada and Tabata (*33*) observed chromosome numbers ranging between 40 and 215 in pith tissues after 5 to 7 days in culture. Aneuploid cells with chromosome numbers ranging between 25 and 192 were observed by Nishiyama and Taira (*28*) in tobacco pith cultures. Shimada (*34*) found that tobacco callus obtained from tobacco root tissue was predominantly diploid after 7 mo, but the chromosome number in 2-year-old cultures varied widely. The designed production of polyploid plants, aneuploid plants, and nullisomic lines has been proposed by Murashige and Nakano (26), Niizeki and Kita (*27*), and Mattingly and Collins (*21*), respectively. Variation in chromosome number and karyotype changes have been observed in suspension cultures (*19*). In these studies the acetocarmine or Feulgen squash techniques described earlier were used. The need for a reliable cytogenetic technique in determining correct chromosome number in a species with such high chromosome numbers and small chromosome is evident.

Meiosis

Meiotic analysis is most efficiently accomplished in *Nicotiana* by analyzing aceto-carmine or aceto-orcein squash preparations of pollen mother cells (PMC's). For this procedure, obtain floral buds containing immature anthers from vigorous, disease-free plants grown in the greenhouse or field. Plants supplying the buds must be unstressed at the time the buds are collected. A common problem encountered in the analysis of PMC's from stressed plants is the clumping together of the meiotic chromosomes apparently caused by the stickiness of the chromosomes. Plant conditions that contribute to chromosome clumping include lack of moisture, nutritional deficiency, fertilizer shock, high temperatures, disease, senescence, and insecticide shock.

Remove buds individually from the plant and place them directly into freshly prepared Carnoy's fixative consisting of 95 percent ethanol:chloroform:glacial acetic acid (6:3:1). Carefully remove the upper portion of both the calyx and corolla from each bud to expose the anthers for rapid penetration of the fixative. Include a range of bud sizes in the collection to insure that the desired meiotic stages are obtained.

Fix buds at least 12 to 24 h at room temperature before staining and observation of the PMC's. Then refrigerate the fixed buds until used. In some cases, the PMC's are easier to spread on the slide after they have been chilled. If the buds are not to be used within a few days of the collection time, transfer from the fixative solution to 70 percent ethanol and store in a freezer.

Remove an individual anther from a bud and place it on a clean microscope slide. Cut the anther in half with a clean scalpel and apply a drop of stain over the tissue. The PMC's contained in each half-anther are best squeezed from the half-anther into the stain by using a blunt, smooth, plastic or glass instrument. Use plastic or teflon-coated instruments to avoid the introduction of excess iron into the stain as this results in dark staining of the cytoplasm. After the PMC's are forced from the anther, use clean forceps to remove the anther wall and tapetal debris from the slide. The amount of stain used corresponds to the quantity required for the area of the cover slip because excess stain results in the loss of numerous PMC's when the stain runs onto the slide beyond the cover slip.

Position a clean cover slip so that contact is made between one side to the edge and the drop of stain. Then

lower the cover slip slowly from the one side such that air bubbles are excluded as contact of the cover slip and stain are completed. Hold two corners of the cover slip securely with the index and middle fingers to prevent movement of the cover slip while gently tapping the area over the PMC's with a blunt instrument to flatten and spread the cells. Blot any excess stain and view the preparation under the microscope for proper meiotic stage. Accomplish additional flattening by applying pressure with the thumbs to the cover slip area after placing several layers of filter paper over the cover slip. Heating slightly over a steam bath or spirit flame will provide additional cell flattening. Evaluate immediately so that additional stain can be added and further flattening done if required. Make permanent or semipermanent seals as in mitotic preparations.

The alcoholic hydrochloric acid-carmine stain solution proposed by Snow (*37*) also has been used for *Nicotiana* meiotic preparations. Snow's method provides PMC's with lightly stained cytoplasm and good detail in the meiotic chromosomes.

The most studied meiotic stages in *Nicotiana* are metaphase I, metaphase II, and the tetrad stage. Observations at prophase I are limited by the large number and small size of *Nicotiana* chromosomes. Chromosome aberrations such as laggards, fragments, and bridges can be observed at the anaphase and telophase stages.

Pollen Staining

An estimate of pollen viability and fertility is often required in the routine evaluation of aneuploid stocks, haploid progenies, interspecific hybrids, and other *Nicotiana* materials. The aceto-carmine procedure is easily performed and provides a rapid evaluation of pollen stainability and size.

For this procedure, collect mature flowers containing undehisced anthers and bring them to the laboratory on the day prior to the observation of the pollen. After the anthers dehisce, dust the pollen onto a clean microscope slide. Add a drop of aceto-carmine stain to the pollen and gently place a cover slip over the stained pollen. Alternatively, obtain pollen from mature, undehisced anthers by placing the anthers on a microscope slide and cutting them in half with a clean scalpel. Force out the pollen into a drop of aceto-carmine stain with a clean, blunt, plastic or glass rod. Remove the anther wall and other extraneous tissue with forceps and place a cover slip over the stained pollen. With both types of preparations, allow the preparation to stain for 15 to 30 min before observing under the microscope.

Viable pollen grains will appear darkly stained in contrast to the empty, unstained, inviable pollen grains. Pollen grains are most accurately classified, using an ocular grid in the microscope eyepiece, by tabulating the stained and unstained grains on a dual-entry desk recorder.

Size differences in pollen grains are conveniently obtained from the same preparation used for counting stained and unstained pollen. An ocular micrometer is satisfactory for obtaining measurements of pollen diameter.

Microspore Cytology

Producing haploid plants from anther cultures requires determining the specific microspore stage in the anthers to obtain optimum plant production from anther cultures of individual *Nicotiana* spp. (ch. 5) (*11, 40, 41*). The aceto-carmine technique described above for pollen staining is applicable for evaluation of microspore stage in anthers to be cultured. The usual procedure is to squash one of the anthers from a floral bud for stage determination. The remaining four anthers are explanted onto the culture medium if the desired microspore stage for culture is indicated by the test anther.

The pollen mitosis stage has been established by Sunderland (*38*) as the optimum stage for culturing anthers of *N. tabacum*. The microspore mitosis can also be used as a convenient stage for the determination of chromosome number in *Nicotiana*. The metaphase stage is especially suitable for such determinations because the chromosomes are contracted even without pretreatment and one encounters the haploid chromosome number that facilitates accurate counts. This latter feature of microspore mitosis is particularly useful in species with high chromosome numbers (ch. 1).

Giemsa Staining

Considerable effort has been expended to characterize the patterns of differentiation along the length of *Nicotiana* chromosomes. Standard karyotypic analysis has included chromosome size, chromosome arm length, centromere location, satellite size and location, chromomere distribution, and heterochromatin distribution (*18*). More recent studies on hetetrochromatin distribution have been completed by Burns (*6*) and Merritt (*22*). Studies have been carried out recently that involve the use of Giemsa staining and quinacrine fluorescence staining in plant species (*30, 42*). In several species the Giemsa bands that have been revealed as lateral differentiated regions along the chromosomes are identical to the heterochromatic segments as revealed in cells that have been cold treated or treated with c-mitotic agents (*30*).

Merritt and Burns (*23, 24*) have used Giemsa procedures to characterize the chromosomes of *N. otophora* especially by comparing Giemsa bands with bands observed in aceto-

carmine stained chromosomes of cells treated with c-mitotic agents. They concluded that all differential staining of chromosomes resulted from differential condensation present in untreated chromosomes. Differential staining procedures revealing specific patterns of banding of chromatin distribution in prophase or metaphase chromosomes are useful cytogenetic techniques for distinguishing between chromosomes in *Nicotiana*. Practical applications include identification of specific chromosomes from different parental species in interspecific or intergeneric hybrids obtained from either sexual crossing or somatic cell hybridization. Identification of individual chromosomes is important for the maintenance and use of the aneuploid series such as the monosomics and nullisomic of *N. tabacum*.

Banks and Evans (*1*) have suggested counting the heterochromatin spots in interphase nuclei of *N. otophora* and its hybrids from cultured cells to determine ploidy level. This technique could be extended to other species that possess blocks of heterochromatin of a sufficient size for accurate observation and counting (chs. 1, 9).

Aneuploid Analysis

The complete monosomic series in *N. tabacum* has been established (*7*), but as Smith (*36*) has noted, few other aneuploids are available in *Nicotiana* (ch. 1). In *N. tabacum*, Gerstel and Parry (*17*) have isolated a nullisomic S line, Mattingly and Collins (*21*) have isolated nullisomic E, and seven different nullisomics have been isolated, but some of these have not been positively identified (Collins and Mattingly, unpublished).

Aneuploid cytogenetic stocks have many important potential uses to researchers. Monosomics are quite useful for locating genes on specific chromosomes or in transferring single chromosomes from one genus, species, or variety to another. Nullisomic lines provide considerable potential for assigning genes to specific chromosomes, determining genic and chromosomal control on chromosomal pairing, establishing homeologies between members of different genomes, assessing major chromosomal effects on specific agronomic traits, and for transferring or substituting specific chromosomes. An extensive discussion of the use of aneuploid techniques in wheat is provided by Morris and Sears (*25*). The complete monosomic, trisomic, nullisomic, and tetrasomic series plus many other aneuploid types have been available in wheat for some time, and the use of these types has been extensive (*31*). Similar studies and significant advances in *Nicotiana* cytogenetics and genetics will be possible if and when complete aneuploid series are available.

Microspectrophotometry

Several types of studies in *Nicotiana* ranging from the determination of nucleic acid and protein content to the determination of ploidy levels of cells in culture can be facilitated by the use of microspectrophotometric methods. Cells to be analyzed may be from somatic or meiotic tissue of plants grown *in vivo* such as we employed in an earlier study (*10*). Recently, there has been extensive use of microspectrophotometric methods in the study of cultured cells. This technique was employed by Bayliss and Gould (*2*) to characterize suspension cell cultures of sycamore maple.

A recent review of microspectrophotometric methods for use in determining deoxyribonucleic acid (DNA) content has been provided by Berlyn and Ceeich (*3*). This article with its excellent bibliography is a good starting point for the scientist interested in doing cytophotometric determinations. All aspects of cytochemistry including many techniques in addition to microspectrophotometry are provided in a chapter on cytochemistry by Berlyn and Miksche (*4*).

Autoradiography

Use of radioisotopes for the localization and study of biochemical events from subcellular to tissue to organ levels is afforded by the technique of autoradiography. Basically, the radioisotopically labelled plant specimen is covered by either a stripping film or liquid nuclear emulsion. With appropriate isotope selection and incorporation, tissue pretreatment and fixation, film and emulsion covering, exposure and development, specific biochemical processes, and activities of cellular structures can be evaluated. The salient features of autoradiographic technique and applications are given by Berlyn and Miksche (*4* pp. 279-290). We have used ^{3}H autoradiography to study DNA synthesis in *Nicotiana* (*9*). There are almost an unlimited number of applications for autoradiography in the study of cells in culture. Autoradiographic methods may also be used at the level of the electron microscope (*8*).

Useful radioisotopes for autoradiography include ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{131}I, ^{59}Fe, and ^{45}Ca. Extensive use of ^{3}H, ^{14}C and ^{35}S has been made because of the good resolution obtained from these weak β particle emitters. Autoradiographic methods are often used in combination with other cytological methods such as microspectrophotometry. Additional detailed information on light microscope autoradiography has been assembled by Sharma and Sharma (*32*) and Priest (*29*).

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3

HYBRIDIZATION

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Introduction

In a majority of instances, there is a close affinity between the extent of meiotic chromosome pairing in an interspecific F_1 hybrid and the degree of taxonomic relationships between the parent species (*16, 20*). The pre-Mendelian hybridizers gave tacit recognition to this relationship in their work on sexuality in plants (*20*). However, in a few instances, the extent of pairing in F_1 hybrids may be higher or lower than the apparent degree of phyletic affinity of the parents.

Intraspecific Hybridization

A cross between plants of the same species is an *intra*specific hybridization regardless of the genetic differences among the parents or the resulting level of heterozygosity of the hybrid. In a monogenically inherited trait, the self-pollination of an *intra*specific hybrid or a test cross should result in genotypic segregations of 1:2:1 or 1:1, respectively. The maternal or paternal position of either parent is unimportant unless one of them possesses a cytoplasmically inherited trait.

Current research on anther or tissue culture (chs. 5, 7), fusion of protoplasts (ch. 9), or biochemical genetics (ch. 10), often involves simply inherited traits. Marker genes that serve to distinguish mutant from normal phenotypes at the seedling stage are particularly useful. Three different seedling markers—white seedling (ws), yellow seedling (ys) and sulfur (Su)—were used recently in a study of genetic expectations following the production of plantlets by means of anther culture (*22*). Marker traits controlled by one or two genes may be assigned to specific chromosome locations in *Nicotiana tabacum* by means of monosomic analyses (*7, 9*).

Yellow-green, commonly referred to as Consolation is a recessive monogenic seedling marker "yg/yg" (*21*). It has more versatility for experimental work than such markers as "ws" or "Su" (*5*) that are lethal in the homozygous condition. The cross normal green (Yg/Yg) by recessive yellow green (yg/yg) will produce an abundance of seeds that are heterozygous (Yg/yg). Sulfur "Su" is a semi-dominant marker that has value for biological investigations. The homozygote Su/Su is lethal and the heterozygote Su/su is viable; although somewhat less vigorous than recessive normal green (su/su). Yellow-green is maintained by selfing plants of the genotype yg/yg and sulfur may be increased by self-pollinating Su/su or backcrossing Su/su × su/su. When growing sulfur seedlings, remove most of the green plants from the pots.

Monogenic seedling marker traits are particularly useful as heterozygotes. Evans and Paddock (*10*) used sulfur to study frequencies of somatic chromosome crossovers. Heterozygotes Su/su or Yg/yg develop twin or single spots on leaves that represent groups of cells of common descent hemizygous at the marker locus; green areas on leaves of sulfur plants are presumably –/su and yellow areas on leaves of Yg/yg plants could be –/yg. Mutagenic treatments presumably increase spotting frequencies. Another monogenic marker that is easy to score is the dominant reaction that produces local lesions on leaves after inoculation with tobacco mosaic virus TMV (ch. 11). The recessive homozygote reacts to inoculation by developing systemic symptoms of infection and plants with the genotypes N/n or N/N produce local lesions.

When using the local lesions trait "N" in experimentation, remove inoculated leaves shortly after the plants have been scored. If it is desired to prevent n/n plants in the population from becoming infected with the virus, detach young leaves, inoculate them, and place them in beakers of water. In this way lesion development can be determined without endangering valuable plant materials. Other potentially useful marker genes may be found in the table of monosomics listed in chapter 1.

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Interspecific Hybridization

F_1 Hybrids and Segregation

The contrast between *intra-* and *inter*specific hybrids can be visualized by the Mendelian behavior of the former and the essentially non-Mendelian behavior of the latter. The difference, or shades of difference, relates to the complete chromosome homology in hybrids between plants of the same species and the partial or nonchromosome homology that characterizes hybrids between plants of different species. The intermediate or gray area between the extremes among interspecific hybrids is seen in the behavior of those involving closely related species. Such species will have the same chromosome number, and the extent of chromosome homologies may be very high. Hybrids of this kind produce seed when self-pollinated and show evidence of Mendelian patterns of segregation for some traits.

Cytological studies of meiosis in some F_1 hybrids may show evidence of chromosome irregularities that reflect the chromosomal differences that mark the parents as different species. A hybrid between distantly related species, or even one between parents in the same section, may show reduced pairing between chromosomes of the different genomes. Meiosis in such hybrids may exhibit the typical chromosome behavior characteristic of monogenomic haploids. Most interspecific F_1 hybrid combinations in the genus *Nicotiana* probably fall into this latter category. Their chromosome doubled counterparts are called amphidiploids, or simply allopolyploids, when a parent of the hybrid may itself be a combination of different ancestral genomes. *N. tabacum* represents a typical example (ch. 1).

The relative difficulty of producing interspecific F_1 hybrids increases in proportion to the decrease in taxonomic relations between their parents (*20*). Although some F_1 hybrids are highly stable when converted to fertile polyploids, even hybrids between distantly related parents share some degree of chromosome homology. There are advantages to maintaining seed stocks of allopolyploids by self-pollination, particularly if the hybrid is difficult to obtain by conventional cross-pollination. Fertile polyploids may also be used directly for experimental purposes for example certain tumor-forming hybrids involving *N. langsdorffii* or other species in section Alatae (ch. 1). If one plans to use an allopolyploid that had been maintained as a seed stock for a number of generations, then verify the chromosome number and test plants from the stock for the presence of the desired trait before using it in a backcross breeding program.

Introgression (Incorporation of Alien Chromosomes or Segments)

Interspecific hybridization and introgression in natural populations of plants and animals is a known source of genetic variation and adaptation. We use the term introgression, or introgressive transfer, of genetic traits in a narrower sense. Our concern is with short-term breeding projects. The goal of experimental interspecific hybridization is usually the introgressive transfer of a specific trait from one species (the nonrecurrent parent) into the genome of another (the recurrent parent). The trait must be expressed with reasonable phenotypic fidelity as a dominant or partial dominant in the F_1 hybrid and all subsequent backcross generations, when nonrecurrent chromosomes are lost at random, otherwise the proposed interspecific transfer could not be made (*6, 17*). Foreign genes from one species can become unstable when translocated into the germplasm of another (*14*).

Chromosome pairing between the foreign genomes in an interspecific hybrid may be extensive or minimal (*16, 20*) although the F_1 hybrid is usually sterile. Fertility may be restored by treating germinating seed of the hybrid in 0.4 percent aqueous colchicine for about 4 h to induce chromosome doubling. Immersion of the seeds in 0.1 percent colchicine for 24 h also is effective. Thereafter, rinse the seed in sterile distilled water and plant them by sowing on the surface of pasteurized soil in glass or plastic, preparation dishes (ch. 4). Transplant the seedlings later to pots of soil in the greenhouse and self-pollinate or backcross mature plants that show good pollen development to the recurrent parent.

Three conventional approaches to interspecific hybridization for the purpose of alien transfer and incorporation of germplasm into stable diploid lines are (a) diploid × diploid then doubling to produce the allopolyploid and backcrossing to produce the sesquidiploid, (b) autotetraploid × autotetraploid to produce the allopolyploid directly, and (c) autotetraploid × diploid to produce the sesquidiploid directly.

To illustrate the mechanics of interspecific introgression of desirable traits from one species to another, designate a hypothetical species as SS (S = the genome of any species other than *N. sylvestris*) and hybridize it with *N. tabacum* (TT). Make the further stipulation that the nonrecurrent parent SS can only be used as a maternal (cytoplasmic) parent. Parent SS has the chromosome complement $2n = 24$ and parent TT has $2n = 48$. The cross SS × TT results in a sterile hybrid S/T ($2n = 36$), and the colchicine-doubled allopolyploid will have the genomic complement SS/TT ($4n = 72$).

In this example, the hybrid possesses the cytoplasm of the SS nonrecurrent parent and will retain this cytoplasm as long as the recurrent parent is used as a paternal parent in backcrossing. The first backcross SS/TT × TT produces S/TT ($3n = 60$), namely, a pentaploid or sesquidiploid. If we had been able to pollinate *N. tabacum* by the SS parent, the pentaploid could probably have been obtained directly by crossing autotetraploid TTTT × SS

= TT/S (*8*). Chromosome pairing in the pentaploid will be largely between homologues of the TT genomes. Therefore, the unpaired chromosomes of the single S genome will be distributed unequally in the resulting meiocytes.

Experience indicates that functional gametes from the sesquidiploid have either many or only a few chromosomes from the unpaired S genome. The morphologically variable progeny that results from the next backcross S/TT × TT is aptly called the breakdown generation.

In the previous example the SS parent was placed in the maternal (cytoplasmic) position to illustrate a problem called male-sterility associated with interspecific hybridization. As one introgresses the chromosome complement of the recurrent parent into a foreign cytoplasm, the loss of a specific chromosome from the SS parent may result in plants that have anomalous anthers and will effectively be male-sterile (MS) (*1*). Repeated pollinations of MS plants and their MS progeny will result in a plant with nonrecurrent cytoplasm and the full complement of the TT parent.

Normal-flowered (NF) plants that occur in the early backcross generations obviously possess a chromosome(s) from the SS parent that effectively controls the formation of normal anthers. Pollination of a MS plant by a NF plant will produce a progeny that contains both MS and NF plants. Introgressing the restorer chromosome, or preferably the *s*-bearing chromosome segment, into the *N. tabacum* genome may be possible in the same manner that other traits are transferred via interspecific hybridization. However, in this example, substitute the cytoplasm of the TT parent for the cytoplasm of SS by using TT as a maternal and pollinate it with a NF plant as soon as possible. Elimination of the foreign (nonrecurrent) cytoplasm effectively circumvents the male-sterility problem.

The number of plants in the breakdown generation that possess a chromosome with a desired trait will vary according to the species used as the nonrecurrent parent. Different transmission frequencies and a useful table of cross compatibilities between *N. tabacum* and other species were presented by Chaplin and Mann (*8*). Selections of plants that bear the desired trait and closely resemble *N. tabacum* (TT) will serve to rapidly isolate those plants that have a minimum of chromosomes from the S genome. If the necessary substitution of cytoplasm has been accomplished and pollen fertility is good, it is best to self the selected plants in an early generation. Consult the summary and conclusion of Chaplin and Mann (*8*) with particular emphasis on the lower rate of univalent transmission with increases in backcross generations and losses of extra chromosomes.

Scoring for the presence of a specific trait "x" among plants of the self-progeny seeks to reveal a frequency of 75 percent with the "x" trait and 25 percent without it. If one also obtains a confirming test cross ratio of 1:1 in a backcross progeny, this provides some assurance that "x" may have been integrated into the T genome. The number of plants that are scored for "x" and selected for further crossing should be as large as possible because an alien segmental substitution is, understandably, a rare event. Even after many generations, one may find that plants selected for a specific trait will not produce Mendelian ratios when selfed or test crossed; usually, the frequency of plants that possess a desired trait will be considerably less than 3:1 or 1:1. This is a function of the lower transmission frequency of gametes with extra chromosomes.

Cytological examination of microsporocytes in plants that produce atypical (non-Mendelian) ratios will show lagging chromosomes, microcytes or micronuclei, or both (ch. 2). Somatic or meiotic chromosome preparations will show the presence of one or more extra chromosomes. However, we assume that the transfer and incorporation of the alien chromosome segment has been accomplished when Mendelian patterns of segregation are obtained, meiosis is normal, and chromosome numbers are equal to the recurrent parent. Thereafter, treat all subsequent crosses involving the plant with the translocation and varieties of the recurrent parent as an *intra*specific cross.

The interspecific breeding sequence mentioned above began with the cross SS × TT. The cross TT × SS is also possible. In crossing diploid by diploid, the product S/T or T/S is sterile, but it can be restored to fertility by means of colchicine treatment. In some instances the chromosome doubling step can be eliminated by crossing autotetraploid versions of the parents TTTT × SSSS to obtain the allopolyploid TT/SS in a single step. If a tetraploid × tetraploid cross is possible (compatible), keep in mind that the autotetraploid parents have four homologous chromosomes of each kind and are subject to multivalent formation that may result in aneuploid gametes; but this is of minor importance when the objective is the introgressive transfer of a unit trait.

Transferring a specific trait from one species into the genome of another is preferable because the final product is a stable true-breeding entity that may be intercrossed with any number of varieties of the recurrent parent. However, because interspecific segmental or gene transfers may be long-term procedures, developing a line that contains an entire alien chromosome may be expedient. This is accomplished by alien chromosome substitution ($2n = 46_t + 2_x$) or by alien chromosome addition ($2n = 48_t + 2_x$).

Holmes (*19*) attempted to develop a line of tobacco with resistance to TMV. His source of resistance was the allopolyploid combination *N. glutinosa* × *N. tabacum* (GG/TT). After a number of backcrosses and self-pollinations, Holmes produced a line designated Holmes' Samsoun. The line resembled *N. tabacum*, possessed 48 chromosomes, exhibited perfect chromosome pairing at meiosis, and reacted

to inoculations with TMV by producing local lesions. Gerstel (*11, 12, 13*) demonstrated that Holmes' Samsoun was an alien substitution because when it was crossed with conventional *N. tabacum*, the F_1 hybrid showed 23 bivalents of *N. tabacum* and a univalent from *N. glutinosa* (Hg) and one from *N. tabacum* (Ht). Current varieties of *N. tabacum* that possess the local lesion response to TMV represent a segmental substitution wherein a portion of the Hg chromosome has been translocated (incorporated) into the Ht chromosome (*15*).

In a study of male-sterility based on the cytoplasm of *N. bigelovii*, a normal-flowered line was produced that had 25 bivalents (*1*). Recently an alien addition line was produced, characterized by the production of normal flowers, in which plants that lacked both alien chromosomes developed distorted male-sterile flowers. In the last named instance, a single alien chromosome controlled development of normal flowers.

The alien addition line was obtained by producing haploid plantlets from anthers of a normal-flowered plant of the chromosome constitution $2n = 48 + 1$. A few normal flowered plants ($n = 24 + 1$) and abnormal-flowered plants ($n = 24 + 0$) were obtained. Colchicine treatment converted the $n = 24 + 1$ plants to $2n = 48 + 2$. These plants had 50 chromosomes but were slightly unstable. Self-pollination of the 50-chromosome lines resulted in progeny with about 5 percent abnormal-flowered plants. This use of anther culture offers a means of obtaining instant homozygosity and also alien addition lines. However, keep in mind that alien substitution or alien addition lines may remain stable only if they are self- or sib-pollinated. Crossing them with conventional diploids will only result in eventual loss of the alien chromosome.

Several ancillary approaches to hybridization exist that can be employed when incompatibilities prevent the development of a viable hybrid. One of these is the bridge-cross method wherein a mutually compatible bridging species is first crossed to one species and, after several backcrosses to the bridge species, the backcross products are crossed to the second species. This was demonstrated in a bridgecross of *N. repanda* through *N. sylvestris* to *N. tabacum* (*2*).

Another variation on interspecific hybridization is to use a monosomic *N. tabacum* ($2n = 47$) as one parent in the cross. Hybrids of *N. tabacum* × *N. langsdorffii* were obtained (*3*) by using monosomics of the former species and diploid forms of the latter. Most of the successful hybrids involved Monosomic "A". Somatic chromosome counts of hybrid cells showed 32 figures. This suggested that the functional gamete from the monosomic parent was deficient for one chromosome.

Technology

A few simple tools, healthy plants, careful emasculation, freedom from contamination by foreign pollen, a reasonable level of cross fertility among the parent plants, and the recording of crosses meet the basic requirements of the plant hybridizer. Use a pair of fine curved-point forceps to slit the corolla and emasculate the blossom. In the case of *N. tabacum*, and many other species, remove the anthers just before the corolla unfolds. Several exceptions apply to this general rule. In the case of *N. repanda*, the anthers are adnate to the stigma, the anther walls usually break down, and the contents of the anther adher to the stigma before the flower matures.

Anthers in the later-formed flowers of *N. nudicaulis* may dehisce before the corolla unfolds and self-pollination will occur. Emasculate the flowers of these species several days before cross-pollinations are made. One can become acquainted with the nature of the self-pollination mechanisms in these species by slitting their corollas and observing the stigma and anthers at various stages in flower development.

Ordinarily, pollinations are made immediately after emasculation, but one can wait from 18 to 24 h after emasculation before applying pollen (*17*). The glistening and sticky surface of the stigma is touched with a newly dehisced anther, or pollen may be applied with the aid of a small artist's brush (size 6/128 to 10/128). Brush pollination aids seed set in some self-fertile species, and sib-pollinations are required to produce seed of self-sterile species (ch. 4). Insert the brush used to transfer pollen between siblings, handle down, in a pot. Inexpensive brushes with plastic handles are preferable to those with wooden handles as microorganismal activity will cause the latter to rot. If pollen of a desired parent is unavailable from living plants, use pollen that was collected at an earlier time and stored frozen in gelatin capsules (*18*). Gwynn[2] has found that frozen pollen from *N. tabacum* may remain viable for as long as 7 yr.

If a parental plant is suspected of harboring a mechanically transmitted virus, sterilize the instruments in 70 percent ethanol. After a pollination has been made, place a length of drinking straw, slightly longer than the pistil, over the stigma and crimp it by folding to prevent contamination by self- or foreign-pollen. Use of straws is probably unnecessary if plants are well spaced on the greenhouse bench, mature blossoms are removed, and neither insects nor excessive air movements present a problem. Furthermore, under most circumstances, a stigma thoroughly covered with the intended pollen offers sufficient protection against contamination. Usually, fertilization is affected within 1 to 2 d. Mark individual crosses with a small, stringed key tag. Moisten the string before

[2]Personal communication.

looping it around the pedicel otherwise the flower may be severed when the string is tightened. Whenever a number of identical crosses are planned, time can be saved by marking the tags in advance of pollination.

Generally, seed of most *Nicotiana* spp. will mature about 28 d after pollination. Seedlings of *N. tabacum* may be obtained earlier by scraping the developing seeds from the carpels about 18 d after pollination and treating them in 0.5 percent sodium hypochlorite (Clorox: water, 1:9) for about 10 min. One or two capsules of *N. tabacum* will contain enough seed to maintain a breeding line, but the seed yield per capsule of other species may be considerably smaller.

Some *Nicotiana* spp. are essentially day neutral whereas others require special temperature and day-length conditions to initiate flowering (ch. 4). Therefore, when planning specific hybridizations, consider the day-length requirements of the species involved, unless you can freeze pollen for use at a later time (*18*). Certain crosses may require a multitude of individual pollinations with the hope of obtaining a single seed among many mature capsules, for example, the cross diploid *N. rustica* × diploid *N. tabacum*. Difficult hybridizations may yield a paucity of shrunken seed of a smaller than average size. Although most seeds of this kind are often inviable, some of them may be induced to germinate if they are sown on soil in covered plastic or glass preparation dishes (ch. 4). When seeds are limited in number, do not treat the seedlings with colchicine. Selecting the most vigorous plants is better for asexual propagation (ch. 4). Later, treat the growing points of the clonal material with 0.3 percent aqueous colchicine three times a day for 3 days to induce chromosome doubling.

Premature blossom drop may be avoided by the use of 0.5 percent indoleacetic acid in lanolin. Apply it by dipping a needle into the preparation and scratching the pedicel at its point of attachment. This treatment will cause the blossom to remain on the plant regardless of embryo development and, therefore, offers no guarantee of a successful hybridization. On the other hand, syngamy serves to prevent abscission.

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4

PLANT PROPAGATION

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Introduction

Many *Nicotiana* spp. are native to South America with the remainder distributed through Central America, western North America, Australia and various islands of the South Pacific (ch. 1) (*2, 9*). Considerable difficulty may be encountered in growing some to maturity in the Eastern United States and similar regions because of their specialized temperature and day-length requirements for growth, flower development, and seed production. Most species are not cultivated. However, *N. tabacum*, and to a lesser extent *N. rustica*, are cultivated; they are called tobacco. Because of this domestication, these two species are adapted to a wide range of environments. Tobacco, primarily *N. tabacum*, is grown world wide from latitude 60° N. to 45° S. Throughout this wide range of climatic conditions the limiting factor for seed production is the number of frost-free days. Optimumly, mature seed can be produced in about 90 days following transplanting but in cooler climates more time is required.

Seed Germination

Extremely small seed and delicate seedlings are characteristics of all *Nicotiana* spp. For example, *N. tabacum* seeds number about 10,500 per gram. The first requirement for growing seedlings is a sterile medium. Vermiculite, a commercial mixture, or a sterilized, greenhouse soil mixture containing sand, loamy soil and sphagnum peat moss may be used (*1*). Forty to 50 seeds are sown per 10-cm clay pot or other suitable container.

Nicotiana seedlings spread out and form short stems unless somewhat crowded. Seed sown on the surface of vermiculite or similar coarse media need not be covered. However, if sowing on the surface of conventional potting soil, cover them with a fine layer of vermiculite. Place pots in deep saucers and add water to them so that it will rise to the level of the seed by capillary action. Water applied from above may cause the seed to be pushed too deeply into the medium or forced from the pot.

Most species will produce visible cotyledonary leaves in 5 to 7 d if the greenhouse temperature is maintained between 21 to 27 C. As soon as the seedlings become established, water them from the top. If the medium consists solely of vermiculite, add about 150 ml of Hoagland's solution (*11*) to the saucers about 1 wk after germination and again about 2 wk later.

Seed of *N. noctiflora* and *N. wigandioides* may take as long as 20 to 30 d to germinate. Plant delicate seed of this kind on soil (see mixture for growing seedlings in greenhouse) contained in glass or plastic preparation dishes. Cover and place them in an environmental chamber (30 C for 12 to 14 h day-length at 4,000 lux).

Seed also may be sown in a protected hot bed or cold frame (*8*) using soil treated with steam or with methyl bromide (*1*). Add about 300 g of a locally recommended complete fertilizer, such as 12-6-6, per square meter. Mix the fertilizer thoroughly in the top 5 to 8 cm of soil before seeding because too high a concentration of fertilizer can inhibit germination. To distribute the seed uniformly on the seed bed, mix them with an inert material such as sterile sand before sowing (0.06 g seed/m²). Cover the plant beds with glass, clear plastic, or cheesecloth (10 by 11 threads/cm²) depending on temperature and location. When using either glass or cheesecloth, carefully water the beds to prevent surface drying. If plastic is used, thoroughly water the beds just before putting on the plastic cover. The beds will generally then need no more water before the seed germinate. Thereafter, water as needed. When plants are exposed to direct sunlight, remove the glass or plastic

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covers during the day to prevent excessive heat buildup. When using plastic covers, perforate them with 0.6-cm holes, 5 cm apart.

Seed dormancy is usually associated with newly harvested seed. To induce germination of seed of *N. gossei*, and other species in section Suaveolentes, treat them with 2 percent sodium hypochlorite (Clorox:water, 2:3) for 15 to 30 min; then rinse and briefly place in acetone (*3*). Seed of such species ordinarily will lose dormancy after about 1 yr of storage. However, *N. spegazzinii* seed has a strong dormancy that may persist longer, but it can be overcome by soaking the seed in 50 ppm gibberellic acid for 10 min followed by 2 percent KNO_3 for 10 min. Seed of *N. noctiflora* may also benefit from the same treatment.

Plant Production

To maintain plants in a vigorous condition and realize maximum growth, transplant seedlings into 5- to 7.5-cm peat pots, 5-cm clay pots, or 5- by 5-cm plant bands or other suitable containers when they are about 4-cm high. The growth medium should be a sterilized greenhouse mixture such as loamy soil, acid peat moss and builders' sand (1:1:1, v:v). One may add supplemental fertilizer but providing Hoagland's solution at weekly intervals until the plants flower is preferable. *Do not overwater.* When the plants have become established, keep them slightly on the dry side as an aid in preventing stem and root diseases.

Depending on the species, again transplant to larger pots about 3 to 5 wk after the first transplanting. Do not transplant diminutive species (table 4-1). These will grow well, flower, and produce seed in the original seedling pot if the stand is thinned to about 6 plants per pot. Also, seedlings of *N. acaulis* are difficult to transplant. Larger species such as *N. tomentosiformis* do better when grown singly in containers 25 cm or larger. These larger containers will suffice to bring most species into flower and permit hybridizations and seed increase (table 4-1). This is particularly important for some of the late-flowering species, requiring 250 d or longer to flower, that must be grown to maturity in a greenhouse.

If seedlings have been produced in a plant bed, transplant them to small pots or plant bands so that a good root system is established before transplanting to field plots. Plants of *N. tabacum* or *N. rustica* will grow well when pulled bare rooted from a plant bed, but most other species will not survive this treatment. Many species do not perform well under field conditions. However, if one must grow them in field plots, plant in well-drained soil to avoid drowning and hand cultivate them to control weeds. Local soil conditions will determine the fertilizer requirement. Apply it in a split application; one-half in bands below and to the side of the roots and one-half as a sidedressing 4 wk after transplanting.

Soilborne diseases and algae can impose severe restrictions on seed germination and survival of seedlings (ch. 12). Airborne spores of *Peronospora tabacina*, the causal agent of blue mold, may pose a problem in the greenhouse or plant bed (*12*). As a general rule, disease and insect problems can be kept under control by strict sanitation practices, by spraying or dusting with appropriate pesticides, and by sterilization of soil and containers (*1, 8, 12*). Because of potential disease problems, confine the vegetative period to a minimum. Susceptibility to stem diseases (bacterial soft rot and sore shin) sometimes makes it difficult to maintain late blooming species such as *N. tomentosiformis, otophora, tomentosa* and *glauca* in the greenhouse for the long periods required to obtain seed. Aphids and white flies, as well as other insects, may be bothersome in the greenhouse and to a lesser extent in field plots.

TABLE 4-1.—*Requirements of floral induction and seed production of* Nicotiana *spp.*

Species	Sowing Date	Flowering Date[1] W-L	C-L	W-S	C-S	Plant Size[2]
acaulis[4]	Mar	—	—	—	—	s
acuminata	Mar	Jun	—	—	—	m-l
	Sep	Dec	Feb	Feb	Apr	
alata[3]	Mar	—	—	Oct	—	m
	Sep	Apr	Mar	Feb	Mar	
amplexicaulis	Mar	Aug	—	—	—	s-m
	Sep	Dec	—	Feb	—	
arentsii[4]	Jun	—	—	Jan	—	l
attenuata	Mar	Aug	—	—	—	m
	Sep	Mar	Apr	Apr	Apr	
benavidesii	Sep	—	—	May	—	l
benthamiana[4]	Mar	Aug	—	—	—	m
	Sep	Dec	—	Dec	—	
bigelovii	Mar	Jul	—	—	—	m
	Sep	Jan	Mar	Feb	Apr	
bonariensis[4]	Mar	—	—	Oct	—	s-m
	Sep	—	—	Feb	—	
cavicola	Mar	Nov	—	—	—	s-m
	Sep	—	—	Mar	—	
clevelandii	Mar	Jul	—	—	—	s-m
	Sep	Jan	Feb	Feb	Feb	
cordifolia[4]	Sep	—	—	Apr	—	m
corymbosa	Feb	Aug	—	—	—	s
	Sep	Mar	Mar	Mar	Mar	
debneyi	Mar	Jun	—	—	—	m
	Sep	Dec	Jan	Dec	Feb	
"eastii" 4n (suaveolens)	Mar	Jul	—	—	—	m
	Sep	Dec	Jan	Dec	Feb	
excelsior	Mar	Jul	—	—	—	m
	Sep	Mar	Mar	Mar	Mar	

See footnotes at end of table.

TABLE 4-1.—*Requirements of floral induction and seed production of* Nicotiana *spp.*—Continued

Species	Sowing Date	Flowering Date[1] W-L	C-L	W-S	C-S	Plant Size[2]
exigua	Mar	Jul	—	—	—	s
	Sep	Dec	Mar	Jan	Apr	
forgetiana[3]	Feb	—	—	Sep	—	m
	Sep	Apr	Apr	Apr	Apr	
fragrans	Mar	Jul	—	—	—	m
	Sep	—	—	Dec	—	
glauca[4]	Mar	—	—	—	Nov	l
	Sep	—	—	Apr	May	
glutinosa	Mar	Jun	—	—	—	m
	Sep	Jan	Feb	Dec	Mar	
goodspeedii	Mar	Jul	—	—	—	m
	Sep	—	—	Mar	Apr	
gossei[4]	Mar	Sep	—	—	—	m
	Sep	Feb	Mar	Mar	Mar	
hesperis	Mar	Aug	—	—	—	s-m
	Sep	Dec	—	—	—	
ingulba	Mar	Sep	—	—	—	s-m
	Sep	—	—	Mar	—	
knightiana[4]	Mar	—	—	Oct	—	m-l
	Sep	—	—	Mar	Mar	
langsdorffii	Mar	Jul	—	—	—	m
	Sep	—	—	Mar	Apr	
linearis	Mar	Jul	—	—	—	s
	Sep	Mar	Mar	Mar	Mar	
longiflora var. *breviflora*	Mar	Aug	—	—	—	m
	Sep	Dec	Mar	Mar	Mar	
longiflora var. *grandifolia*[3]	Mar	Aug	—	—	—	m
	Sep	—	—	Mar	—	
maritima	Mar	—	—	Nov	—	m
	Sep	May	Mar	May	May	
megalosiphon	Mar	Jul	—	—	—	m
	Sep	Dec	Jan	Dec	Feb	
miersii	Mar	Jul	—	—	—	s
	Sep	Jan	Dec	—	—	
nesophila	Mar	Aug	—	—	—	m
	Sep	Mar	Apr	Mar	Apr	
noctiflora[3]	Mar	Sep	—	—	—	m
	Sep	Apr	Jun	Apr	Jun	
nudicaulis	Mar	Jul	—	—	—	m
	Sep	May	Jul	—	—	
occidentalis	Mar	Jul	—	—	—	m
	Sep	Feb	Feb	Mar	Mar	
otophora[4]	Mar	—	—	Dec	—	l
	Sep	—	—	Feb	—	
palmeri	Mar	—	—	Nov	—	m
	Sep	Feb	Apr	Feb	May	
paniculata[4]	Mar	Jul	—	—	—	m-l
	Sep	Jan	Mar	Feb	Mar	
pauciflora[4]	Mar	—	—	Oct	—	m
	Sep	Apr	May	—	—	
petunioides[3]	Mar	Aug	—	—	—	s-m
	Sep	Apr	May	—	May	
plumbaginifolia	Mar	Jul	—	—	—	m
	Sep	Dec	Mar	Feb	Mar	
raimondii[4]	Jun	—	—	Apr	—	m-l
	Sep	Jul	—	—	—	
repanda	Mar	Jun	—	—	—	m
	Sep	Dec	Apr	May	Apr	
rosulata	Mar	Sep	—	—	—	s-m
	Sep	—	—	Mar	Apr	
rotundifolia	Mar	Jul	—	—	—	m
	Sep	Dec	Jan	Mar	Mar	
rustica var. *brasilia*	Mar	Jul	—	—	—	m-l
	Sep	Jan	—	Jan	—	
rustica var. *pavonii*	Mar	—	—	Dec	—	m
	Sep	—	—	Feb	—	
rustica var. *pumila*	Mar	Jul	—	—	—	s-m
	Sep	Feb	—	Feb	—	
sanderae[3]	Mar	Aug	—	—	—	m
	Sep	—	—	Jan	—	
setchellii[4]	Mar	—	—	Feb	—	l
	Sep	—	—	Apr	—	
simulans	Mar	Aug	—	—	—	s-m
	Sep	Dec	—	—	—	
solanifolia	Mar	Sep	—	Nov	—	m-l
	Sep	—	—	May	—	
spegazzinii	Sep	—	—	Mar	—	s-m
stocktonii	Mar	—	—	Nov	—	m
	Sep	Mar	Apr	Mar	Apr	
suaveolens	Mar	Jul	—	—	—	m
	Sep	Feb	Mar	Feb	Mar	
sylvestris	Mar	Jul	—	—	—	m
	Sep	Jan	Feb	—	—	
tabacum	Mar	May	May	May	May	m-l
	Sep	Jan	Dec	Jan	Dec	
thyrsiflora	Aug	—	—	May	—	m-l
tomentosa[4]	Mar	—	—	Apr	—	l
	Sep	—	—	Apr	Mar	
tomentosiformis[4]	Mar	—	—	Mar	—	l
	Sep	—	—	Mar	Mar	
trigonophylla	Mar	Jul	—	—	—	m
	Sep	Dec	Jan	Feb	May	
umbratica	Mar	Jun	—	—	—	s-m
undulata	Mar	—	—	Jan	—	m-l
	Sep	—	—	Mar	—	
velutina[4]	Mar	Jul	—	—	—	m
	Sep	—	—	Mar	—	
wigandioides[4]	Mar	—	—	Feb	—	l
	Sep	—	—	Feb	—	

[1] The month that flowering occurred is indicated under the respective conditions of temperature and day length: warm-long (W-L), cold-long (C-L), warm-short (W-S) and cold-short (C-S).

[2] Size designation at maturity: s = small, s-m = small to medium, m = medium, m-l = medium to large and l = large. The s and s-m plants may be grown in 10-cm pots but it is more economical to grow several plants in the same pot; m plants also may be grown in 10-cm pots. Plants of m-l size should be grown in pots 18 cm or less. Most l-species will grow well and produce an abundance of flowers when grown in clay pots 18 cm or larger diameter. Plants of *N. thyrsiflora* benefit from soil containers about 30 cm diameter.

[3] Self-sterile species that must be sibbed.

[4] Species in which brush pollination is helpful.

Flower Induction

Most *Nicotiana* spp. are day neutral and do not pose a problem in flower initiation, but some will only flower when grown under rather specific conditions of day-length and temperature (*4, 14, 15*) (table 4-1). Excepting *N. glutinosa*,

other members of section Tomentosae flower from January through March at latitude 36° N., provided they are maintained in a vigorous vegetative condition for at least 4 months before flowering. For *N. acaulis*, an artificial day-length of 22 h is optimal for flower induction (*5*). The conditions required to induce flowering in *N. thyrsiflora* are equally bizarre. One must start seedlings in August or September, maintain them in a vigorous condition, and provide a day-length of 8 h during April to induce flowering in May (*4*). These conditions must be strictly adhered to if flowering is to be induced in greenhouse-grown plants. An artificial regime using a controlled environment chamber may also be used (*5*).

Sowing seeds of *N. wigandioides* in September is preferable to sowing them in March (*4*) because the plants will only flower in February under the cooler temperatures and shorter day-length of that period of the year. Although under most circumstances *N. tabacum* is considered to be day neutral, certain accessions such as the mammoth type will only flower during short days. Under short-day conditions species belonging to the section Alatae, *N. repanda* and a few others will form a rosette and not begin to flower until March or April. They can be induced to flower earlier, however, by applying two drops of a 10-ppm gibberellic acid solution to the center of the rosette at 2-wk intervals.

Seed Production and Storage

Self-incompatibility among certain Nicotiana species (*alata, bonariensis, forgetiana, longiflora* var. *grandifolia, noctiflora, petunioides*, and *sanderae*) imposes a limitation on seed production (*4*). Populations of these species must contain plants of different mating types, and sib-pollinations must be made among them to obtain a seed increase (*7*). Grow 10 or more plants of these self-incompatible species, and periodically cross-pollinate them with an artist's brush in a random manner so that a mixture of pollen is distributed among all the mature flowers (ch. 3). Pollination of some self-fertile species also is aided by using a brush to transfer pollen. They include *N. acaulis, arentsii, benthamiana, cordifolia, glauca, gossei, knightiana, otophora, paniculata, pauciflora, raimondii, setchellii, tomentosa, tomentosiformis, velutina*, and *wigandioides*. Special problems exist with respect to the yellow- and red-flowered forms of *N. glauca* (*13*) and the *grandifolia* variety of *N. longiflora;* that is, the latter species is self-incompatible when grown at Beltsville, Md. (39° N.) and self-compatible at Oxford, N.C. (36° N.).

A seed increase of *N. tabacum* or species with a similar growth habit is made in the following manner: Allow the flower head to produce from 20 to 30 flowers so that the stalk is strengthened, remove all mature flowers and developing capsules, dust the flower head with an insecticide such as carbaryl (1-naphthyl methylcarbamate), and cover it with a 5- or 7-kg wet-strength paper bag. The bag may be stapled around the stalk or tied if one wishes to remove the bag to make individual crosses. Harvest the seed between 45 to 55 d after bagging. In the greenhouse, isolate a single plant and allow it to self-pollinate naturally, taking precautions against pollen contamination if the greenhouse is unscreened or crowded (ch. 3). The capsule will turn brown and the seeds will be mature about 28 d after pollination. In about 40 d, the capsule will be completely dry.

A low relative humidity (RH) is necessary when seeds are stored for more than 1 yr. Viable seed have been maintained for 10 yr or longer under controlled conditions. Seed stocks should be maintained in a controlled environment room at 4 to 5 C° and 40 to 42 percent RH. Store seed of breeding lines and marker stocks that are used frequently in heavy duty coin envelopes when short-term storage is contemplated. When longer times are required, small glass vials are more desirable.

Vegetative Propagation

Most *Nicotiana* spp. can be asexually propagated by stem cuttings or axillary shoots. In the first case, remove a portion of the stalk and cut it into pieces so that each has at least one node and that an entire leaf or large portion thereof remains on the cutting. Plunge the cutting into moist vermiculite and place the container in a shaded location. Treat axillary shoots or suckers in the same manner. If not present in sufficient numbers, they can be induced by removing the apical meristem.

A systematic study of root regeneration and shoot development in all species has not been made. Thus, for some species use rooting hormones, such as indole-3-butyric acid, and place the cuttings or shoot tips under mist (*10*). Experience has indicated that *N. acaulis* is very difficult to propagate vegetatively. For some species, when limited plant material is available, a shoot may be obtained by excising an axillary bud and setting it in soil contained in a glass or plastic preparation dish. Cover the dish to provide a humid and protective environment for the buds.

In leaf propagation, an entire leaf or small portion thereof is cut to include a portion of the midrib or secondary vascular strand. The vascular portion of the leaf, proximal to the stem end, is dipped in 0.5 percent indoleacetic acid suspended in talcum. The treated leaves or portions of leaves are planted in shaded rooting beds containing an absorbent soil mix equivalent to the formulas

for the John Innes "Seed Compost", or Cornell "Peat-lit Mix C" (*10*). The petiolar end of the vascular strand on the leaf disc, leaf part, or entire leaf responds by forming a callus and initiating roots in 6 to 8 d (*6*). Shoots emerge in about 30 d.

The method of choice for rapid propagation by asexual means is from axillary shoots because the size and vigor of the detached branch assures rapid rooting and top growth. Use of axillary shoots is also mandatory if one wishes to preserve an existing chimera. Propagation by means of adventitious shoots is useful when one desires to separate a chimerical plant into its component cell types.

Grafting

Approach or cleft grafting of *Nicotiana* spp. is relatively simple. The approach graft involves relatively tender seedlings that receive longitudinal cuts 2 to 3 cm long on their adjacent sides so that approximately half of the stem is removed from each plant. Press the cut surfaces together to obtain a good fit of their respective vascular systems (*10*). Hold the grafted area together by a loosely wound length of rubber band. In some instances, join the cut faces on the stems with the aid of a spring-type, wooden clothespin. The shoot of the stock donor is removed immediately, and the stem of the intended scion is cut free in about 14 days. Evidence of callus development in the region of the graft indicates the time to remove the rubber band or clothespin.

The cleft graft has the advantage over the approach graft that plants do not have to be matched precisely for size. Cut the stock transversely and split the stub to a depth of 3 cm or more (*10*). Cut the scion to a wedge-shaped point and insert it in the split of the stock. Match the vascular systems of the stock and scion. The cleft graft need not be supported by a stake if the scion is relatively short. Hold the graft together by wrapping it with a strip of nylon stocking or similar elastic mesh that is found in certain types of sterile bandages. A disadvantage of the cleft graft is that it requires protection against drying conditions. However, this can be overcome by placing a plastic bag over the grafted plant and fastening it around the base of the pot. Remove the bag in about 6 days. Another way to prevent moisture loss from the scions is to place the grafted plants in a mist chamber for about 6 days.

Additional types of grafts are described in chapter 11.

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5

HAPLOID PLANT PRODUCTION AND USE

M. J. Kasperbauer and H. M. Wilson

Anther Culture[1]

Introduction

Haploid plant production from immature anthers of *Nicotiana tabacum* is relatively easy. Haploids can be cultured in large numbers, screened for disease resistance, plant form, chemical content, and so forth, and then diploidized to produce homozygous lines.

The primary requirements for successful anther culture are haploid cells that divide and organize and a suitable culture environment. Different genetic lines sometimes differ in response to a given chemical and physical environment. Consequently, methods used for production of haploids and diploidization may differ slightly according to the plant materials and laboratory objectives. Pollen and anther culture have been reviewed by Nitsch (*13*) and Sunderland and Dunwell (*22*). This section is based on the use of anther and tissue culture in tobacco physiology and genetics by SEA scientists at Lexington, Ky.

Media Preparation

Stock solution components and final contents of the media—A, B, and R—are shown in table 5-1. The only differences between anther, budding, and rooting media are in the content of auxin and cytokinin.

Prepare stock solutions by dissolving chemicals in glass-distilled water and store them in glass-stoppered bottles in darkness at 5 C. Only the sucrose, agar, and inositol are weighed out each time. Add the stock solutions and weighed components to distilled water and bring to the desired volume with additional distilled water. Then heat the medium, stirring constantly, until the agar melts. Adjust to pH 5.8, using 2 *N* NaOH, before autoclaving at 121 C for 15 min. The medium can be autoclaved directly in culture flasks or test tubes, or in larger flasks, followed by pouring to Petri dishes. If the latter procedure is used, place the autoclaved flasks in a sterile transfer chamber. When cool enough to handle, pour 50-ml aliquots of medium into sterile, 20- × 100-mm plastic Petri dishes. This size dish is convenient to use because 10 or more anthers can easily be cultured in each, and plantlets can be removed easily. Stack the dishes on laboratory shelves to cool overnight and then cover with plastic bags to reduce evaporation.

Haploid Plant Production

Select floral buds containing anthers at first pollen division from healthy plants. Greater success is usually attained with anthers from the first floral buds than from buds developed on plants that have been flowering for some time. We prefer to excise the entire inflorescence and immediately place the cut end in a flask of water. The floral buds can be processed immediately or stored at 5 C in a refrigerator for 24 to 48 h. Some investigators report increased plantlet production following chill treatment (*13*). Usually, floral buds with the corolla visible just beyond the calyx will contain anthers at the appropriate stage of development, although various genetic lines differ in correlation between meiotic stage and external bud appearance. This stage is described as "Stage-2" by Nitsch (*18*) and as "Stage-4" by Sunderland and Dunwell (*22*). When large numbers of haploid plants are desired from a given line, select numerous buds of about the size described above and culture extra anthers rather than pursue the

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TABLE 5-1.—*Composition of media for culture of haploids*

Constituent	Stock solution		Culture Medium[1] Stock	Culture Medium[1] Final concentration
		Grams per liter	*Milliliters per liter*	*Milligrams per liter*
	Anther Culture Medium (A-Medium)[2]			
EDTA	A	0.80	28	22.4
$Fe_2(SO_4)_3$		.38		10.6
NH_4NO_3	B	82.50	20	1650
KNO_3		95.00		1900
H_3BO_3	C	1.24	5	6.2
KH_2PO_4		34.00		170.0
KI		.166		.83
$Na_2MoO_4 \cdot 2H_2O$		.050		.25
$CoCl_2 \cdot 6H_2O$		.005		.025
$MgSO_4 \cdot 7H_2O$	D	74.00	5	370
$MnSO_4 \cdot H_2O$		4.46		22.3
$ZnSO_4 \cdot 7H_2O$		1.72		8.6
$CuSO_4 \cdot 5H_2O$		.005		.025
$CaCl_2 \cdot 2H_2O$	E	88.00	5	440
Thiamine·HCl	F	.02	5	.1
Nicotinic acid		.10		.5
Pyridoxine·HCl		.10		.5
Glycine		.40		2.0
NAA[3]	NAA	.10	1.0	.1
Kinetin[3]	KIN	.40	.5	.2
Sucrose	—	—	—	20,000
Agar	—	—	—	6,000
myo-Inositol	—	—	—	100
	Budding Medium (B-Medium)			

Composition is identical with that of A-Medium, except that 5 ml of the kinetin stock solution is used giving a final concentration of 2 mg/L.

Rooting Medium (R-Medium)

Composition is identical with that of A-Medium except that no kinetin or NAA is used, agar is reduced to 5,000 mg and all other components are half strength.

[1]Bring components to volume with glass-distilled water. Adjust pH to 5.8 with 2 *N* NaOH before autoclaving at 121 C for 15 min.

[2]Medium's modified from that of Murashige and Skoog (ch. 7), (*12*), as used by Kasperbauer and Collins (*10*).

[3]Smaller amounts of naphthalene acetic acid (NAA) and kinetin stock solutions are normally prepared because of the small volume used.

time-consuming procedure of microscopically examining one and culturing the other four anthers of each bud. However, the latter approach is desirable when the objective is to maximize the percentage of anthers that produce plantlets.

Decontaminate the plant material in a sterile transfer chamber. If the inflorescence is too large to conveniently handle, cut it into smaller segments. Remove floral buds that are larger or smaller than described above and wash the inflorescence in 70 percent ethanol for 30 sec followed by 0.8 percent sodium hypochlorite (Clorox:water, 3:17) for 5 min. Dip all forceps and scalpels in ethanol, flame, and cool them before contacting plant material. Transfer the plant material from the sodium hypochlorite solution to sterile water for a final rinse. Remove the buds and transfer them to a sterile Petri dish for excision of anthers. As quickly as possible, transfer the anthers to A-Medium (table 5-1) in Petri dishes, flasks, or test tubes, depending on the choice of the investigator. We use disposable plastic Petri dishes because they are easy to handle and can be stacked to conserve space.

After the anthers are plated, tape the Petri dish cover and place a loose-fitting transparent plastic bag over each stack of dishes. The plastic bags help maintain humidity around the anthers and reduce the possibility of fungal contamination when the dishes are examined in an open laboratory. The anthers can be cultured successfully under conditions ranging from a desk drawer to an elaborate culture chamber (*9*). Temperatures within the normal range for tobacco seed germination are suitable. However, all other conditions being equal, plantlet emergence is earlier at 26 C than at 21 C.

Light is acceptable but not essential for the first several weeks of culture. Nevertheless, light is essential to prevent etiolation of the emerging plantlets and to initiate chlorophyll formation. For anther culture, use continuous illumination from cool-white fluorescent lamps (300 lux) and a temperature of 25 C. Anthers can be given a dark treatment in this illuminated chamber by covering dishes with aluminum foil. Plantlets begin to emerge from cultured anthers after about 4 or 5 wk, depending on the genetic line, temperature, and medium composition.

Although production of haploid plantlets from tobacco anthers does not require auxin and cytokinin in the culture medium, we include these components because they retard root formation on the emerging plantlets. The rootless plantlets are easy to separate, especially if they originate directly from individual microspores.

The first-emerged, easily separable plantlets are nearly always haploids; whereas plantlets emerging later may originate from callus that might have originated from parental tissue containing nonhaploid cells. Therefore, discard anthers after removing the first-emerged, easily separable plantlets. These plantlets are transferred to R-Medium (table 5-1) when they are about 3-mm long.

During the rooting period, grow the plantlets in a growth chamber (22 C and 12-h light at 5,000 lux from white fluorescent lamps). Do not use incandescent lamps as they may cause excessive heating. After approximately 10 d under these conditions, code each plantlet and collect root tips for

cytological evaluation of ploidy (ch. 2) (*2*). This verification of haploidy is extremely important if subsequent work involves diploidizing selected haploids.

When the work requires haploid plants to be observed under greenhouse or field conditions, first transfer the plants from the R-Medium to a soil-peat mixture (1:1, v:v) in small pots. Start them at 22 C and 12-h days using cool-white fluorescent lamps (5,000 lux). Transparent plastic covers can be used to obtain high humidity. After several days, raise the plastic to reduce humidity. During the next week, gradually increase the light intensity to full sunlight. After the plants have attained suitable size, transplant them to the field or to larger pots in the greenhouse for further screening and evaluation.

A two-step procedure may be required for haploid plant production if undifferentiated callus, rather than plantlets, emerge from the cultured anthers. This occurs with anthers from some *Nicotiana* spp., including some genetic lines of *N. tabacum*. Place callus on B-Medium to induce plantlet formation. Transfer the plantlets to R-Medium for root formation. Root tips must be studied cytologically because a higher percentage of nonhaploid plants develop by this two-step procedure than by the one-step procedure described above. Nevertheless, the two-step procedure offers the possibility of obtaining haploids from some difficult-to-culture genetic material. Because the callus may have originated from one or only a few microspores, haploid plantlets derived by the two-step procedure should not be used to study genetic ratios.

Use of Haploid Plants

Anther-derived haploids offer a unique method of gamete selection in the form of whole plants. Theoretically, obtaining and evaluating haploid plants of all genetic combinations that a tobacco plant is capable of producing in its male gametes is possible. However, some genetic combinations may be self-destructive and not capable of forming plantlets. If this is suspected, be cautious in interpreting apparent genetic ratios.

Many characteristics such as leaf shape, stem color, and relative level of certain chemical constituents can be selected in haploids derived from F_1 hybrids between plants differing in the characteristic under consideration. In this approach, the haploid plants under selection must be grown in an environment that allows segregation for the characteristic whether it is physical or chemical.

As an example, we produced a large number of haploid plants from anthers of F_1 hybrids between a low-alkaloid and a normal alkaloid cultivar of burley tobacco. The haploid plants were grown in a greenhouse under 16-h, high-intensity light periods that ended with red light. This environmental combination favored alkaloid accumulation in tobacco. When the plants were about 80-cm tall, the apical buds and small upper leaves were removed. All axillary buds were removed by hand each day for 3 wk before leaf samples were collected, processed, and analyzed for alkaloids. The favorable environment allowed accumulation of alkaloid levels corresponding to a two-gene genetic control. Thus, four populations for alkaloid production were found among the haploid plants. Selected haploids were diploidized by the leaf midvein technique described below. Several homozygous lines were produced for each of the four populations (*3*). This material was useful in studies of alkaloid biosynthesis (*6*), alkaloid relationship with insect resistance (*25*), and so forth. The same general principles of haploid selection under an optimal environment followed by diploidization of selected haploids should be equally useful for other physical and chemical characters.

Another potential use of haploids, that of selection at the cell level, has been described by Melchers (*11*). The haploid plants are first grown under environmental conditions to select the most suitable plants. Protoplast cultures can be established from leaf mesophyll cells (ch. 8) (*11*). The haploid cell cultures can be exposed to mutagens and then to a selective medium to allow expression of desirable mutants (ch. 10). Plants can be regenerated from the protoplast cultures and further evaluated, diploidized, and self-pollinated to produce homozygous lines.

At present, haploidizing, screening, and chromosome doubling techniques can be combined to rapidly develop homozygous lines to study genetic-environmental control systems, provide materials for physiological and biochemical studies of synthetic pathways, and provide a source of material for breeding programs. Other uses for haploids and haploid-derived cultures undoubtedly will develop. However, each new approach should be thoroughly examined and evaluated against the more traditional approaches in plant breeding and physiology.

Reconstitution of Diploids from Haploids

Several techniques can be used to obtain diploids from haploid plants. However, haploidy of the plant should be confirmed before the diploidization is attempted (ch. 2). Without cytological confirmation, the plant to be "diploidized" may already be a diploid, possibly derived from connective tissue in the anther. Assuming that such a plant is a diploidized haploid could thoroughly confuse a research program in genetics and plant breeding.

Sometimes regenerating diploids from a selected haploid while retaining the haploid plant for further study is desirable. In such cases, a procedure is necessary that does not destroy nor chemically alter the haploid plant. A tissue culture technique works well for this purpose (ch. 7) (*10*). Another factor in favor of this technique is that it uses essentially the same equipment as is used for the production of anther-derived haploids.

Diploid plants can be reconstituted from aged leaf tissue from selected, cytologically verified haploids. Select a leaf that has retained a healthy appearance for several weeks

after attaining full expansion. Discard the lamina and surface sterilize the midvein by washing it in 70 percent ethanol for 2 min. Next, immerse it in 0.8 percent sodium hypochlorite (Clorox:water, 3:17) for 6 min. Wash the surface-decontaminated midveins several times in sterile distilled water and then cut into 5-mm segments. Place the midvein segments on B-Medium (table 5-1) in Petri dishes. Tape the dishes, stack, and cover them with a transparent plastic bag to retain humidity around the tissue. Incubate the midvein segments at 25 C with continuous illumination from cool-white, fluorescent lamps (300 lux). Leafy shoots will begin to develop from the midvein tissue in about 3 wk. When the shoots are about 5 mm long, excise and transfer the normal-appearing ones to R-Medium. Use root tip cytology to determine ploidy of the regenerated plants (ch. 2). Ploidy of these plants is correlated with the age of the leaf from which they originate. Very young leaves produce haploid plants. Leaves that are fully expanded and of healthy appearance produce both haploids and diploids. Leaves that are beginning to senesce produce many aneuploids and an unpredictable mixture of tetraploids, diploids, and haploids.

Sometimes field-grown plants contain microbial contaminants in the tissue of aged leaves but not in very young leaves. Such contaminants cannot be removed easily without destroying the plant tissue. Therefore, we have developed an alternate two-step procedure to regenerate diploids from such haploid plants.

Culture midveins from young leaves of the cytologically verified haploid on B-Medium. These are usually free of the contaminant present in the older levels. Leafy shoots develop from the cultured tissue in about 3 wk. Excise and place them on R-Medium. Study root tips of the regenerated plants cytologically. If any of the regenerated plants are diploid, the problem is solved. However, if all are haploids, they can be grown in the aseptic rooting medium for several weeks to develop some aged root tissue. Transfer root segments, excised 2- to 4-cm from the tip, directly to B-Medium and handle them the same as described for midvein cultures. Leafy shoots develop from the root tissue. After regenerated shoots are about 5 mm long, excise and transfer normal-appearing ones to R-Medium. Use root tip cytology to verify ploidy of these regenerated plants. We have obtained about 75 percent normal diploids from root segments by this two-step (or second chance) procedure.

The root culture procedure is also useful as a primary diploidizing procedure. This requires that the characteristic under selection (for example leaf color) is readily identifiable in the original haploid plantlets while they are still growing under aseptic conditions. Stem tissue from young haploid plants also can be cultured to produce diploids; however, the procedure does not allow the plant to be used in extensive screening before the stem is excised. Pith explants from older haploid plants are usually unsatisfactory because the regenerated plants include many aneuploids (*10*).

Some investigators advocate use of colchicine to produce doubled haploids. Techniques with small plantlets and grown haploid plants have been established (*1*).

Pollen Culture[2]

In 1973 Nitsch and Norreel (*17*) demonstrated that isolated pollen of *Datura innoxia* formed plantlets when cultured on a simple medium supplemented with a boiling water extract from cultured *D. innoxia* anthers. Pelletier (*19*) reported, in the same year, the regeneration of plantlets from isolated *Nicotiana tabacum* pollen by using *Petunia hybrida* callus as a nurse tissue. Defined media have since been developed to achieve this purpose (*13*); the technique is known today as pollen culture.

Species of *Nicotiana*, particularly some cultivars of *N. tabacum*, have proved responsive, although in terms of application and exploitation pollen culture remains in its infancy. Difficulties have been encountered in obtaining repeatable results, and anther culture generally gives higher yields in terms of numbers of plants recovered. However, for convenience of handling in mutation studies and for a more direct approach to the study of the nutritional requirements of pollen cultured for haploid plant regeneration, the use of isolated pollen is clearly advantageous. In addition, the culture of isolated pollen removes the element of doubt, inherent in anther culture, concerning the gametic origin of regenerated plants which have the somatic number of chromosomes or are polyploid.

Culture Media

The media used for tobacco pollen culture are derived mainly from those developed for anther culture. Most authors agree that phytohormones are not required though there is controversy regarding the addition of certain amino acids. On the basis of an amino acid analysis of anther tissues supporting pollen embryogenesis, Nitsch (*14*) recommended the addition of 100 mg/L of L-serine. Wernicke and Kohlenbach (*26*) and Horner (*7*), on the other hand, were unable to detect serine in amino acid analyses of similar extracts. The latter author found that asparagine, glutamine, and proline were present in the largest amounts but that media containing these amino acids were unable to sustain growth of pollen directly isolated from anthers. Nitsch (*15*) and Wernicke and Kohlenbach (*26*) have demonstrated the requirement for the addition of 800 mg/L glutamine to the culture medium and Nitsch (*15*) reported the

[2]Prepared by H. M. Wilson, Department of Plant Pathology, University of Wisconsin, Madison, Wis. 53706.

beneficial effect of increasing the amount of *myo*-inositol from 60 mg to 5 gm per L.

Physiological Status of Donor Plants

No data are available relating directly to pollen culture, but the physiological status of donor plants is of major importance in determining the response of tobacco pollen cultured within the anther. Responses are greater in anthers taken towards the beginning of the flowering period than at the end (*21*) and are about 60 percent greater when the donor plants are grown under an 8-h day, high light intensity regime (*5*).

Selection and Pretreatment of Anthers for Pollen Extraction

Buds with anthers containing pollen just before, during, or just after first pollen mitosis are most suitable as starting material. This means, in the case of *N. tabacum* cv. White Burley, selecting buds with petals between 17 and 21 mm in length. Before every experiment the pollen stage should be correlated exactly with petal length by making squash preparations of anther samples in acetocarmine (ch. 2).

Nitsch (*13*) advises that the detached buds be cold-treated at this stage. This treatment is given by placing them in a dark, humid environment at 5 C for 2 d. This treatment is less effective when given to the whole plant, excised anthers or isolated microspores (16). Reinert, Bajaj, and Heberle (*20*) cold-treated buds for 3 d whereas Wernicke and Kohlenbach (*26*) obtained promising results in the absence of exposure to a cold environment.

Immediately before dissecting out the anthers, surface-sterilize the buds by immersion in 7 percent calcium hypochlorite for 2 min, followed by five washes in sterile water. In the procedure followed by Nitsch (*15*) the five anthers in each bud are removed aseptically and floated on the surface of 5 ml of liquid culture medium I (table 5-2) in a plastic Petri dish (6-cm diameter). The dishes are sealed with Parafilm and incubated at 25 C under Gro-Lux tubes (500 lux) with a 12-h day-length. After between 4 and 6 d incubation, pollen within these anthers is suitable for extraction and culture.

Other procedures used for pretreatment of the anthers are similar in outline (*23, 26*). In these cases, however, detailed accounts are best given along with the particular pollen extraction technique employed.

Pollen Extraction Techniques and Culture

To calculate the final number of pollen grains per milliliter of culture medium it should be remembered that each anther of *N. tabacum* contains about 40,000 grains (*24*). Three separate methods of extracting pollen for isolated culture have been described:

(a) The simplest approach is that of Sunderland and Roberts (*23*). They observed that anthers floating on liquid medium in plastic Petri dishes dehisced soon after inoculation and released pollen into the medium. This pollen continued to grow if the anthers were removed from the dishes. Chilling of the buds for 12 d at 7 to 8 C is an important requirement for early dehiscence of tobacco anthers. For best results chill buds when they reach a corolla length of 21 to 23 mm (just after first pollen mitosis). After this period, dissect out the anthers from three buds and float them on the surface of 5 ml of liquid medium II (table 5-3). Then transfer the anthers to fresh medium of the same composition after 6, 10, and 14 d. Seal dishes with Parafilm and incubate at 28 C in darkness for the first 14 d. Then transfer them to 25 C in the light (Gro-Lux, 500 lux, 12-h day-length). The pollen released into the medium at 6, 10, and 14 d continues to grow and give rise to haploid embryos and eventually plants.

(b) The current interest in culturing isolated pollen to produce large numbers of haploid plants has been generated principally by the work of Nitsch and her colleagues (*13, 14, 15*). The technique they developed for liberating pollen into the culture medium depends upon the mechanical disintegration of the anther. For this method place approxi-

TABLE 5-2.—*Anther culture medium I*[1]

	Milligrams per liter
KNO_3	950
NH_4NO_3	725
$MgSO_4 \cdot 7H_2O$	185
$CaCl_2$	166
KH_2PO_4	68
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
Glutamine	800
Serine	100
myo-Inositol	5,000
Sucrose	20,000

Adjust pH to 5.8 and sterilize by filtration.

[1]Nitsch (*13*).

TABLE 5-3.—*Anther culture medium II*[1]

	Milligrams per liter
KNO_3	950
NH_4NO_3	825
$MgSO_4 \cdot 7H_2O$	185
$CaCl_2$	220
KH_2PO_4	85
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
Sucrose	20,000

Adjust pH to 5.5 and sterilize by autoclaving (15 psi for 15 min at 121 C).

[1]Sunderland and Roberts (*23*).

mately 50 anthers in a 100-ml beaker containing 20 ml of culture medium I (table 5-2). Use the piston of a 30-ml glass syringe to burst the anthers by crushing them against the sides of the beaker. Remove the debris by filtering through nylon mesh (40-μ pore size) and then centrifuge the pollen suspension filtrate at 100 g for 5 min. Wash the pollen pellet twice in fresh medium by resuspension and centrifugation, then dilute with culture medium to give a density of 10,000 grains/ml. Pipet the final suspension in 2.5-ml aliquots into 5-cm Petri dishes, seal the dishes with Parafilm and incubate at 27 to 30 C in the light (500 lux). In successful experiments using this technique, 5 percent of the pollen grains are reported to give rise to haploid plants (*15*).

(c) Wernicke and Kohlenbach (*26*) were able to achieve only limited success with Nitsch's method. Similar problems have been encountered in other laboratories, and evidence suggests that the random damage to the anther walls, which occurs when they are crushed, can inhibit pollen embryogenesis (*8*). The approach taken by Wernicke and Kohlenbach (*26*) is aimed at minimizing anther damage during pollen extraction. Allow the anthers to float on 5 ml of culture medium III (table 5-4) for 4, 8, and 16 d. For anthers containing uninucleate pollen at the beginning of the culture period, 16 d pre-extraction incubation, are required; for those in which pollen is just completing first mitosis, only 4 d are necessary.

To remove the pollen, gently hold the anthers with forceps while making incisions using a fine scalpel blade. Squeeze out the contents of the anthers into the culture medium, discard the anthers and seal the Petri dish with Parafilm. Using culture medium conditioned in 5-ml aliquots by incubation with two anthers for the appropriate period is important. For best results the final concentration of pollen in the conditioned medium should be about 100,000/ml. Culture the extracted pollen either under the same conditions as the anthers, namely a 12-h photoperiod (800 lux) at 25 C, or in the dark. The time required for haploid embryogenesis is shortened by 4 to 6 d when the pollen is grown in the dark. In the most successful experiments 1 percent of the pollen, isolated after 10 d culture within the anther, gave rise to haploid plantlets.

TABLE 5-4.—*Anther culture medium III*[1]

	Milligrams per liter
KNO_3	950
NH_4NO_3	720
$MgSO_4 \cdot 7H_2O$	185
$CaCl_2$	166
KH_2PO_4	68
$MnSO_4 \cdot 4H_2O$	25
H_3BO_3	10
$ZnSO_4 \cdot 7H_2O$	10
$Na_2MoO_4 \cdot 2H_2O$	.25
$CuSO_4 \cdot 5H_2O$	.025
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.8
Nicotinic acid	5
Glycine	2
Pyridoxine-HCl	.5
Thiamine-HCl	.5
Folic acid	.5
Biotin	.05
myo-Inositol	100
Sucrose	20,000
Adjust pH to 5.5 and sterilize by filtration.	

[1]Wernicke and Kohlenbach (*26*).

Recovery of Haploid Plants

Once the plantlets have clearly defined leaves, they can be removed from the Petri dish and transferred to an agar medium with a low salt concentration (half-strength Murashige and Skoog's mineral salts (ch. 7) (*12*) to induce root formation. The resultant whole plants are then potted in compost or a mixture of peat and sand and covered with plastic bags to maintain humidity until they are well established. Make chromosome counts on the root tips of the regenerated plants as soon as an adequate root system has developed (ch. 2).

Discussion

In briefly appraising the three approaches to pollen culture described above, consider the purposes for which such cultures will be needed. Owing to the present difficulties in maintaining stable cultures of haploid cells, there is great interest in the use of isolated pollen for mutation studies. Although obtaining mutants is possible by X-irradiation of *N. tabacum* flower buds and the subsequent regeneration of plants through anther culture (*4*), cultures of isolated pollen have greater potential for mutation screening and facilitate the application of chemical mutagens.

The stage at which the pollen is isolated is of particular importance in this context. To avoid chimaerism the pollen should be treated with mutagens at a stage when, in subsequent embryogenesis, it is functionally uninucleate. The youngest pollen at present, which responds to culture in isolation, is early bicellular. This pollen would be suitable if normally differentiated into a vegetative and a generative cell because the latter degenerates rapidly during the course of pollen embryogenesis. However, in the case of Nitsch's technique, the presence of abnormal pollen with two identical nuclei before isolated culture is considered essential to success (*15*). This technique would, therefore, appear to be inappropriate for use in mutation studies.

The difficulties in obtaining reproducible results can probably be traced to the use of donor plants with different physiological statuses and, in the case of pollen extraction through mechanical disruption of the anther, to differing degrees of anther wall damage (*8*).

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6

ORGAN CULTURE

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Introduction

Although all tissue cultures originate from organs or their sections, the progenitor organization need not always be retained during *in vitro* development. But an organ culture has as its aim the achievement of an organized structure, the morphology and physiology of which are identifiable with the specified organ. It usually begins with a primordium explant.

The first successful cultures of excised plant parts involved organ cultures, not cell or tissue cultures. In 1904, Hannig (*25*) obtained crucifer seedlings by culturing embryos excised from immature fruits. The potentially unlimited growth *in vitro* of isolated tomato roots demonstrated by White (*100*) also preceded by several years the first success with cell or callus cultures.

Organ cultures were most significant between the early 1940's and the mid-1960's. Interest in them has declined since. Today, with the exception of the shoot apex cultures that are being used to achieve rapid clonal propagation and pathogen-free plants, virtually no activity is associated with organ cultures. The diminished interest should not be interpreted as signifying a lesser need of organ culture investigations. With many plants organ cultures remain unattainable.

Organ cultures are helpful in development studies, inasmuch as many correlative factors that characterize the attached state can be minimized. Nutritional and environmental requirements of an organ, as well as differentiation and functioning of specialized tissues, can be explored more precisely.

Vegetative Organs

Root Culture

1. **Background and characteristics.** Kotte (*39*) and Robbins (*73*) were first to explore the culturability of excised roots. Their experiments with several legumes and cereals did not yield indefinitely culturable clones. Potentially unlimited growth of roots was eventually realized by White (*100*), who experimented with tomato and employed a nutrient solution that contained mineral salts according to Uspenski and Uspenskaia, sucrose and yeast extract.

White (*101*) was able to extend his success with tomato to root cultures of certain species of *Nicotiana* as *N. langsdorfii* and *N. tabacum*. Excised roots of *N. glutinosa*, *rustica*, and *sylvestris* were not maintainable beyond a few passages. White's successes with *Nicotiana* were subsequently confirmed by Dawson (*19*); Levine (*45*); Solt (*79*); Solt, Dawson and Christman (*80*); and Kisaki, Mizusaki and Tamaki (*37*).

Roots cultured in isolation generally possess the same morphological characteristics as those that are attached to the plant. The quiescent center can be observed in the root apex (*63*). The vascular pattern typical of the species is also retained (*89*). Lateral branching occurs and is manipulatable by certain nutrient addenda (*88*) or illumination (*22*). Many physiological phenomena that typify roots *in vivo* can be reproduced *in vitro*, that is, undiminished production of the alkaloids, anabasine and nicotine, by *Nicotiana* root cultures (*19*, *37*, *79*, *80*). This observation is significant because cells and callus cultures are often associated with reduced synthesis of secondary metabolites.

Some cultured roots may not retain the progenitor geno-

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type. P. R. White[2] had noted that his tomato root cultures were tetraploid when examined after 30 years. Whether this signified a ploidy change that had occurred during culture *in vitro* or a chance selection of a tetraploid progenitor has not been established. However, alteration of chromosome numbers during the course of repeated subculture has been encountered in other instances such as root cultures of certain cereals (*9*).

2. **Requirements of isolated roots.** The degree of ease or difficulty of culturing isolated roots is not predictable on taxonomic grounds. Although much of the successes have been associated with herbaceous dicots, to generalize that monocot roots are more intractable than those of dicots or that root cultures of woody genera are less probable than those of herbaceous plants is misleading. Cultivars within a species can show significant variations. Street noted even intraclonal variations (*15*, *83*). Excised roots are normally grown in gently agitated, liquid media. Maximum development is attained when all essential mineral elements, including micronutrients, are provided. Of curious interest has been White's observation that tomato roots are stimulated by I^-, an ion whose essentiality to plants remains unconfirmed. Iron must be provided in the chelated or other organically complexed form. The term "staling" has been applied to media that have lost their capacity to support root growth because of diminished iron supply (*83*).

Sucrose has served as the main carbon source of most root cultures and, when tried, it has not been replaceable by an equivalent of fructose and glucose. Glucose may be superior to sucrose for root cultures of some grasses.

The yeast extract of White's tomato medium has been replaceable by a combination of the vitamins, thiamine, pyridoxine, and nicotinic acid, as well as the amino acid, glycine. Experience with a variety of *in vitro* cultures has established that thiamine is a critical addendum. Pyridoxine and nicotinic acid enhance growth but are usually not essential, and glycine is probably unnecessary.

Inositol may have significant nutritional effects on some excised roots (*24*). Asparagine, glutamine, and arginine may also be beneficial. No evidence has been shown of an exogenous cytokinin requirement. Presumably it is synthesized in sufficient quantities by most roots. Gibberellin has been shown to stimulate cultured roots of genetically dwarf cultivars but not of normal plants (*49*).

Street (*15*, *83*) has categorized plant cultivars according to the response of their root cultures to supplied auxin. Roots of some cultivars are unaffected or are inhibited by auxin as in tomato; others are enhanced by an auxin provision as in pine; in still others the root cultures may be dependent on an external source of auxin as in rye. The initiation of lateral roots is promoted by an auxin addendum (*88*).

The pH optimum for elongation of roots *in vitro* is in the range 5.0 to 5.5 (*99*), whereas that of initiating lateral roots is in the range of 6.0 to 6.5 (*88*).

Low levels of illumination may enhance growth of some roots (*83*). However, other root cultures are inhibited by exposure to light. The reduced growth of excised wheat roots in light has been correlated with the presence of iron, suggesting an involvement of chlorophyll (*14*). Lateral root initiation is repressed by red light; this effect could be reversed by far red light (*22*).

In the absence of data concerning the temperature requirements of cultured roots, the practice has been to provide an environment that approximates one where the cultivar is normally found. Many root cultures can be maintained at constant temperatures in the neighborhood of 25 to 27 C.

Disinfestation is usually impossible with roots obtained from plants grown under greenhouse or field conditions. Fungi and bacteria are often lodged in crevices of the root and sometimes found residing intracellularly. Eliminating them may require excessive injury to the explant. The usual practice in root cultures is to obtain explants from aseptically produced seedlings. Seeds are surface disinfested and germinated in sterile medium. Satisfactory disinfestation sometimes requires removal of all seed coats. Germination is fastest in constant darkness.

Adventitious roots that arise in callus or organ segments could also be used as sources of clean explants.

A convenient length of the root tip is severed and placed in nutrient solution. After 7 to 14 days, sectors are subcultured. The original apex is normally not recultured, except in instances where lateral root initiation is rare. Segments of subapical regions, each containing one to few newly emerged laterals, are transferred to freshly prepared nutrient solution. The subculturing process is repeated at suitable intervals and clones are quickly established. All culture requirements can be considered fulfilled when the prescribed conditions enable indefinite subculturability. A progressively diminishing growth of subcultures, on the other hand, reflects a still inadequate situation.

3. **Sample procedures.**

(a) Aseptically germinating seeds:

(1) Dispense 50-ml aliquots of White's nutrient solution (table 6-1) into 250-ml Erlenmyer flasks. Take two circles, together, of 9-cm diameter Whatman No. 42 filter paper and make several, small, accordion folds along the periphery. Perforate the center with a sharp instrument to enable air passage. Place filter paper in nutrient flasks with folds facing down. A platform, with the supporting surface remaining slightly above the nutrient level, should result. Plug flasks and autoclave 15 min at 121 C.

(2) Place seeds to be disinfested in a small

[2]Personal communication.

TABLE 6-1.—*White's nutrient solution for root tip culture*[1]

$MgSO_4 \cdot 7H_2O$	milligrams per liter	720
$Ca(NO_3)_2 \cdot 4H_2O$	do.	300
Na_2SO_4	do.	200
KNO_3	do.	80
KCl	do.	65
KH_2PO_4	do.	20
$MnSO_4 \cdot H_2O$	do.	5.3
$ZnSO_4 \cdot 7H_2O$	do.	3
H_3BO_3	do.	1.5
KI	do.	.75
Glycine	do.	3
Nicotinic acid	do.	.5
Pyridoxine·HCl	do.	.1
Thiamine·HCl	do.	.1
Sucrose	grams per liter	20

Supply iron by adding 10 ml/L of a stock solution containing 2.784 g/L $FeSO_4 \cdot 7H_2O$ and 3.724 g/L Na_2EDTA
Adjust pH to 5.0

[1] White (*100*).

vial. Add a generous quantity of 0.25 percent sodium hypochlorite (Clorox:water, 1:19) and a small amount of detergent, cap vials, and let stand 10 min. Decant disinfestant and rinse seeds with sterile water three times. Using a sterilized microspatula, transfer a small sample of seeds to nutrient flasks, distributing seeds neatly over the filter paper platform. Do not crowd seeds because crowding causes enmeshing of roots and difficulty in obtaining explants. Allow germination to occur in darkness at 27 C.

(b) **Initiating root culture:**

(1) Prepare nutrient medium of the same composition as above and dispense 25-ml aliquots into 125-ml DeLong flasks (Erlenmeyer flasks may suffice, in which case cotton plugs or other similar closures will be necessary). Cap Delong flasks with Morton stainless steel closures and autoclave 15 min at 121 C.

(2) In excising and planting root tips, handle seedlings delicately. No alcohol or hot instruments should be allowed to touch roots. Using a pair of long, thin tweezers, transfer seedlings to sterile Petri dishes that have been lined with moist, Whatman No. 50 filter paper. Do not grasp seedling at its roots. With a surgeon's scalpel fitted with a No. 10 or No. 15 disposable blade, sever 1 cm of the tip of the primary root. Grasp excised tip at its cut basal region with fine-tipped forceps and transfer to nutrient flask at one explant per flask.

(3) Incubate cultures by placing freshly planted flasks on a gyratory shaker (80 rpm) and incubate in darkness at 27 C.

(c) **Subculturing and establishing clones:**

(1) To prepare sectors for subculturing, after 7 d when the initially cultured root has elongated considerably and produced numerous laterals, remove it to a sterile Petri dish and divide into sectors, each possessing one or more, 1 cm or longer, lateral roots. Place sectors in freshly prepared nutrient solution of the same composition and reincubate as above.

(2) Growth measuring process of subcultured sectors should be repeated every 7 to 10 d. Successful establishment of a clone will be evident in undiminishing growth from one subculture to next. Measurements that might be recorded at the end of each subculture are total increase in length of lateral roots and number of newly emerged laterals.

Shoot Apex Culture

1. **Applications and background.** The earlier efforts with shoot apex cultures were directed primarily at their use in morphogenetic investigations. The experiences, particularly of Ball (*5, 6*), Wetmore and Morel (*97*), and Wetmore (*96*), led to the concept that the apical meristems of ferns and angiosperms differed in their capacities for autonomous development. The shoot apical meristem of ferns could be isolated and cultured to produce rooted plants in a relatively simple salt-sugar medium. In contrast, that of the higher plants was unable to develop more than a few leaves *in vitro*, even when provided with a complex of nutritional supplements. Rooted plants from angiosperm meristems were obtained only when the explant contained a few primordial leaves and some subjacent stem. The concept has been invalidated by the more recent demonstration of Smith and Murashige (*78*), who produced complete plants from apical dome explants, with no subjacent leaf or stem tissues, of several angiosperms.

Several investigators have employed shoot apex cultures to explore flowering phenomena (*4, 26, 31, 65*). In some instances, inducing flowering *in vitro* is possible with shoot tips serving as sensing organs of florigenic stimuli.

Shoot tip cultures are finding extensive horticultural applications. The procedure has been identified in some circles by the unfortunate misnomer "meristem culture." The term meristem culture should be reserved for cases where the explant has been restricted to the apical tissue distal to the youngest leaf primordium. All others involving the shoot apex region should be more appropriately identified as shoot apex or shoot tip cultures.

Currently, shoot tip cultures are finding major uses in (a) recovery of pathogen-free stock and (b) rapid clonal multiplication. Potential benefits from their use have been demonstrated in cryogenic preservation of germplasm (*75*) and in international transport of genetic stocks (*32*).

The recovery of pathogen-free plants through shoot tip cultures is based on the premise that pathogen concentration is not uniform throughout the infected plant. Frequently, the apex of a rapidly elongating stem remains uninvaded by pathogens. Its isolation and culture *in vitro* could result in a pathogen-free plant. Morel and Martin (*52*) were first to verify this principle by obtaining virus-free dahlias from infected plants. Establishing pathogen-free stock through shoot tip cultures is now standard practice with many clonally propagated crops (*54*).

Morel was also first to demonstrate the effectiveness of shoot tip cultures as a rapid cloning procedure (*51*). His effort to recover virus-free Cymbidiums from infected plants resulted in exclusion of virus, as well as in clonal increase of plants, at rates that were substantially higher than those achieved by traditional propagation methods. Today a wide variety of crops, especially ornamentals, are being propagated commercially through an extension of Morel's findings (*55*). Rapid clonal propagation of *N. rustica* through shoot tip explants has been demonstrated by Walkey and Woolfitt (*94*).

2. **Requirements.** The *in vitro* requirements of shoot apices vary with the dimensions of the explants, the intended use of the culture, and the genotype. Explants comprised of the meristem dome alone apparently require hormonal supplements, whereas those consisting of the dome together with a few primordial and emerging leaves do not (*76*). The leaf structures, particularly those that are emerging and enlarging, are the major sources of hormones in the shoot tip.

Shoot tip explants for the recovery of pathogen-free plants should be relatively small, 0.1- to 0.3-mm tall, to ensure high frequencies of exclusion of pathogens, especially viruses and viroids. In contrast, those intended for rapid clonal multiplication can be quite large, several millimeters in many instances; the survival frequency, growth, and propagule multiplication rates are often related directly to the explant size.

Variations in culturability may be encountered even among cultivars of a species. But successful shoot tip cultures, resulting in rooted plants, can now be expected of any cultivar, whether fern or angiosperm, monocot or dicot, herbaceous annual or woody perennial. In addition to the usual concern for nutrient medium and culture environment, particular attention should be paid to certain characteristics that are associated with the explant and its source.

Smith and Murashige's (*78*) success with cultures of angiosperm apical meristems is possibly attributable to their use of a nutrient formula rich in inorganic salts. Certain desirable, high level mineral ions, especially potassium, have been observed elsewhere (*53*). Sucrose in a 3 percent concentration has been satisfactory for shoot apex cultures. Inclusion of thiamine•HCl and inositol may be desirable. For explants constituted solely by apical meristem tissue, the medium should contain both auxin and cytokinin (*76*). These hormones may not be critical to larger explants, but even then they could be employed advantageously. They often enhance growth of all cultures and, furthermore, the pattern of organogenesis in shoot tip cultures can be manipulated by varying the levels of the two hormones in the nutrient medium. A multitude of shoots, a desired feature in clonal multiplication, is obtained by providing high levels of cytokinin. Exogenous gibberellin is rarely necessary, although attaining rooted plants from relatively small shoot tips has been enhanced in some instances (*53*).

Agar-gelled media are most often used in shoot tip cultures. Gently agitated, liquid formulations are sometimes helpful with cultures that are intended for rapid propagation as in certain orchids. Unagitated liquid media can also be used, but for small explants this might require a filter paper bridge or platform supports.

Small, shoot tip explants should not be exposed to high, light intensities. An intensity of 1,000 lux is about optimum for initiating and maintaining most shoot tip cultures. However, providing exposures to higher light intensities, 3,000 to 10,000 lux, may be necessary to harden rooted plants before transferring to soil. Fluorescent lamps of the Gro-Lux type are most widely used, although no disadvantage has been observed with the ordinary, cool-white types. With the above light intensities, a daily exposure of 16 h has been satisfactory.

Perhaps the diurnal temperatures of a cultivar's natural habitat should serve as guide to the *in vitro* requirements of its shoot tip cultures. The frequent practice, however, has been to maintain a constant temperature at about 27 C. With many perennials, especially those of the temperate climate, consider seasonal dormancy as an important requirement when obtaining explants. Prior exposure of source plants or organs to chilling temperatures or appropriate photoperiods, or both, may be necessary.

3. **Sample procedure.** The procedure below distinguishes between shoot tip cultures that are intended for recovering pathogen-free plants and those for rapid, clonal propagation.

(a) **Nutrient media:**

(1) Prepare a basal medium containing Murashige and Skoog's mineral salts (ch. 7), and the following in milligrams per liter: sucrose, 30,000; thiamine•HCl, 0.4; *i*-inositol, 100; and Phytagar, 8,000.

(2) Obtain individually rooted plants from the substantially smaller shoot tips, as are desired when recovering pathogen-free plants by supplementing the basal medium with about 0.3 mg/L IAA and 0.3-1 mg/L kinetin.

TABLE 6-2.—*Nutrient medium for leaf culture*

Stock[2]	Salt		
STOCK SOLUTIONS FOR HELLER'S SALTS[1]			
A	KCl	grams per liter	100
B	$NaNO_3$	do.	100
C	$MgSO_4 \cdot 7H_2O$	do.	100
D	$NaH_2PO_4 \cdot H_2O$	do.	10
E	$CaCl_2 \cdot 2H_2O$	do.	10
F	$FeCl_3 \cdot 6H_2O$	do.	1
G	$ZnSO_4 \cdot 7H_2O$	do.	1
	H_3BO_3	do.	1
	$CuSO_4 \cdot 5H_2O$	milligrams per liter	30
	$AlCl_3$	do.	30
	$NiCl_2 \cdot 6H_2O$	do.	30
	KI	do.	10
	$MnSO_4 \cdot 4H_2O$	do.	10
ORGANICS[3]			
Sucrose		grams per liter	10
Thiamine·HCl		milligrams per liter	20
Inositol		do.	10
Nicotinic acid		do.	5
Calcium pantothenate		do.	5
Pyridoxine·HCl		do.	1
Choline chloride		do.	1
para-aminobenzoic acid		do.	.5
Riboflavin		do.	.5
Folic acid		do.	.1
Biotin		do.	.01
Phytagar		grams per liter	8
Adjust pH to 5.7 before adding agar.			

[1]Heller (*28*).

[2]Use/liter of medium: stock A, 7.5 ml; stock B, 6 ml; stock C, 2.5 ml; stock D, 12.5 ml; stock E, 7.5 ml; stock F, 1 ml; and stock G, 1 ml.

[3]Steeves Gabriel and Steeves (*81*).

(3) Use 2 mg/L each of IAA and kinetin, 80 mg/L adenine sulfate·$2H_2O$ and 170 mg/L $NaH_2PO_4 \cdot H_2O$ for rapid clonal multiplication through proliferation of shoots.

(4) Adjust pH of both media to 5.7 before adding agar.

(5) Dispense media in 25-ml aliquots into 25- × 150-mm culture tubes. Cap tubes with polypropylene closures, autoclave 15 min at 121 C, and cool as 45° slants.

(b) Preparing explants and planting:

(1) Remove large leaves near the stem apex and sever a terminal segment containing 1 cm of stem and small leaves.

(2) Disinfest by immersing in 0.5 percent sodium hypochlorite (Clorox:water, 1:9) containing a few drops of concentrated detergent for 10 min.

(3) Rinse three times and transfer the segment to a sterile Petri dish for excision of the shoot tip.

(4) Excise the meristem dome alone or the 0.1 to 0.3 mm shoot tip with the aid of a quality dissecting microscope. Hold explant source erect with a pair of small forceps and remove all emerging and expanding leaves with a sharp scalpel (surgeon's scalpel fitted with a No. 11 blade), until the shiny meristem dome and its two to three immediately subjacent leaf primordia become visible. If a true meristem culture is desired, obtain explant by removing only the tissue distal to the youngest leaf primordium. Sever the dome at its base by a transverse incision, using the very tip of the scalpel blade. Allow explant to come to rest on the tip of blade and transfer it immediately to nutrient tube. Slivers of brittle razor blades, inserted into small dowels, might be used if the excision requires scalpels with finer tips. Virus- and viroid-free plants are obtainable without resorting to true, meristem cultures. The explant must nevertheless be sufficiently small. Sever shoot apex transversely at the base of the second or third leaf primordium to obtain a structure 0.1 to 0.3 mm tall. Once again lift explant with the tip of the scalpel blade and transfer it to nutrient tube.

(5) For rapid, clonal propagation, trim the disinfested stem terminal superficially by removing one or more of the outer leaves and a small portion of the basal stem tissue. This should still leave a large explant. Transfer the entirety to a culture tube containing shoot multiplying medium.

(c) Incubating and handling of the smaller, shoot apex culture:

(1) Place cultures under Gro-Lux or cool-white, fluorescent lamps (1,000 lux), for 16 h daily at constant 27 C.

(2) Transfer rooted plants from the shoot apex explants, when about 1 cm, to 10,000-lux illumination and culture a further 2 wk.

(3) Remove from nutrient tube and transplant into soil. Providing high humidity and shading may be desirable during a short period immediately after transplanting.

(d) Incubating and handling larger explants:

(1) Incubate larger explants at 27 C under fluorescent lamps (1,000 lux) for 16 h daily.

(2) Separate individual shoots from the multitude of shoots that arise after about 4 wk.

(3) Subculture each shoot in freshly prepared nutrient tubes.

(4) Repeat subculturing at monthly intervals.

(5) Transfer individual shoots to a nutrient medium of composition similar to that employed

above in rooting the small shoot-apex explants after the desired number of shoots is attained. This enables regeneration of roots. Several shoots can be placed in each of fairly large culture vessels during the rooting step. Provide a higher light intensity (10,000 lux) in the rooting step.

(6) Transplant rooted shoots in soil and place in high humidity and shade during the first few days.

When the intent is to exclude pathogens, each of the plants produced *in vitro* should be tested appropriately, and the pathogens eliminated should be identified. Moreover, the plants obtained through *in vitro* cultures should be ascertained of reproduction of cultivar characteristics; any variability should be noted. Minimizing subculturing of highly variable plants is advisable, inasmuch as the proportion of variants has been observed to increase with each passage.

Leaf Culture

1. **Background.** Much of the work on leaf cultures has been confined to ferns. In 1953, Sussex and Steeves (*84*) described the first success in achieving development from leaf primordia of *Osmunda cinnamomea*. They observed that the ultimate size attained by isolated leaves was smaller than of leaves that developed while attached to the plant. Extensive studies with fern leaf cultures have shown further that explants of the young primordia can produce shoots as well as leaves, whereas those of the older primordia emerged only as leaves (*81*). Evidence of a leaf-determining substance has been obtained (*40*). Cultured fern leaves have also been shown to produce functional sporangia and spores (*18, 27*).

Steeves, Gabriel, and Steeves (*82*) have reported the only instance of angiosperm leaf culture. Their investigation was focused on *Helianthus* and *Nicotiana*. In contrast to the ferns, the excised leaf primordia of angiosperms developed only as leaves. The ultimate size attained by angiosperm leaves *in vitro* was also smaller than that *in vivo*.

2. **Requirements.** Not much is known regarding the requirements of isolated leaves. All reported cases have employed media of relatively simple compositions. Among the significant observations of the fern experiments have been the direct relationship between available sugar and ultimate leaf size (*16, 85*) and the maximum initiation of sporangia in constant darkness (*27*).

3. **Sample procedure.** Steeves, Gabriel and Steeves (*81*) described the following procedure for their *Helianthus* and *Nicotiana* leaf cultures:

(a) **Nutrient medium:**

(1) Dispense medium (table 6-2) in 25-ml aliquots in 25- × 150-mm culture tubes. Cap tubes with polypropylene closures, autoclave 15 min at 121 C and cool as 45° slants.

(b) **Preparing explants:**

(1) Remove outer leaves of apical bud to expose leaf primordia.

(2) Excise and lift out primordia 1 to 5, numbered from youngest to oldest, with fine-tipped scalpels (razor blade slivers may be desirable), and transfer to nutrient tubes. No prior disinfesting of apical bud usually is necessary.

(c) **Incubating conditions:**

Light and temperature requirements and other environmental conditions *in vitro* have not been specified. Perhaps those that are satisfactory for *Nicotiana* shoot tip cultures would be applicable.

Reproductive Organs

Embryo Culture

1. **History and applications.** As noted earlier, the first plant embryo culture was reported in 1904 by Hannig (*25*). Since then, the *in vitro* method has been used extensively by plant breeders to resolve certain problems encountered in rearing such hybrid seedlings as in *Linum* (*41*) and *Prunus persica* (*92*). The germination of orchid seeds, which is currently accomplished routinely *in vitro*, is, essentially, applying embryo culture methods (*38*).

Although many papers have been published under the heading of embryo culture (*56, 71*), only a small portion can be identified that deals with true embryo cultures. In most instances, fully differentiated but undersized structures had been placed in culture, and the development attained was simply that of aseptic germination. The technique of true embryo culture uses a small explant, and its aim is to achieve an autotrophically viable entity from one that is not. The globular embryo, comprised of about 50 cells, has been the smallest isolate that has been cultured successfully *in vitro*. The feat of isolating the zygote of a higher plant and accomplishing its differentiation into an embryo *in vitro* remains unattained.

2. **Requirements.** The germination *in vitro* of fully differentiated but undersized embryos is possible in simple nutrient media containing primarily salts and sugar. In contrast, the requirements *in vitro* of relatively undifferentiated isolates are more demanding. A complex natural supplement, such as coconut endosperm, may be necessary (*20, 93*). Sometimes this requirement can be satisfied by providing high concentrations of sugar, that is, 8 to 12 percent sucrose (*72*), or by supplying suitable osmotica (*48*). In still other instances the complex need has been fulfilled by providing suitable balances of exogenous hormones, especially auxin, cytokinin, and gibberellin (*66*). Small cereal embryos have been reared successfully in a chemi-

cally defined nutrient medium containing mineral salts, sugar, mixtures of vitamins and amino acids, and malic acid (*62*).

The usual practice in plant embryo culture has been to employ nutrient media solidified with small amounts of agar.

Embryo cultures are normally exposed to temperatures comparable to those of the cultivar's natural environment. Sometimes it may be necessary to satisfy seasonal temperature needs. Relatively mature embryos of many perennial species of the temperate climate, for example, must be given chilling treatments to ensure their development into normal plants (*36, 43, 44*). Embryo cultures are usually maintained under the same illumination conditions as those of shoot trip cultures.

3. **Sample procedure.** No published report on embryo cultures of *Nicotiana* has been located. Perhaps this is expected because no obvious advantage is seen in the use of embryo cultures in *Nicotiana* hybridization. *Nicotiana* embryos are furthermore very small and difficult to use in morphogenetic experimentation. The procedure outlined below is derived from Norstog's work with barley (*62*) and has not been tested specifically with *Nicotiana;* it may prove satisfactory, nevertheless.

(a) **Preparing medium:**

(1) Prepare nutrient medium (table 6-3).

(2) Sterilize ingredients except agar by filtration.

(3) Autoclave the agar solution 15 min at 121 C.

(4) Combine agar and filter-sterilized ingredients and pour into 15- × 100-mm Petri dishes at 20 ml/dish. Seal dishes with Parafilm to minimize evaporation.

(b) **Isolating and planting tobacco embryos:**

(1) Obtain immature tobacco seed pods and disinfest with 0.5 percent sodium hypochlorite (Clorox:water, 1:9).

(2) Transfer to a sterile Petri dish, make incisions longitudinally, and spread open carpels to expose ovules.

(3) Remove ovules individually and transfer to another sterile Petri dish.

(4) Excise embryos from the ovules with a quality dissecting microscope. Grasp ovule with a pair of fine-tip tweezers and make shallow longitudinal incisions through its integuments with a sharp scalpel.

(5) Pry open the ovule to reveal the embryo, which is visible as a tiny, glossy structure in the micropylar end of the embryo sac.

(6) Bisect embryo sac and, with the tip of the scalpel, lift out embryo and transfer it to nutrient medium.

TABLE 6-3.—*Nutrient medium for embryo culture*[1]

	Milligrams per liter
KH_2PO_4	910
KCl	750
$MgSO_4 \cdot 7H_2O$	740
$CaCl_2 \cdot 2H_2O$	740
$MnSO_4 \cdot H_2O$	3
H_3BO_3	.5
$ZnSO_4 \cdot 7H_2O$	.5
$CoCl_2 \cdot 6H_2O$	.025
$CuSO_4 \cdot 5H_2O$	.025
$NaMoO_4$	.025
Fe-citrate	10
meso-Inositol	50
Thiamine·HCl	.25
Ca-pantothenate	.25
Pyridoxine·HCl	.25
L-glutamine	400
L-alanine	50
L-cysteine	20
L-arginine	10
L-leucine	10
L-phenylalanine	10
L-tyrosine	10
Malic acid	1,000
Sucrose	34,200
Purified agar	6,000

Adjust pH to 4.9. Sterilize all ingredients except agar by filtration.

[1] Norstog (*62*).

(c) **Incubating embryos** *in vitro:*

Place cultures at 27 C under Gro-Lux lamps 1,000 lux for 16 h daily. Leaving the cultures in constant darkness the first few days may be helpful.

Ovary Culture

1. **Background.** LaRue was perhaps the first to attempt cultures of excised ovaries, or pistils. In 1942, he described results with 92 species of monocots and dicots (*42*). He noted that some explants rooted; others showed swelling of the ovary. The more systematic studies of ovary cultures were performed by Nitsch (*61*), who revealed that ovaries from unpollinated flowers required exogenous auxin for their enlargement into fruits. Following Nitsch's investigations, a flurry of activity with ovary cultures was apparent, especially among botanists in India. Unfortunately the activity lasted only 10 yr. Today, there is hardly a major, ovary culture investigation.

Successful cultures of excised ovaries have been limited to species that are characterized by morphologically simple and rapidly maturing fruits. In most instances, the fruits obtained *in vitro* have been substantially smaller than those that are left attached to the plant. Nevertheless, the enlargement pattern of the ovary has usually paralleled that *in vivo*. The fruits ripened without noticeable abnormalities, and those arising from pollinated flowers contained viable seeds although fewer than in fruits maturing *in vivo*.

The intent of most ovary cultures has been to study fruit morphogenesis. Unfortunately, the limited investigations *in vitro* have served simply to confirm findings disclosed by more traditional, experimental methods. An interesting application of ovary cultures might be in plant hybridization (*30*). Ovary cultures can ensure the attainment of seeds in instances where premature fruit abscission is a problem.

2. **Requirements of excised ovaries.** Better growth has been attained when the calyx is left attached to the explant. Hormonal supplementation of the nutrient medium is usually not necessary with ovaries excised a few days after the flower has been pollinated. In contrast, ovaries from unpollinated flowers must be provided some auxin and perhaps also cytokinin or gibberellin, or both (*17, 61, 74*). Most ovary cultures are allowed to develop at a temperature of 25 C and with some illumination.

3. **Previous work.** Nitsch (*61*) included *N. tabacum* in his initial investigation. He noted some growth among fertilized tobacco ovaries, but they produced no seeds. The pistils of *N. rustica* were subsequently cultured successfully by Rao (*69, 70*) using a nutrient medium containing mineral salts and sucrose. They were excised from unpollinated flowers and fertilization was accomplished by applying pollen *in vitro*. Dulieu (*21*) was able to accomplish successful test tube fertilization with *N. tabacum* by applying pollen *in vitro* to cultured ovaries.

4. **Sample procedure.** This method has been used by Rao and Rangaswamy (*70*) to culture *N. rustica* ovaries:

(a) **Preparing medium:**

Dispense the nutrient medium (table 6-4) at 25 ml/tube into 25- × 150-mm culture tubes. Cap with polypropylene closures, autoclave for 15 min at 121 C and cool as 45° slants.

(b) **Preparation of explants and implantation:**

(1) Obtain flowers or flower buds, trim their pedicels, and remove stamens and petals.

(2) After dipping pistils in 70 percent ethanol, immerse in 0.5 percent sodium hypochlorite (Clorox:water, 1:9) for 10 min and rinse with sterile water.

(3) Transfer pistils to nutrient agar.

(4) If the pistils must be pollinated *in vitro*, avoid wetting the stigma during the disinfestation process. Obtain pollen from anthers that had been excised a day before anthesis and allowed to dehisce in a sterile vial. Collect pollen on a sterilized micro-spatula and transfer onto pistil.

(c) **The culture environment:**

Incubate at 27 C temperature under Gro-Lux lamps (1,000 lux) for 16 h daily. Successful cultures should produce enlarged fruits that contain viable seeds. The seeds subsequently may germinate within the ovary.

TABLE 6-4.—*Nutrient medium for ovary culture*[1]

	Milligrams per liter
$Ca(NO_3)_2 \cdot 4H_2O$	500
KNO_3	125
$MgSO_4 \cdot H_2O$	125
KH_2PO_4	125
$MnSO_4 \cdot 4H_2O$	3
$ZnSO_4 \cdot 7H_2O$	.5
H_3BO_3	.5
$CuSO_4 \cdot 5H_2O$	.025
Na_2MoO_4	.025
$CoCl_2$	.025
Fe-citrate	10
Glycine	7.5
Nicotinic acid	1.25
Thiamine·HCl	.25
Ca-pantothenate	.025
Pyridoxine·HCl	.025
Casein hydrolysate	500
Sucrose	40,000
Phytagar	8,000
Adjust pH to 5.8 before adding agar.	

[1]Rao and Rangaswamy (*70*).

Ovule Culture

1. **Background.** Ovule cultures are often used as an alternative to embryo cultures when the excision must be performed before the embryo has attained sufficient size to enable successful isolation and culture. The ovules are isolated a few days after the flower had been pollinated but before the zygote had divided, such as in orchids (*98*) and *Iberis* (*47*). The practice of pollinating ovules *in vitro*, or test-tube pollination (*34*) has useful applications. Viable seeds have been obtained by applying the method to such normally self-incompatible cases as *Petunia* (*57, 68*) and *Brassica* (*33*). Ovule cultures have also been used to study fiber differentiation in cotton (*7, 8*).

2. **Characteristics of ovule development** *in vitro*. The successful culture of an ovule is characterized by the formation of a viable seed. Polyembryony may be encountered, as a result of irregular proliferations of the zygote or zygotic embryo, or through embryo initiation in certain ovular cells as nucellus (*67*).

3. **Requirements and general culture procedure.** Developing viable seeds from fertilized ovules of *Zephyranthus* required supplementing with complexes such as coconut milk or hydrolyzed casein (*35*). The amino acids histidine, arginine, and leucine were able to replace the casein hydrolysate. Viable seed produced from fertilized ovules attached to placental tissue of *Petunia hybrida* was enhanced by the presence of high NH_4^+ and BO_3^{-3} in the medium (*58, 59*). The efficiency of BO_3^{-3} was dependent upon the form of iron and the kind of sugar provided (*59*). Iron as Fe EDTA enabled the development of *Petunia* ovules in the absence of placental tissue (*60*). The pH requirement of *Petunia* ovules *in vitro* furthermore depended on the iron source (*59*).

TABLE 6-5.—*Nutrient medium for ovule culture*[1]

	Milligrams per liter
$Ca(NO_3)_2 \cdot 4H_2O$	500
KH_2PO_4	125
KNO_3	125
$MgSO_4 \cdot H_2O$	125
$MnSO_4 \cdot 4H_2O$	3
H_3BO_3	.5
$ZnSO_4 \cdot 7H_2O$	.5
Na_2MoO_4	.025
$CuSO_4 \cdot 5H_2O$	.025
Fe-citrate	10
Glycine	7.5
Nicotinic acid	1.25
Ca-panthothenate	.25
Pyridoxine·HCl	.25
Thiamine·HCl	.25
Casein hydrolysate (optional)	500
Sucrose	50,000
Phytagar	7,000
Adjust pH to 5.7 before adding agar.	

[1]Kanta and Maheshwari (*34*).

The precise requirements of nutrients, light, and temperature have not been established for *Nicotiana* ovule cultures. In most cases, the medium used was that developed by Nitsch (*61*) and supplemented with such natural complexes as coconut endosperm or casein hydrolysate (*34, 68*). Light and temperature conditions have been similar to those provided for other types of tissue cultures.

In performing test-tube pollination, the pollen may be applied directly to the isolated ovules, or placed on the placenta or ovules, or both, of explants comprised of ovules attached to placental sections. The pollen may be dusted onto the explant or spread over it as a suspension in a solution of boric acid.

4. **Sample procedures.** Excising and culturing ovules from pollinated flowers is extremely simple; thus it will not be treated specifically. Instead, two methods of performing test-tube pollination or fertilization are suggested below. The nutrient medium and incubation conditions will apply equally to ovules from pollinated flowers.

One method was used by Kanta and Maheshwari (*34*) to accomplish test-tube fertilization with *N. rustica* and *N. tabacum* and by Siddiqui (*77*) with *N. tabacum*:

(a) **Preparing nutrient medium:**

Dispense nutrient medium (table 6-5) at a rate of 25 ml/tube in 25- × 150-mm culture tubes. Cap with polypropylene closures, autoclave 15 min at 121 C and cool as 45° slants.

(b) **Preparing, excising, and planting explants:**

(1) Emasculate flower buds before anthesis.

(2) Isolate ovaries at anthesis from the flowers and disinfest with 0.5 percent sodium hypochlorite (Clorox:water, 1:9).

(3) Dissect ovary and scoop out ovules with a sterile scalpel.

(4) Transfer ovules to nutrient agar slants.

(5) Excise flower buds 1 to 2 h before anthesis and disinfest in dilute sodium hypochlorite solution.

(6) Remove anthers, scoop out their pollen, and spread them onto and around the ovules, which have just been placed on nutrient agar.

(c) **Incubating cultures:**

Incubate cultures at 27 C and in either darkness or under Gro-Lux or cool-white fluorescent lamps (1,000 lux) for 16 h daily.

A second method was developed for *N. tabacum* by Balatkova and Tupy (*2, 3*) and modified slightly by Zdruikovskaya-Rikhter and Babasyuk (*102*). Sections of the placenta with adhering ovules are used instead of isolated ovules.

(a) **Preparing nutrient medium:**

Prepare a nutrient medium similar in composition to that employed in the first method.

(b) **Preparing, excising, and planting explants:**

(1) Collect emasculated flowers one day after anthesis.

(2) Disinfest pistils in 0.5 percent sodium hypochlorite (Clorox:water, 1:9).

(3) Remove the external ovary walls by making several longitudinal incisions but without injuring the ovules or placenta.

(4) Bisect the placenta longitudinally and transfer each half, together with the adhering ovules, to the nutrient agar, with the cut surface in contact with the agar.

(5) Collect pollen aseptically as in the previous method and dust pollen onto a portion of the placenta.

(c) **Incubating cultures:**

Incubate the pollinated material at 27 C and in darkness or under Gro-Lux lamps (1,000 lux) for 16 h daily.

Flower Bud Culture

1. **Background.** In dealing with flowers *in vitro*, distinction must be made of their development from (a) excised flower buds, (b) flowering induced *in vitro* in excised shoot apices and (c) initiation of flowers in cultured sections of stem, leaf, root, and other materials. As noted previously, one of the experimental applications of shoot apex cultures has been in the induction of flowering by photoperiod treatments *in vitro*. Sections of various organs and tissues of some species, excised when plants are in flowering condition, have been observed to generate flowers directly from explants or from their callus (*1, 46, 90, 95*). Our discussion will be confined to cultures of explants that are already in the primordial or further stage of flower development.

Flower bud cultures are particularly useful in examining the conditions that enable differentiation of such specific floral parts as stamens, pistils, petals, and so forth. They are also useful in exploring sex expression and other reproductive phenomena.

2. **Characteristics of cultured flower buds.** Developing reproductively functional flowers has been achieved in only a few instances, for example, melon (*64*) and *N. tabacum* (*28*). Usually, incomplete or nonfunctional flowers have resulted: *Aquilegia* (*10, 11, 12, 86, 87*), chrysanthemum (*99*), and *Kalanchoe* (*50*). Perhaps noteworthy, flowers resultant from buds that are differentiated *in vitro* develop into functional structures, producing a high frequency of viable seeds: *Browallia* (*23*), *Chicorium* (*46*), and *Nicotiana* (*1*).

3. **Requirements.** The precise, nutritional needs of excised flower buds remain unestablished. The beneficial effects of exogenously supplied hormonal substances have been confirmed (*10, 11, 12, 29*). The importance of association with developing leaves has been observed consistently. Blake (*13*) noted that *Visceria* flower buds developed into mature flowers *in vitro* only when the explant had one pair of leaf primordia attached to the young explant. Without the leaf structures, the explants completely developed only if they had already initiated sepal primordia (*13*). Hicks and Sussex (*29*) found that kinetin could replace the leaves in *Nicotiana* flower primordium cultures.

4. **Sample procedure.** Following is the method Hicks and Sussex (*28*) used with *N. tabacum* Havana 38 (Wis 38).

(a) **Preparing nutrient medium:**

(1) Prepare a nutrient medium containing Murashige and Skoog's mineral salts (ch. 7) and, in milligrams per liter: thiamine•HCl, 0.4; *myo*-inositol, 100; sucrose, 40,000; and kinetin, 0.1-1.0.

(2) Adjust pH of medium to 5.5 before autoclaving.

(3) Dispense media in 25- × 150-mm culture tubes at 15 ml/tube or in 125-ml Erlenmeyer flasks in 45 ml aliquots. Cap or plug the culture vessels and autoclave at 121 C or 15 min.

(b) **Excising and planting explants:**

(1) Detach immature inflorescences from the tobacco plants.

(2) Hold small portions of each inflorescence under a dissecting microscope in a sterile transfer hood and expose individual flower primordia by removing the floral bract that subtends each primordia. Disinfesting the plant material is not usually necessary.

(3) Using a fine surgeon's scalpel (No. 11 blade), excise the individual primordia and transfer to nutrient medium, one per culture vessel. Allow the primordia to float on the surface of the nutrient solution.

(c) **Incubating cultures:**

Incubate cultures at 27 C under Gro-Lux lamps (1,000 to 4,000 lux) for 16 h daily.

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7

TISSUE AND CELL SUSPENSION CULTURE

J. P. Helgeson[1]

Introduction

In 1939, White (*36*) reported that tissues from the hybrid *Nicotiana glauca* × *N. langsdorfii* could be cultured indefinitely on a nutrient medium. His experiments, together with those by Nobecourt and by Gautheret who used carrot, were the first real successes at growing masses of undifferentiated tissues *in vitro*. Thus, from the beginning, *Nicotiana* spp. have played a central role in the development of plant tissue culture methods. Early accounts of tissue culture work have been presented by White (*37*) and Gautheret (*6*).

Nicotiana spp., particularly *N. tabacum*, are controllable in tissue culture. Many procedures, for example, growth of haploid plants from pollen grains (ch. 5), have either been developed with or were initially successful with *N. tabacum*. Thus, as a model for development of techniques with other plant species and as experimental systems for studies of basic phenomena in plants, culture of *Nicotiana* spp. can be useful. Today, experimentation with *Nicotiana* tissue cultures has many facets ranging from basic studies on the synthesis of macro-molecules to differentiation of plants with new agronomic capabilities. The experimenter should be aware, however, that there are limitations both to the extension of methods to other systems and to correlation of results with events in intact plants. Readers who desire more information are directed to the laboratory manual by Gamborg and Wetter (*5*) and to the extensive treatises on tissue culture such as the one by Street (*33*).

Potentials and Limitations of Tissue and Cell Suspension Cultures

Growth Substance Assays

Jablonski and Skoog (*16*) noted that *N. tabacum* pith tissue would grow if placed in contact with or closely adjacent to pieces of cambial tissue. This observation led to a cell division assay for cytokinins, which was useful for the isolation of kinetin (*20*) and subsequent isolations of cytokinins from natural sources (*29* and references therein). Murashige and Skoog (*22*) developed a tissue culture medium (MS medium) that, because of its high salt content, screens out promoting effects of salts in natural extracts.

The quantitative nature of the response to exogenous cytokinin has permitted extensive studies on the structure/activity relationships of cytokinins and the development of inhibitory cytokinin analogs (*7, 31*). Although the total yields of tissue are dependent on the carbohydrate supply

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(*35*), the logarithmic growth rate of *N. tabacum* tissues are programmed by the cytokinin concentration in the medium (*8, 13*). Tissue growth is stimulated by gibberellic acid (*14*) and thus natural gibberellins in extracts may affect bioassay results. The tissues are markedly inhibited by toxic materials in crude extracts and by a fungal toxin (*11*). Skoog and Armstrong (*30*) have recently published their bioassay procedures as a laboratory exercise.

Plant Metabolism

The availability of actively growing tissues on defined media has permitted extensive studies on plant metabolism. Both suspension cultures and cultures grown on solid media have been used. For example, enzymatic reactions in sugar metabolism have been examined by Scott and others (*27*) and Thorpe and Laishley (*34*). Berlin and Widholm (*1*) have studied altered phenolic metabolism of *N. tabacum* suspension cultures that are resistant to *p*-fluorophenylalanine.

In 1965, using suspension cultures of *N. tabacum*, Filner (*3*) demonstrated that deoxyribonucleic acid synthesis in cells was semiconservative. More recently, Kemp and his coworkers have done extensive studies on protein synthesis (*17*) and nucleic acid synthesis (*18*) in agar-grown cultures of *N. tabacum*.

Experimental Manipulation of Growth Form

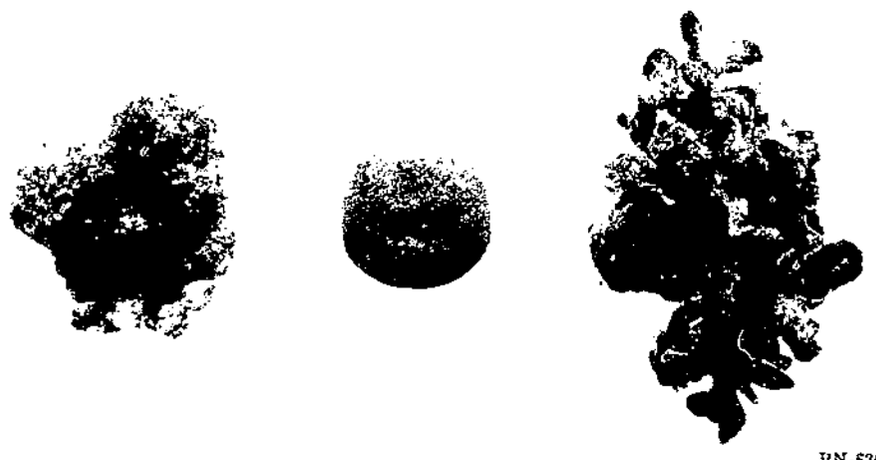
Skoog and Miller (*32*) showed that the balance of auxin and cytokinin controls differentiation of *N. tabacum*. The facile manipulation of growth form (fig. 7-1) has since been used in several ways. With high levels of cytokinin relative to auxin, buds can be obtained which, if detached and rooted, can yield whole new plants. With intermediate levels of auxin and cytokinin, for example, 11.5 μM indoleacetic acid (IAA) and 1 μM kinetin in MS medium, *N. tabacum* tissues will grow as tight hemispherical masses ideally suited for radioactivity labeling and other manipulations (*17*). Finally, if the auxin level is high relative to cytokinin, friable masses of cells ideally suited for growth substance bioassays are obtained (*8, 13, 25*).

The levels of growth substances required by various species may differ and modifications of phytohormone addenda may be required to achieve the desired results. For example, good yields of buds have been obtained with *N. acuminata, goodspeedii, megalosiphon, plumbaginifolia, suaveolens,* and *tabacum* using MS medium supplemented with 1 m*M* phosphate, 1 μ*M* IAA and 10 μ*M* 6-(3-methyl-2-butenylamino) purine (2iP). In contrast, no buds were obtained with *N. benavidesii, gosei, knightiana,* and *paniculata* on this medium (Helgeson, unpublished).

Pathological Studies

Plant tissue culture media also support growth of most bacteria and fungi. Thus, introducing a given pathogen and knowing that *only* that organism is present in addition to the *Nicotiana* spp. is relatively easy. One such model system employs *N. tabacum* tissues containing a gene for black shank resistance that was extracted from *N. plumbaginifolia* (*10, 12*) (fig. 7-2). Responses of the tissues can be studied under controlled conditions (both physical and chemical) where cell types are relatively uniform, and where wounding of tissues to bypass particular barriers is not required.

Another pathological system that uses *Nicotiana* tissue cultures is crown gall. Galls are induced on tobacco stems by inoculation with the bacterium *Agrobacterium tumefaciens*. When pieces of these galls are removed from the plant, plated on culture medium, and freed of bacteria, they no longer require auxin or cytokinin for growth. Today, there is considerable interest in the study of this system to determine the mechanism by which the bacteria can transform normal cells into tumor cells (ch. 1) (*2*).



PN-6387

Figure 7-1.—Morphological forms of *N. tabacum* cv. Havana 38 (Wis 38) tissue cultures grown on Linsmaier and Skogg's medium containing (left to right) 11.5 μ*M* IAA and 0.1 μ*M* kinetin; 11.5 μ*M* IAA and 1 μ*M* kinetin; and 5.75 μ*M* IAA and 5 μ*M* N^6-(3-methyl-2-butenylamino) purine.

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Figure 7-2.—The appearance of *N. tabacum* tissue cultures from a resistant plant (left) and a susceptible plant (right) 7 d after inoculation with zoospores of race O of *Phytopthora parasitica* var. *nicotianae*.

Tissue Cultures and Crop Improvement

Tissue and cell suspension culture techniques are important for obtaining new plants from single cells. Nitsch and Nitsch (*24*) used passage through tissue culture to obtain haploid plants from pollen grains (ch. 5). Nagata and Takebe (*23*) relied on tissue culture media to keep their protoplasts alive. Recently, Shepherd and Totten (*28*) obtained successful results with the isolation, growth, and differentiation of protoplasts from *N. tabacum* (ch. 8).

Limitations

Although tissue and cell suspension cultures have proven useful, the techniques have limitations (for example, species and cultivar differences, changes in culture, and differences in morphology or biochemical processes between intact plants and tissue cultures).

Basically, tissue cultures are not whole plants but, when actively growing, resemble only the meristematic areas of the plant. Cell types may be considerably decreased in the culture and some metabolic processes normally occurring in different parts of the plant may not be operating in tissue cultures. For example, thiamine is required for tissue culture growth although intact plants are self-sufficient. For this reason it is useful, if possible, to compare intact plants and tissue cultures. For example, direct comparisons of rooted shoots and callus tissues showed that the gene for resistance to black shank is expressed in both cultures and intact plants (*10*).

Another major problem with tissue cultures is species variation in response to media addenda. Thus, although general techniques may be described, the precise details for success with a given species or cultivar may require considerable experimentation. Conditions that give good budding of *N. tabacum*, for example, are completely unsuccessful with soybean or corn tissues. When fourteen *Nicotiana* spp. were cultivated using 11.5 μM IAA and 1 μM kinetin (the standard conditions for compact callus with *N. tabacum*), I observed results ranging from budding of tissues through tight, compact and loose, friable growth and death (Helgeson, unpublished).

A major problem with tissue cultures may be genetic instability after extended periods in culture. Thus, one has no assurance that the tissue in culture has more than a superficial resemblance to the original explant. Murashige and Nakano (*21*) examined this problem using *N. tabacum* and found that tissue ploidy became highly variable (ch. 2). In other cases, tissues may lose their requirements for growth substances and become "habituated." Thus, if possible, the original plant that yielded the original culture should be maintained clonally to permit reisolation. Periodic checks of the cultures should be made to ascertain that they have not changed substantially.

Cultivation on Solid Media

Media

The MS medium developed by Murashige and Skoog (*22*) can serve as a basis for studies of cells growing on solid media. It was developed to optimize growth of *N. tabacum* tissues. Linsmaier and Skoog (*19*) modified the medium slightly, omitting some organic constituents. In my laboratory this latter medium supports good growth of 10 *Nictoiana* spp. (*N. acuminata, cordifolia, goodspeedii, gossei, knightiana, megalosiphon, plumbaginifolia, rustica, suaveolens* and *tabacum*). A few species (*N. benavidesii, paniculata, raimondii,* and *solanifolia*) appear to grow less well but can be maintained. Whether these tissues will require a different basic medium, additional organic supplements, or different phytohormone balances is not yet known. A useful discussion of the above and other commonly used media has been published recently by Gamborg and others (*4*).

Medium Preparation

The ingredients of MS medium are listed in table 7-1. In general, use the highest quality of salts and other constituents and glass-distilled water in preparing media. Bacto-agar is satisfactory; some ingredients in other agar preparations can be toxic to some tissues. The cost of commercially prepared media mixes is often considerably higher than if the medium is prepared from the individual ingredients (appendix). Keep stock solutions of mineral salts in Nalgene bottles in a refrigerator. Alternatively, divide the stocks into small quantities and store the units in a freezer. Add dry organic ingredients such as sucrose and vitamins during medium preparation. Cytokinin solutions are conveniently standardized by UV absorption maxima. Auxins such as 2,4-D or naphthaleneacetic acid are often prepared in concentrated stock solutions. However, as IAA is rather unstable, add it by dissolving in ethanol (about 2

TABLE 7-1.—*Murashige and Skogg's medium*[1]

STOCK SOLUTIONS FOR MINERAL SALTS

Stock	*Salt*	*Grams per liter*
A[2]	NH_4NO_3	82.5
B[2]	KNO_3	95
C[3]	$CaCl_2 \cdot 2H_2O$	88
D[3]	KH_2PO_4	34
E[3]	H_3BO_3	1.24
	$Na_2MoO_4 \cdot 2H_2O$	.05
	$CoCl_2 \cdot 6H_2O$	.005
	KI	.166
F[3]	$MnSO_4 \cdot H_2O$	3.38
	$MgSO_4 \cdot 7H_2O$	74
	$CuSO_4 \cdot 5H_2O$	.005
	$ZnSO_4 \cdot 7H_2O$	1.725
G[3]	$FeSO_4 \cdot 7H_2O$	1.39
	Na_2EDTA	1.865

FINAL MINERAL SALT CONCENTRATION OF MEDIUM

Salt	*Milligrams per liter*	*μM*	*Salt*	*Milligrams per liter*	*μM*
NH_4NO_3	1650	N 41.2	H_3BO_3	6.2	100
KNO_3	1900	18.8	$MnSO_4 \cdot H_2O$	16.9	100
$CaCl_2 \cdot 2H_2O$	440	3.0	$ZnSO_4 \cdot 7H_2O$	8.63	30
$MgSO_4 \cdot 7H_2O$	370	1.5	KI	.83	5
KH_2PO_4	170	1.25	$Na_2MoO_4 \cdot 2H_2O$	.25	1
Na_2EDTA	37.3 Na	.2	$CuSO_4 \cdot 5H_2O$	.025	.1
$FeSO_4 \cdot 7H_2O$	27.8	.1	$CoCl_2 \cdot 6H_2O$	.025	.1

ORGANICS COMMONLY ADDED TO MEDIUM

Sucrose	grams per liter	30
Glycine	milligrams per liter	2
Indoleacetic acid	do	1-30
Kinetin	do	0.04-10
myo-Inositol	do	100
Nicotinic acid	do	.5
Pyridoxine·HCl	do	.5
Thiamine·HCl	do	.1
Agar	grams per liter	10

[1]Murashige and Skoog (*22*).
[2]Use 20 ml/L medium.
[3]Use 5 ml/L medium.

mg/L) and then adding the ethanol solution to the diluted medium.

Following is a step-by-step procedure for preparing 1 liter of MS medium containing 11.5 μ*M* IAA and 1 μ*M* kinetin: Place approximately 500 ml of glass-distilled water in a 1-L Erlenmeyer flask containing a magnetic stirring bar. Add the following dry ingredients to the stirring solution: 30 g sucrose, 100 mg *myo*-inositol, 0.1 mg thiamine:HCl, 0.5 mg pyridoxine:HCl, 2 mg glycine, 0.5 mg nicotinic acid. Add 20 ml of stock solutions A, B, and G and 5 ml each of stock solutions C, D, E, and F (table 7-1). Dissolve, with slight warming, 2 mg of IAA in 1 ml or less of absolute ethanol and transfer this solution to the medium with several rinses from a squirt bottle containing glass-distilled water. Add 20 ml of a 50 μ*M* aqueous solution of kinetin (OD of 0.95 at 267 nm). Make the solution to a volume of about 900 ml with glass-distilled water and, with constant stirring, adjust to pH 5.8 ± 0.2 with 1 *N* NaOH or KOH.

Remove the stirring bar and pour the contents of the flask into a 1-L graduate cylinder and adjust the volume to 1 L. Pour the contents of the graduate into a 2-L Erlenmeyer flask, add 10 g of Bacto-agar and steam the solution in an autoclave at 100 C for 25 min (be sure to use a flask about twice the volume of the solution to avoid loss of medium to boiling over during steaming). After steaming, allow the solution to cool to about 90 C, and then swirl the solution vigorously to insure complete mixing of the melted agar in the solution. (**Caution!** Do not swirl solution immediately after removal from autoclave.) The medium is now ready for distribution in appropriate quantities to individual containers.

If heat-labile addenda are used, make the medium slightly more concentrated than above. Then dilute it with cold-sterilized addenda in water to proper volume just before the agar solidifies after autoclaving. For some applications, for example, the addition of small quantities of cytokinins, the dimethylsulfoxide (DMSO) method described by Schmitz and Skoog (*26*) may be used. Use particular care not to spill any DMSO on your skin as it facilitates rapid penetration of the dissolved material.

Generally, including antibiotics in media is not necessary. However, if required (for example, to kill bacteria when isolating crown gall tissues) include (cold-sterilized) 50 mg/L of neomycin and streptomycin sulfate, 80 mg/L of penicillin G, and about 50,000 units of polymixin B.

Containers for Growing Tissues

Many early experiments were done with 125-ml Erlenmeyer flasks or with prescription bottles. The flasks can be stoppered with foam or cotton stoppers. When tissues are grown in light, or when ambient air is not filtered, flasks or capped prescription bottles are preferable. However, when tissues are grown in incubators, Petri dishes (100 by 20 mm) are more convenient. Culture dishes (100 by 80 mm) are particularly convenient for growing budding tissues or for rooting shoots derived from tissue cultures.

Use the following procedure for adding media to Petri dishes: Swirl the melted medium vigorously and pour it into a dispensing apparatus with a 50-ml head (Kontes K-67550). Place the Petri dish bottoms in a wire tray and dispense the medium in 50-ml portions in the dishes. Place the covers on the Petri dishes and sterilize the medium and container for 15 min at 121 C. Remove the dishes as soon as the autoclave has returned to 100 C. Let the medium cool at room temperature until the agar solidifies. Use the medium immediately after solidification. Otherwise, store it so that no direct light hits the medium. Do not use media over 2 d old and avoid remelting media.

Isolation of Tissues

Stem tissues are particularly useful for obtaining tissue explants. Certain species have thick stems with a well-delineated pith region. Others have much thinner stems and isolating pith tissue is inconvenient. The methods described below yield viable callus lines from a number of *Nicotiana* spp.

1. **Isolation and culture of pith tissues.** Isolate callus tissues from such plants as *N. tabacum* by the following procedure: Remove stems of nonflowering plants about 1-m tall (make cuts 10 cm or so above the soil surface so the plant will regenerate by sprouting of lower buds. In this way, tissue cultures and intact plants that are clonally related can be obtained.) Cut the middle one-third of the stem into 3- to 5-cm segments. Immerse the segments in 70 percent ethanol for 30 sec and then in 1 percent sodium hypochlorite (Clorox:water, 1:4) for 10 to 15 min. Rinse the segments with sterile, distilled water. Remove the pith with a cork borer (No. 1 or No. 2 size). Use a sterile, glass rod, which fits inside the barrel of the cork borer, to extrude the pith segment into a sterile Petri dish. Remove the end portions (about 5 mm) with a sterile scalpel and cut the remaining pith into cylinders about 3-mm long. Place these pieces on MS medium containing 11.5 μM IAA and 1 μM kinetin.

2. **Isolation of callus from small stems.** If a large stem with ample pith is not available, an alternative procedure using young stem or petiole sections is often successful. Use the procedures described above for sterilizing stem segments. Exercise particular care in the sterilizing procedure so that the tissues are exposed to hypochlorite for the proper time. Too long an exposure can kill the tissues whereas too short a treatment may not kill microbial contaminants. After sterilization, thoroughly rinse the tissue with sterile, distilled water. Transfer the segments to sterile, dry Petri dishes for dissection. Cut the segments into 3- to 4-mm segments. Split the segments down the middle and place the cut surface of the half-cylinder on the medium. Again, MS medium containing 11.5 μM IAA and 1 μM kinetin appears satisfactory for many *Nicotiana* spp.

All operations after the first immersion in ethanol are best done under a transfer hood or in a transfer room. Exercise particular care to sterilize all instruments (dip in 95 percent ethanol and flame) and to allow sufficient time for the instrument to cool before dissection. An ample supply of sterile, dry Petri dishes (preferably glass) should be available to provide cutting surfaces.

3. **Growth conditions.** Grow callus tissues in the dark in biological incubators at 26 to 28 C. If the incubator is reasonably full of Petri dishes, an RH of about 70 percent is obtained by water evaporation from the plates. However, if only a few dishes are present, place a pan of water on one of the shelves to prevent excessive drying of the medium. At times, high airflow rates (such as those in most plant growth chambers equipped with lights) can cause excessive contamination of the cultures. If this occurs, place an air filter over the fan intake.

Cultivation of Tissues After Initial Isolation

1. **Subculture from the initial callus.** Within 3 to 5 wk after initial isolation of plant tissues, considerable growth of pith explants usually occurs. In some cases, the new tissues closely resemble callus lines that have been through a number of subcultures. Alternatively, the growth may be loose and friable. The latter growth type often occurs when intact, small stem segments were used as the tissue source. Generally, 20- to 50-mg (fresh weight) pieces of the newly formed callus tissues can be dissected from the old tissue, put on identical media, and grown into uniform callus lines. In some cases, growth declines after the initial isolation and finally loss of the callus line occurs. In this case additional supplements may be required in the medium (*4*).

2. **Maintaining the cell line.** Once established, transfer the callus tissues frequently. As a selection of cell types will take place upon subsequent transfers, transfer a new line several times before doing critical experiments (see discussion in *4*). The transfer intervals required will depend on the growth rate of the tissue and the size of the explant used for subculture (see below for growth measurements). For example, loose friable tissues of *N. tabacum* cv. Havana 38 (Wis 38) grows logarithmically until about half of the sugar in the medium is depleted (*15, 35*). Transferring the tissues before their leaving log phase will permit a continuous supply of healthy, logarithmically growing cells for bioassays and metabolic studies.

Measurement of Callus Growth

Some quantitative measurement of callus growth is particularly useful for insuring uniformity in sampling of tissues. Techniques for dry weight measurement and cell mitotic indices are given by Gamborg and Wetter (*5*) (see also ch. 2). The procedure given below has been used for measuring growth kinetics (*13*). For this procedure, aluminum moisture dishes and a toploading balance are convenient.

A typical growth curve for *N. tabacum* cv. Havana 38 (Wis 38) is shown in figure 7-3. For a curve such as this, use approximately 40 Petri dishes containing 6 pieces of tissue per dish. An experienced person can cut pieces freehand so that a standard error of 3 to 5 percent is obtained per 24 pieces of tissue on the initial planting. Transfer all tissues to the test medium and then harvest the contents of 4 (24 pieces) for each weight point. Label moisture dishes on the bottom with the experiment number and harvest date and then dry in an oven (65 C) until the empty dishes reach constant weight. Record this tare weight. Add pieces (carefully removed from the media without includ-

ing any agar) one by one and record weights to give a running cumulative total. Data reductions are then made conveniently with a computer program (table 7-2). This procedure will provide recording of individual weights of pieces, the average weight for the point, the standard deviation for the point, and the standard error for the point. Repeat the weighings at intervals appropriate for the period of growth to be examined.

Cultivation of Suspension Cultures

The cultivation of cells on agar media is useful for a number of experimental purposes and for maintaining cell lines. At times, however, cultivations of single cells or small clumps of cells in suspension culture is more desirable. This technique simplifies the study of materials that are elaborated into the medium and assures good contact of individual cells to various additions to the culture media.

Media

The MS medium is used as the initial starting medium. Prepare the medium, adjust the pH and sterilize it without the addition of agar. Useful phytohormone addenda are 1 μM 2,4-D and 0.1 μM kinetin. Species may differ in their requirements for various salts and sugars and also for such organic addenda as vitamins and amino acids. Therefore, after obtaining a callus culture, modify the medium so it will support adequate growth.

A critical aspect of suspension culture is adequate stirring to provide oxygen. Suspension cultures are fragile and mechanical stirring may be too harsh for some lines. A rotary shaker (60 to 120 rpm) located in a 26- to 28-C controlled room works well. Cultures can be grown conveniently in Erlenmeyer flasks or, if large quantities are desired, in Fernbach flasks. As long as the quantity of the medium is kept to about one-fifth of the total volume of the flask, reasonable aeration is maintained on the shaker. Reciprocal shakers are less satisfactory and may cause considerable splashing of the medium on the cotton or foam rubber plugs used to stopper the flasks.

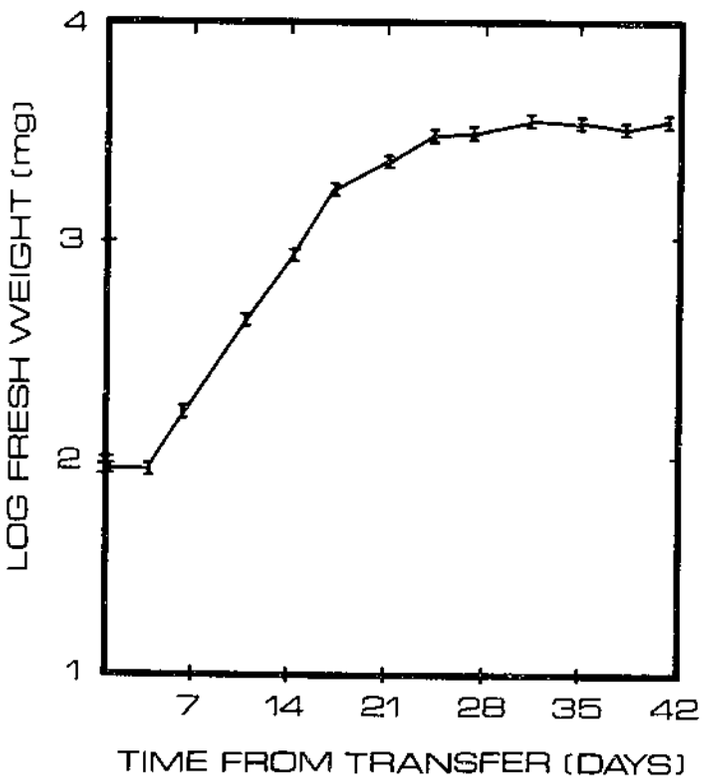

Figure 7-3.—Computer plot of tobacco tissue culture growth curve. Each weight point is the logarithm (base 10) of the average fresh weight. The vertical bars designate the limits for one standard error of the mean.

Preparing the Initial Culture

Tissue cultures grown in a loose, friable form usually form good suspension cultures quickly. With *N. tabacum*, this tissue form can be obtained by subculturing the stock tissue on MS medium containing 11.5 μM IAA and 0.1 μM kinetin. The following is a step-by-step procedure. Working under a sterile transfer hood, remove the plug from a 500-ml Erlenmeyer flask containing 100 ml of MS medium. Flame sterilize the lip of the flask and, with sterile forceps, drop 2 to 3 g of loose friable tissue into the medium. Take care that the tissue does not come in contact with the upper part of the flask. Again, flame the lip of the flask, replace the plug, and immediately put the flask on a rotary shaker. The shaking action will fragment the pieces adequately so that cutting of the tissues is unnecessary. After 2 to 3 wk, a suspension of many small clumps of cells and a few individual cells is usually obtained.

At this point, filter the cells through a nylon net or some other nontoxic mesh to remove larger cell clumps. A convenient procedure is to fold a circle of nylon net of the desired pore size into a cone as one would fold filter paper. With a paper clip, hold the cone in its folded form, place it in a beaker, and then autoclave it. The heat will permanently mold the net into a cone shape that will fit conveniently into a sterile funnel for transfer of cells to new flasks. It can be autoclaved repeatedly in a beaker.

After initiation of the culture, subculturing the line several times is often necessary until a uniform cell suspension is obtained. Also providing a minimum quantity of old "conditioned" medium may be necessary to successfully maintain the culture. As a rule, early in the development of the cell line, avoid diluting the cell concentrations to less than one-fourth the original value.

Maintaining a Primary Cell Line

A well-established culture of *N. tabacum* cells will double its cell weight every 40 to 60 h (*9*). For uniform main-

TABLE 7-2.—*Computer program in* BASIC *for weight calculations*

```
10 DIM X(120)
20 DIM A(120)
30 PRINT HEX(03)
40 PRINT "STD ERR"
50 PRINT "EXPT";
60 FOR I=1 TO 120
70 X(I)=0
80 NEXT I
90 INPUT A$
100 PRINT "ENTER TARE";
110 INPUT Z3
120 S,T,N=0
130 PRINT "ENTER -1 TO END, -2 TO DELETE LAST DATUM"
140 PRINT "ENTER DATA"
150 PRINT "RAW DATA","WT"
160 INPUT X
170 IF X=-1 THEN 330
180 IF X=-2 THEN 280
190 N=N+1
200 A(N)=X
210 X=X-Z3
220 S=S+X
230 X(N)=X
240 PRINT TAB(10),HEX(0C),X(N), "OBS#=";N
250 T=T+X↑2
260 Z3=Z3+X
270 GOTO 160
280 S=S-X(N)
290 T=T-(X(N))↑2
300 Z3=Z3-X(N)
310 N=N-1
320 GOTO 160
330 PRINT "INSERT DATA SHEET IN TYPEWRITER"
340 PRINT "PUSH CONTINUE"
350 STOP
360 SELECT PRINT 211
370 PRINT "  EXP:  ";A$
380 FOR I=1 TO N STEP 6
390 PRINT "RAW DATA";
400 B=0
410 N1=I+5
420 IF N1<>=N THEN 440
430 N1=N
440 FOR J=I TO N1
450 B=B+1
460 PRINT TAB(12+7*B);A(J);
470 NEXT J
480 PRINT
490 PRINT "INDIVIDUAL WEIGHTS";
500 B=0
510 FOR J=I TO N1
520 B=B+1
530 PRINT TAB(12+7*B);X(J);
540 NEXT J
550 PRINT
560 PRINT
570 NEXT I
580 M=S/N
590 V=(T-(S↑2/N))/(N-1)
600 PRINT
610 PRINT "NUMBER OF OBSERVATIONS =";N
620 PRINT "MEAN=";M
630 PRINT "VARIANCE=";V
640 PRINT "ST.DEV.=";SQR(V)
650 PRINT "ST. ERR.=";SQR(V)/SQR(N)
660 PRINT "% STD ERR =";(SQR(V)/SQR(N))/M*100
670 SELECT PRINT 005
680 END
```

tenance of cell lines, determine the doubling time of the particular culture as a guide for transfers. If cell concentrations exceed 40 mg fresh weight per milliliter medium, transfers with no appreciable lag before the logarithmic phase will be obtained. To avoid serious oxygen deficiency, maintain cultures within 40 to 400 mg fresh weight per milliliter. They become so thick that they almost solidify if the fresh weight per milliliter exceeds 400 mg. Generally, weekly transfer of the cells is advisable and more frequent transfers may be required for a rapidly growing line. To transfer cells, swirl flask and pour cells and medium into a sterile graduate cylinder. Add the contents of this cylinder to new medium. Alternately, pipet (remove the tip of the pipet to prevent clogging by tissues or use a Cornwall pipet with a size 12 or larger needle) a portion of the old medium into the new medium. This latter procedure is particularly convenient for adding uniform samples to small flasks.

The cell line established in suspension culture can also be transformed back into a line grown on agar. Pipet 2 to 3 ml of cell suspension onto agar-solidified MS medium containing 11.5 μM IAA and 0.1 μM kinetin. A "lawn" of loose, friable tissue is obtained by this method.

Measuring Culture Growth

The growth of the culture can be followed in several different ways. The cell number or mitotic index can be determined (ch. 2) (*5*) or the weight of a given volume can be determined. In the latter case, remove a uniform sample by pipet and place it on a small circle of Miracloth in the center of a Hirsch funnel. Apply vacuum until the medium is drawn off and then scrape the cells onto a tared aluminum moisture dish. Determine the fresh weight of the sample on the dish. Determine dry weight after 48 h drying at 65 C. For critical experiments, use duplicate or triplicate samples from duplicate or triplicate flasks.

Automated Procedures

The methods described above will yield batch cultures of cells. If standardized with care, the results can be reproducible. Batch cultures can have serious limitations, however. Both cell population and nutrient supply are changing continuously and, in some cases, these changes are highly undesirable. To stabilize either nutrient supply or cell population, various automated procedures have been developed (*5*, *33*). In general, a batch culture is used to charge the automated system and both air supply and nutrient supply are carefully controlled. Cells can be removed at various intervals or removed continuously so that a stable growth rate of cells can be maintained under stable nutrient conditions.

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8

PROTOPLAST ISOLATION AND CULTURE

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Introduction

As early as 1892, Klercker succeeded in isolating protoplasts from plasmolyzed cells of the water warrior (*Stratiotes aloides*) by mechanical means (*2*). Early physiological studies on such protoplasts were hampered by the small numbers obtainable. In 1919 Giaja isolated protoplasts in large numbers from yeast by removing the cell wall with snail enzymes. However, not until recent years were bulk preparations of viable protoplasts obtained from cells of higher plants.

Cocking (*1*) was the first to isolate protoplasts from tomato root tips by exogenous application of enzymes that macerated the tissue and degraded cell walls. Since then, cellulytic and pectic enzymes have been used successfully to isolate protoplasts in large numbers from many higher plants including *Nicotiana* spp. Takebe and others (*3*) isolated protoplasts from leaves of *N. tabacum* cv. Bright Yellow and demonstrated that they were metabolically active insofar as they supported multiplication of tobacco mosaic virus (TMV). Since then, naked tobacco leaf protoplasts have been used for a range of physiological, biochemical, and genetic studies including virus infection, deoxyribonucleic acid (DNA) uptake, particle uptake, somatic hybridization, cell wall formation, and membrane function.

Isolation Procedures

Species and Cultivars

Several species and cultivars of *Nicotiana* have been used as protoplast sources. Among these are *N. glutinosa*, *N. otophora*, and the following cultivars of *N. tabacum:* Turkish, White Burley, Burley 21, Bright Yellow, Xanthi, Xanthi nc, Maryland, and Samsoun. Considering the range of families and genera of plants from which viable protoplasts have been isolated, probably many other *Nicotiana* spp. and cultivars will be found suitable. The procedures, however, for growing plants, isolating protoplasts, and growing them may have to be modified for each.

Growth Conditions Before Protoplast Isolation

The ability consistently to obtain preparations of viable protoplasts depends to a large extent on the disposition of the source plants before protoplast isolation. Optimal growing conditions may vary depending on the purpose for which protoplasts are to be used, the type of plant, or even the individual experimenter. Usually, experimenting with different growing conditions is necessary until ones are found that allow isolation of viable protoplasts. Once such conditions have been determined, they should be strictly retained.

Shepard and Totten (*4*) found that the following conditions were best for growing *N. tabacum* cv. Xanthi nc before protoplast isolation under low osmotic conditions for cell and plant regeneration: maintain plants in controlled growth rooms in 30-cm pots at constant 23 C under cool, white fluorescent light (1,500 ft-c) for 16 h and 70 to 75 percent RH. Use heavy, loam soil containing peat moss (2:1) and water with a solution containing 1 g/L of soluble 20-20-20 Peters fertilizer. Take care to prevent the soil from being continually saturated. Collect leaves when they reach approximately 25 cm in length from plants that are less than 0.7-m tall and which have not yet begun to flower.

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Asepsis

If plants are to be regenerated from isolated protoplasts, carrying out all steps under aseptic conditions is essential from initial isolation of protoplasts until the time regenerated plants are potted in soil. Before extracting protoplasts, surface sterilize tobacco leaves by submerging them in 0.5 percent sodium hypochlorite (Clorox-water, 1:9) for 15 min. Then rinse with sterile, distilled water. Place them in a sterile, laminar flow hood until visible, free moisture has evaporated. Protoplasts and cells can most easily be maintained without microbial contamination if all subsequent manipulations are carried out in the hood. Either autoclave, or pass through a micropore filter, all solutions.

When incubating chloroplasts under sterile conditions is not essential, as in virus infection studies, microbial contamination can be held to a minimum by adding antibiotics such as rimocidin (10 mg/L) to the incubation medium.

Protoplast Isolation

Protoplasts may be obtained from either cell suspensions prepared from tobacco callus cultures (ch. 7) or intact leaves. Two approaches have been used to isolate tobacco leaf protoplasts. In one, leaf tissue is macerated under high osmotic conditions using mannitol as an osmoticum. This approach has been widely used for preparing protoplasts to be inoculated with viruses. The other approach uses sucrose as an osmoticum under comparatively low osmotic conditions. It has been particularly useful for regeneration studies.

Cell suspensions. The following method was used by Uchimiya and Murashige (*5*) to isolate protoplasts from cell suspensions grown in liquid nutrient medium (ch. 7) derived from callus cultures of *N. tabacum* cv. Bright Yellow. Centrifuge the cell suspension at 300 g for 10 min. Resuspend 500 mg of cells in 5 ml of enzyme solution in a 50-ml DeLong flask. The enzyme solution is composed as follows: 0.2 percent Macerozyme R-10, 1 percent Cellulase Onozuka R-10, and 0.8 *M* mannitol. Adjust to pH 5.7 with 0.1 *N* HCl or NaOH. Place on a rotary shaker (50 to 100 rpm) for 3 to 4 h at 27 C. Filter suspension through nylon cloth (150- to 200-μm mesh). Centrifuge at 100 g for 2 min and resuspend the protoplasts in 0.7 *M* mannitol. Wash protoplasts three more times in 0.7 *M* mannitol.

Leaves—high osmotic conditions. This procedure was initially used by Takebe and colleagues (*3*) to prepare protoplasts for virus infection studies. It provides a relatively uniform population of protoplasts derived from leaf pallisade cells from several cultivars of *N. tabacum*. Remove the lower epidermis of the leaf by stripping with fine tweezers. Place 2 g of tissue in a 150-ml evacuation flask containing 20 ml of solution composed as follows: 0.1 percent Macerozyme R-10, 1 percent potassium dextran sulfate, and 0.7 *M* mannitol. Adjust to pH 5.8 with 0.1 *N* HCl. Vacuum infiltrate the tissue for 4 min. Place the flask on a reciprocating shaker (100 excursions per min) at room temperature. After 11 min, pour off the enzyme solution, add 20 ml of fresh Macerozyme solution, and shake as before for 1 h. Pour the tissue suspension (containing some macerated cells) through several layers of cheesecloth and set aside the cell suspension.

Place the undigested tissue in the flask with 20 ml of fresh Macerozyme solution, shake for 1 h, and again filter the suspension. Repeat this operation a third time pooling the three, 20-ml cell suspensions. Wash the cells twice in 0.7 *M* mannitol containing 0.1 m*M* $CaCl_2$ by centrifuging at 100 g for 2 min, then resuspend the cell in the same solution. Centrifuge as above and resuspend the cells in 40 ml of cellulase suspension having the following composition: 0.5 percent Cellulase R-10 and 0.7 *M* mannitol. Adjust to pH 5.4 with 0.01 *N* KOH. Incubate in a water bath at 37 C for 1 to 3 h, swirling occasionally. When most of the cells have been converted to free protoplasts (as determined by microscopic examination), wash the protoplasts twice in 0.7 *M* mannitol and 0.1 m*M* $CaCl_2$ by centrifugation and resuspend in the same solution. If protoplasts prepared in this manner are to be used for virus infection studies, immediately inoculate them (ch. 11).

Leaves—low osmotic conditions. The success of this

TABLE 8-1.—*Composition of media for protoplast preparation and cell regeneration*[1]

Constituent	Medium I	Medium II	Medium III
		Milligrams per liter	
$Ca(NO_3)_2 \cdot 4H_2O$		180	
NH_4NO_3	825	82.5	825
KNO_3	950	167	950
$CaCl_2 \cdot 2H_2O$	220	22	222
$MgSO_4 \cdot 7H_2O$	1223	447.3	1223
Na_2SO_4		180	
KH_2PO_4	680	68	680
$NaH_2PO_4 \cdot H_2O$		14.9	
KCl		58.5	
Na_2EDTA	37.3	3.7	37.3
$FeSO_4 \cdot 7H_2O$	27.8	2.8	27.8
$Fe_2(SO_4)_3$		2.3	
KI	.83	.76	.83
H_3BO_3	6.2	2	6.2
$MnSO_4 \cdot 4H_2O$		4.1	
$MnCl_2 \cdot 4H_2O$	19.8	2	19.8
$ZnSO_4 \cdot 7H_2O$	9.2	2.3	9.2
$Na_2MoO_4 \cdot 2H_2O$	.25	.03	.25
$CuSO_4 \cdot 5H_2O$	.025	.003	.025
$CoSO_4 \cdot 7H_2O$	.03	.003	.03
meso-Inositol	100	39.7	100
Thiamine·HCl	1	.19	1
Glycine		2.7	
Nicotinic acid		.45	
Pyridoxine·HCl		.1	
α-Naphthaleneacetic acid	3	1.2	
Indoleacetic acid			4
6-Benzylaminopurine	1	.4	1
Kinetin			2.56
Sucrose	Variable	Variable	0.05-0.3 *M*
pH	5.6	5.6	5.6

[1]Shepard and Totten (*4*).

method (*4*) depends on rigid adherance to the conditions for growing plants before protoplast extraction (see **Growing conditions before protoplast isolation**). Surface sterilize leaves of *N. tabacum* cv. Xanthi nc and place them in sterile paper towels overnight in a refrigerator. Do all subsequent steps aseptically. To facilitate enzyme penetration, remove the lower epidermis by stripping with fine, sterile tweezers. Alternatively, cut the leaves into thin strips (about 1 mm). Place 2 g of tissue in a 250-ml evacuation flask containing 100 ml of the following sterile solution: 0.1 percent Macerozyme R-10, 0.5 percent Cellulase R-10, 2 percent polyvinylpyrrolidone (mol. wt. 10,000) and 0.2 *M* sucrose. Adjust to pH 5.4.

Vacuum infiltrate and incubate at 30 C on a rotary shaker (maximum of 40 cycles per min) for 4 to 6 h. Collect protoplasts by passing the suspension through four layers of cheesecloth into 16.5-cm graduated, Babcock milk test bottles. Centrifuge at 400 g for 5 min in a Babcock centrifuge. During this step, viable protoplasts will migrate centripetally forming a green band in the neck of the bottle. Withdraw the protoplasts with a Pasteur pipette and place them in Babcock bottles containing a rinse solution of 0.25 to 0.4 *M* sucrose and one-tenth concentration of medium I (table 8-1). At this point the protoplasts should be suitable for regeneration of cells and plants (*4*).

Regeneration of Cells and Plants

Two procedures for regenerating cells and intact tobacco plants from isolated protoplasts are given here, the first by Uchimiya and Murashige (*6*). Obtain protoplasts of *N. tabacum* cv. Xanthi or Bright Yellow using the procedure outlined in **Cell suspension.** Transfer protoplasts to 50-ml DeLong culture flasks containing 5 ml protoplast culture medium (table 8-2). Each flask should contain about 5×10^5 protoplasts. Incubate without agitation at 27 C under Gro-Lux lamps (1,000 lux) for 16 h daily. After 1 to 2 wk, add an equal volume of fresh culture medium containing 1.6 percent Difco Bacto-agar. This medium is prepared using 0.3 *M* sucrose in place of mannitol. Transfer 2 to 3 ml of the suspension to 10 × 55-mm sterile, disposable Petri dishes. Incubate 1 to 2 mo. By now callus masses (2- to 3-mm diameter) should be obtained. Transfer callus masses individually to 25 × 150-mm culture tubes containing 25 ml of shoot-inducing nutrient agar (table 8-3). When shoots form, transfer to culture tubes containing rooting medium (table 8-4). Incubate under Gro-Lux lamps (10,000 lux) for an additional 2 to 4 wk. Transfer rooted plants to pasteurized soil and grow in the greenhouse.

The second procedure is that of Shepard and Totten (*4*). Isolate protoplasts under low osmotic conditions (see **Leaves—low osmotic conditions**) from leaves of *N. tabacum* L. cv. Xanthi nc. Suspend protoplasts in culture medium II (table 8-1) containing 0.59 percent ionagar No. 2 and 0.4 *M* sucrose at a density of 1 to 1.5 × 10^4 protoplasts/ml. Add 5 ml of the protoplast-agar culture medium mixture to 230-ml glass prescription bottles. Form thin agar layers by placing the bottles in a horizontal position. Incubate at 21 C under white fluorescent light (400 lux). After 27 h increase the light intensity to 700 lux. After 2 wk, add 2 ml of culture medium II (lacking agar) and tilt the bottles slightly to prevent the liquid from covering the agar surface.

Begin dilution plating from the bottles after about 4 wk of culture. This is done by first disrupting the soft agar layer with a glass rod, then disrupting the colonies by gentle repipetting. Dilute the colonies tenfold by mixing in 10-mm Petri dishes 1 ml of the colony suspension with 9 ml of medium I (table 8-1) containing 0.05 *M* sucrose and 0.7 percent agar. Seal dishes with Parafilm and incubate in clear polystyrene boxes at 24 to 26 C under fluorescent light (1,500 lux). When most colonies exceed 1-mm diameter and have turned green, transfer them with a fine-pointed scalpel to Petri dishes containing medium

TABLE 8-2.—*Medium for protoplast culture*

Murashige and Skoog's mineral salts (ch. 7)	*Milligrams per liter*
Sucrose	15,000
Mannitol	110,000
α-Naphthaleneacetic acid	.6
Kinetin	.1
Thiamine-HCl	10
Pyridoxine-HCl	10
Nicotinic acid	5
myo-Inositol	100
Glycine	2

TABLE 8-3.—*Shoot-inducing nutrient agar medium*[1]

Murashige and Skoog's mineral salts (ch. 7)		
Sucrose	grams per liter	103
Thiamine·HCl	milligrams per liter	.4
myo-Inositol	do	100
Kinetin	do	2
Indoleacetic acid	do	2
Adenine sulfate·$2H_2O$	do	80
$NaH_2PO_4 \cdot H_2O$	do	170
L-tyrosine	do	50
Bacto-agar	do	800

[1]Uchimiya and Murashige (*6*).

TABLE 8-4.—*Rooting medium*[1]

Murashige and Skoog's mineral salts (ch. 7)		
Sucrose	grams per liter	30
Thiamine·HCl	milligrams per liter	.5
myo-Inositol	do	100
Indoleacetic acid	do	2
Bacto-agar	grams per liter	8

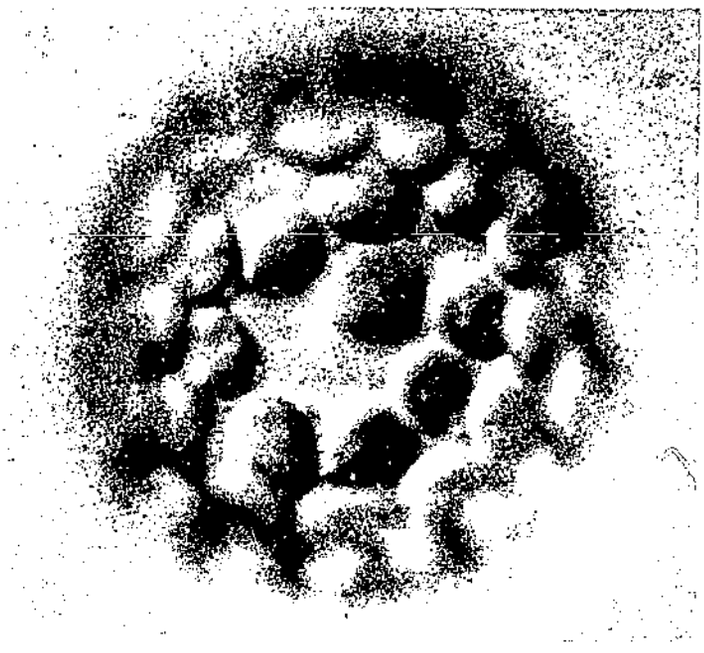
[1]Uchimiya and Murashige (*6*).

III (table 8-1). Colonies should grow rapidly and differentiate shoots and leaves on this medium. When colonies have exceeded 5 mm diameter and have initiated primordial shoots, transfer them with tissue forceps into Pyrex casserole dishes (Corning No. 681) containing 150 ml of 1/2 concentration medium I (table 8-1), 0.025 *M* sucrose, 0.6 percent ionagar, and no hormones. Seal the dishes with Parafilm and incubate under white fluorescent light (7000 lux). When shoots are well developed and root formation has begun, remove the plants from the dishes, transplant into solid, peat growing blocks, and place them in an environmental chamber at greater than 85 percent RH. When the root system has become fully developed and shoot growth has resumed, transplant the plants into soil.

Microscopic Methods

For various reasons, examining protoplasts microscopically is often necessary during or following their isolation. As an example, in the fusion procedure to be discussed, light microscopy can be used to detect heterkaryons as a criterion of fusion between protoplasts from two different *Nicotiana* spp. However, by far the most common use of light microscopy is simply to determine the condition or viability of individual protoplasts and cells in suspension. Criteria for assessing whether or not a protoplast is alive will be discussed together with procedures for preparing protoplasts for study with the electron microscope. Antibody labeling techniques, particularly useful in virus infection studies, are given in chapter 11.

The simplest way of telling whether protoplasts are alive is to place a drop of the suspension on a microscope slide, add a cover slip, and examine the preparation with phase contrast optics. Living protoplasts prepared under low osmotic conditions (see **Leaves—low osmotic conditions**) clearly exhibit protoplasmic streaming, that is, orderly movement of mitochondria and microbodies about the protoplast. In dead protoplasts, or cells, mitochondria and microbodies exhibit brownian movement, shaking about at random. Protoplasmic streaming is less easy to detect in protoplasts prepared under high osmotic conditions (see **Cell suspensions** and **Leaves—high osmotic conditions**) because the chloroplasts form a nearly continuous shell at the periphery of the spherical protoplast, occluding the inner contents (fig. 8-1). However, the fact that the protoplasts are spherical and the chloroplasts form a continuous shell around the periphery are themselves indications that the protoplasts are alive. Dead protoplasts usually are irregularly shaped, and the chloroplasts become clumped and disoriented.



PN-6309

Figure 8-1.—Leaf protoplast of *N. tabacum* isolated under low osmotic conditions showing the peripheral orientation of the chloroplast. Nomarski differential interference-contrast optics, magnification 1900×.

Another means of determining whether protoplasts are living is their ability to take up vital stains such as neutral red. Such staining is not necessary, however, if one has access to a phase-contrast microscope for detecting protoplasmic streaming.

Thin sections of protoplasts may be prepared for ultrastructural study in the manner used for intact tissues except that it is necessary that the primary fixative (glutaraldehyde) contains an osmoticum. Also, protoplasts will withstand more rapid dehydration before infiltration with epoxy resin than will intact tissues.

The following procedure works well for protoplasts that have been isolated under high osmotic conditions (see **Leaves—high osmotic conditions**). Place 1 ml of protoplast suspension in a BEEM capsule (cylindrical tip, size 00) and pellet the protoplasts by centrifugation at 100 g for 2 min. The initial concentration of protoplasts should be such that after centrifugation the pellet of protoplasts should not occupy more than 1 mm at the tip of the capsule. Pour off the supernatant fluid and fill the capsule with 3 percent glutaraldehyde in 0.05 *M* potassium phosphate buffer, pH 6.8, and 0.7 *M* mannitol. Fix for 1 h at room temperature.

Wash twice with phosphate buffer (without mannitol) and post-fix an additional hour with 2 percent OsO_4 in the same buffer without mannitol. Wash twice with buffer and

TABLE 8.5—*Spurr's low viscosity epoxy resin*

	Grams
Vinylcyclohexene dioxide (VCD)[1]	150
Diglycidyl ether of polypropelne glycol (DER 736)	9
Nonenylsuccinic andrydride (NSA)	39
Dimethylaminoethanol (S-1)	.6

[1]Mix components at room temperature in the order listed.

once with distilled water for 1 h. Centrifuge at 10,000 g for 20 min to pack the protoplasts tightly in the bottom of the capsule. Pour off the superantant fluid and fill the capsule with 95 percent acetone (EM grade). After 15 min, make three changes of 100 percent acetone during 1 h.

Then, using a Pasteur pipet, withdraw half of the acetone from the capsule and fill the capsule with Spurr's low viscosity epoxy resin (table 8-5). Stir gently with a glass rod, taking care not to disturb the pellet. Let stand for 30 min. Withdraw half of the acetone-resin mixture and again fill the capsule with epoxy resin. Let stand for 4 h. Withdraw all of the resin from the capsule and refill the capsule with fresh resin. Let stand at room temperature overnight. Pour off the resin and refill the capsule with fresh resin. Cure at 70 C for 8 h or longer. Protoplasts embedded in this manner may then be sectioned and stained with lead citrate and uranyl acetate in the usual manner.

Applications

DNA Uptake

Owing to a lack of a cell wall, protoplasts provide a potential means whereby genetic material can be transferred from one plant to another. Under proper conditions isolated protoplasts will incorporate exogenously applied DNA. If plants are subsequently regenerated from protoplasts that incorporate DNA isolated from other plant species or cultivars, their genetic constitution can be altered. The following procedures were found most effective for uptake of DNA by protoplasts of *N. tabacum* cv. Xanthi, a systemic host of TMV (*6*).

Quantitative measure of absorbed DNA. Protoplasts of *N. tabacum* cv. Xanthi are obtained from cells using the procedure outlined in **Cell suspensions.** Suspend the protoplasts in protoplast culture medium (table 8-2) at a density of 5×10^6 protoplasts per milliliter. Add ^{3}H-DNA (specific radioactivity 1.9×10^3 cpm/μg) in 0.015 *M* trisodium citrate and 0.15 *M* NaCl, pH 7, to a concentration of 7.26 μg/ml. Incubation is conveniently done in 10- × 75-mm culture tubes containing 270 μL protoplast suspension and 30 μL DNA. Seal tubes with Parafilm and incubate at 27 C for 5 h. Collect the protoplasts by centrifugation at 480 g for 5 min and resuspend in fresh protoplast culture medium lacking DNA. Wash twice more in fresh culture medium. Remove DNA adhering to the protoplast surface after the final centrifugation by resuspending the protoplasts in 450 μL of protoplast culture medium and 50 μL of DNase solution (1 mg/ml DNase in 0.03 M $MgCl_2$). Incubate at 37 C for 5 min and wash protoplasts three times by centrifugation with culture medium. Lyse protoplasts by suspending them in 450 μL of the trisodium citrate-NaCl solution containing sodium dodecyl sulfate (10 percent by wt/vol). Add an equal volume of cold 10 percent trichloroacetic acid (TCA) containing 10 percent bovine serum albumin. Collect the resulting precipitate on a Millipore membrane filter (0.45 μm) and wash with 30 ml cold 5 percent TCA, then 5 ml cold 95 percent ethanol. Dry and measure radioactivity using 5 ml toluene containing 4 mg/ml Omnifluor (New England Nuclear) as a carrier. Using this procedure, about 3.5 percent of the initial radioactivity was taken up by the protoplasts (Shalla, unpublished).

Gene transfer studies. The following procedure was used by Uchimiya and Murashiga (*7*) to transfer (TMV) local lesion genes from *N. tabacum* cv. Xanthi nc to *N. tabacum* cv. Xanthi. Suspend protoplasts in 2- to 3-ml protoplast culture medium (table 8-2) at a density of 10^5 to 10^6 protoplasts per milliliter in 15- × 115-mm centrifuge tubes. Add unlabeled DNA (in the trisodium citrate-NaCl solution) to a final concentration of 20 to 30 μg/ml. Seal tubes with aluminum foil and incubate at 37 C for 5 to 10 h. Wash three times by centrifugation at 300 g for 2 min, resuspending the protoplasts each time in fresh protoplast culture medium. Protoplasts may then be manipulated to promote regeneration of plants (*6*).

Protoplast Fusion

Fusion of protoplasts from different species or genera provides a potential means of combining genetic material to form artificial hybrids. To date, most such attempts have been unsuccessful, possibly because of difficulties in working with the protoplasts of many species, particularly of cereal origin. Nevertheless, the potential is present, and interspecific fusion has been accomplished with some systems, notably different *Nicotiana* spp. (ch. 9).

Virus Infection

Infecting isolated protoplasts synchronously is possible with plant viruses. This provides a system wherein the sequential steps in viral infection, and pathogenesis may be studied (ch. 11).

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9

PROTOPLAST FUSION AND ORGANELLE TRANSFER

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Introduction

Past successes in cell fusion and organelle uptake have proven that hybrid cells can be made and novel cytoplasms created. Methods to promote the stable cofunctioning of the mixed nuclei and cytoplasms must be developed to extend the range of hybridization beyond that which has been achieved. Understanding how genes are regulated and how the nucleus is organized is crucial to this endeavor.

Several approaches to somatically altering the genetic content of plant cells are being taken. They include (a) fusing protoplasts within species, between species, between monocots and dicots, and between plants and animals (*16, 28, 38, 46, 48*), (b) inserting organelles such as nuclei, plastids, mitochondria and chromosomes into protoplasts (*8, 22, 63, 71, 74*), (c) inserting microorganisms such as blue-green algae, bacteria and viruses into protoplasts (*12, 21, 36*), and (d) transferring nucleic acids, which may be infective, transforming, or vector-borne into protoplasts, meristems, stem wounds, or ovaries (*27, 42, 45, 54, 57, 62, 73, 75*). Descriptions of the successes and potential of introducing genes from various sources into plant cells have recently appeared (*15, 23, 26, 37, 41, 71*).

Much of this work has been significantly aided by the amenability of *Nicotiana* spp. to protoplast technology. Indeed, the outstanding creations have been the new chromosomal variants and cytoplasmically altered phenotypes obtained in the progeny of somatic hybrids of *Nicotiana* spp. (*4, 71, 72*). Four of the six (*4, 30, 67, 71, 72*) successful attempts to somatic hybridizing from which fertile plants were obtained used *Nicotiana* species *N. glauca* + *langsdorffii* (*13, 72*), *N. tabacum* + *N. debneyi* (*4*), and two different intraspecific *N. tabacum* combinations (*38, 60*).

Cells containing transferred organelles are called cytoplasmic hybrids or heteroplasts. Cells containing nonidentical nuclei are referred to as heterokaryons or heterokaryocytes; when these nuclei fuse, a hybrid cell is formed.

Agents and Mechanisms of Protoplast Fusion and Organelle Uptake

The first step in protoplast fusion and organelle uptake requires the aggregation or agglutination of cells or organelles with cells. Although immune sera (*39*), poly-L-ornithine (*53*), or concanavalin A (*11*) can agglutinate protoplasts, they do not induce fusion.

Membrane fusion between plant protoplasts is promoted by sodium nitrate (*65*), polyethylene glycol (PEG) (*17, 50*), environmental conditions of high pH, high Ca^{+2} concentration, and elevated temperature (*52*), and lysolecithin combined with PEG (*17*). Using these facts, two methods, which give high frequencies of protoplast fusion, have been devised by Keller and Melchers (*52, 60*) and Kao and collaborators (*50, 51*). The methods can be used with highly vacuolized mesophyll cells and meristematic cells from culture. After treatment the fused cells recover and subsequently divide. The first method combines high pH (10.5), high Ca^{+2} (50 m*M* $CaCl_2 \cdot 2H_2O$), and high temperature (37 C) treatment that promotes fusion at frequencies up to 20 to 50 percent (*52, 60*). Centrifuging cells during the 40 to 45 min treatment may be necessary to induce fusion with certain tobacco protoplasts (*52*). The second method uses PEG (15 to 50 percent) and Ca^{+2} (10.5 m*M* $CaCl_2 \cdot 2H_2O$) and osmoticum (*17, 49, 50*). When followed by elution with a high pH/high Ca^{+2} solution this method has been successfully used to fuse protoplasts of many species (*28, 46, 48, 67, 72*). Interspecific fusion events range between 4 and 39 percent.

PEG is widely used to induce both protoplast fusion and organelle uptake. Its general formula is $HOCH_2(CH_2-$

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$O-CH_2)_n$ CH_2OH (*50*); the ether linkages make the molecule slightly negative. In addition to its effectiveness with plant protoplasts, PEG promotes the fusion of bacterial protoplasts (*69*) and animal cells (*24*). PEG may not work with all cell combinations. For example, although PEG induced yeast protoplast fusion (*20*), it did not promote fusion of yeast protoplasts with Boston ivy, *Parthenocissus tricuspidata* (*21*). Rather, the yeast protoplasts were taken into the cell in vesicles formed by the *Parthenocissus* plasmalemma.

The molecular weight and concentration of PEG (*10, 21, 31, 50, 51*), as well as that of Ca^{+2} (*50, 52*), are critical to achieve both aggregation/fusion and uptake. The most effective molecular weight classes are 1,540, 4,000 and 6,000. When applied to *Daucus carota* protoplasts, the optimum concentration of PEG for inducing the greatest number of binucleate cells (25 percent) and the least number of multinucleate cells (about 3 percent) was 0.2 *M* (*31*). The percentage of heterokaryocyte formation induced by PEG also varies with the biological system used; for example 0.2 percent for plant + animal cells (*28, 46*), 4 percent for *Petunia parodii* + *P. hybrida* (67), and 39 percent for *N. glauca* + soybean (*48*).

To optimize adhesion, the concentrations of protoplasts and organelles need to be regulated. A concentration of 2 to 3×10^5 cells/ml yielded 9 percent heterokaryons (*50*). Extensive protoplast aggregation in dense protoplast suspensions (4×10^6 cells/ml) promoted a higher incidence of protoplasts with incorporated chloroplasts (*8*). With a chloroplast to protoplast ratio of 10:1, chloroplast incorporation frequencies of 10 to 16 percent were observed (*10*).

Ahkong, Lucy, and collaborators (*1, 2*) have proposed a model of membrane fusion compatible with evidence obtained from treating animal cells with various fusogenic chemicals, including PEG and Ca^{+2}, and high temperature. They suggest that fusogens and heat perturb the bilayer structure of the cellular membrane that leads to an increase in the fluidity of the lipid region. Protein/glycoprotein particles then become aggregated within the perturbed membranes leaving regions of the membrane free of protein. Calcium also may aggregate intramembraneous proteins. These protein-free regions on closely apposed or agglutinated membranes are probably the sites of fusion as the lipids interact and intermix. Recently, a new class of biologically active compounds, called membrane mobility agents, have been described which promote local disorder within membranes (*55*). They actively promote fusion of animal cells; however, their effects on plant protoplasts are not well documented.

Cell plasmolysis and deplasmolysis occur during fusing and uptake treatments. PEG plasmolyzes protoplasts making the plasmalemma available to invaginate around externally adhering organelles (*8, 21*). When agglutinated protoplasts are washed free of PEG, the cells deplasmolyze and fusion occurs. In the high pH/high Ca^{2+}/high temperature fusion method, cell fusion occurs in mildly hypotonic, deplasmolyzing conditions (*52*). The membrane perturbing effects of PEG are probably achieved by raising the temperature to 37 C (*2, 52*).

Electron microscopic studies of cell + cell fusion induced by sodium nitrate (*78*) or PEG (*33, 74, 77*) indicated that agglutinated plasmalemma first fuse in small, localized areas forming cytoplasmic bridges. This process begins after PEG has been eluted. Continuing fusion of the agglutinated membranes leaves plasmalemma-bounded vesicles in the cytoplasm. Organelles then migrate to the periphery of the fused cell and mix, leaving the vacuoles in close contact. Cytoplasmic mixing is complete when the tonoplasts fuse. In certain heterokaryons, the organelles of one species retain normal structure whereas those of the other species appear to degenerate (*34*).

Ultrastructural studies by Davey and colleagues (*21, 22*) showed invaginated protoplast membranes fusing around yeast protoplasts and chloroplasts, thereby releasing filled membrane-bound vesicles into the cytoplasm. Eventually the chloroplast membranes or yeast plasmalemma fuse with the vesicle membrane, releasing the vesicle contents into the cytoplasm. Apparently, uptake of blue-green algae requires development of deep invaginations of the plasmalemma. Davey (*21*) ascribed the lack of uptake by *N. tabacum* cv. Xanthi nc mesophyll protoplasts to chloroplasts packing the peripheral cytoplasm and keeping the plasmalemma taut. Intact double-membraned chloroplasts can fuse directly with the plasmalemma similar to what occurs in cell + cell fusion (*21*). *Vaucheria* dichotoms chloroplast lamellae may be transferred into carrot protoplasts in this manner. The photosynthetic lamellae are present in cytoplasm of carrot protoplasts 30 min after PEG treatment without their own limiting membrane and without carrot plasmalemma surrounding them (*8*).

Efficiency of Organelle Uptake and Survival

The efficiency of uptake of microorganisms by plant protoplasts varies. After treatment with PEG 6000 (40 percent wt/vol), 20 percent of Boston ivy protoplasts contained from one to many cells of *Anacystis nidulans*, a blue-green algae. *N. tabacum* cv. Xanthi nc protoplasts, on the other hand, did not take up *A. nidulans* readily (*21*). Following PEG 4000 treatment (52 percent wt/vol), 1 percent of *N. tabacum* cv. Havana 38 (Wis 38) protoplasts harbored a single cell of *Gloeocapsa*, a nitrogen-fixing, photosynthetic, nontoxin producing blue-green alga (*12*). Isolated organelles may be treated with membrane modifiers, such as lysozyme (*63*) or sodium nitrate, to encourage uptake, along with slow centrifugation (*63*). One to 5 extra nuclei and 1 to 20 extra chloroplasts were

transferred into 0.5 percent of the recipient protoplasts, including *Nicotiana* protoplasts, using these methods (*63*). Chloroplasts from the alga, *V. dichotoma*, are taken up by 9 to 24 percent of carrot protoplasts during PEG 1540 or 4000 (28 percent wt/vol) treatment (*8, 10*). In these experiments, PEG induced protoplasts, but not chloroplasts, to aggregate and fuse. Chloroplasts were not taken up by protoplasts when they were centrifuged together if PEG was not added (*10*).

Subprotoplasts are fragmented protoplasts that often lack a particular group of organelles—nuclei, chloroplasts, mitochondria, or nucleus and mitochondria. They may be an alternate way to transfer organelles into cells (*5, 6*). Fusing subprotoplasts with each other or with protoplasts could have two advantages. Organelles may be protected from damage during isolation, and the technique would allow the subprotoplasts to be directly released into the common cytoplasm unencumbered by an enclosing plasmalemma vesicle.

To assure that successful intracellular colonization occurs, the organelle or organism must be isolated and presented to the recipient protoplast in a viable condition. Rathnam and Edwards (*68*), Blaschek and others (*7*) and Mennes and others (*61*) have perfected methods for isolating viable, metabolically active chloroplasts and nuclei from tobacco. Plant protoplasts are a convenient source from which to harvest organelles (*5, 7, 63, 68*). Leaf protoplasts retain about 100 percent of their photosynthetic activity, measured by CO_2-fixing ability, for up to 20 h when stored on ice (*68*). Chloroplasts isolated from these protoplasts also retained more than 80 percent CO_2-fixing ability after 20 h. In short-term culture, Bonnett and Banks (*9*) detected electron transport in algal chloroplasts that had been transferred into higher plant protoplasts.

Stable incorporation of the transferred organelles or algae, which implies continued division and function of the introduced genetic material, generally has not been achieved. However, there are some notable exceptions.

Persistent nitrogen fixation and acetylene reduction has been detected in colonies of the fungus *Rhizopogon*, which developed from protoplasts that had incorporated the nitrogen-fixing bacteria, *Azotobacter vinelandii* (*36*). Albino *N. tabacum* protoplasts seem to have incorporated and maintained nuclei and chloroplasts from green *N. suaveolens* (*56*). The *N. tabacum* plant obtained was variegated, had supernumerary chromosomes, and synthesized small and large subunit molecules of Fraction I protein characteristic of both *N. tabacum* and *N. suaveolens*. In contrast, with rare exceptions, somatic hybrid plants produced by cell fusion between *N. glauca* + *N. langsdorffii* and their offspring up to the F_3 generation contained chloroplasts of either *N. glauca* or *N. langsdorffii* (*14*).

Efficiency of Protoplast Fusion and Hybrid Cell Recovery

Identification of fused cells is commonly based on differential staining of heterokaryons (*18, 53*), distinctive chromosome morphologies (*48, 51*), and chloroplasts that differ in chlorophyll content, size, grana or stroma structure and inclusion bodies (*34, 50*). Crucial events that follow cell fusion or organelle uptake and lead to hybrid cell formation are nuclear fusion, cytoplasmic mixing, and stable expression and replication of the combined nuclear or cytoplasmic genomes.

Interphase nuclei have been observed to fuse in heterokaryocytes (*18, 29, 48*). Nuclear consolidation may also occur during the first mitotic division after protoplast fusion in heterokaryocyte combinations in which division is synchronized (*51*). Overlapping spindles can lead to inclusion of both sets of chromosomes within the new nuclear membrane. Alternatively, a nucleus from an interphase cell, for example, leaf mesophyll, may undergo precocious chromosome condensation if fused with a highly mitotic cell, from cultured cell lines (*66*). Hybrid mitotic cells that occur immediately after fusion may be of this type (*51*). Because the precociously condensed chromosomes are not attached to a spindle, they may be easily lost. The impact of post-fusion events is illustrated by the fate of *N. glauca* + *N. langsdorffii* heterokaryocytes. Although expected to be compatible, only 13.5 percent of the heterokaryocytes underwent nuclear fusion and formed hybrid cell colonies (*51*).

Heterokaryons commonly lose nuclei, chromosomes and organelles, especially if selective pressure is withdrawn or none is applied (*34, 51, 66*). Certain phenomena suggest that eukaryotic cells may have mechanisms for destroying foreign, non-self deoxyribonucleic acid similar to the restriction enzyme systems of bacteria (*25*). When these phenomena are sufficiently understood they may become assets in experimental design. Use of restriction enzymes to clone genes is one example (*40*) and the rapid advances in mapping the human genome with man and mouse somatic cell hybrids is another (*59*).

Various selection schemes have been used to recover hybrid cells. They are applied at different times after fusion, namely, immediately after fusion but before cell division has occurred (*67*); after division has started; or when calli are visible (*51, 60, 67, 72*). Power and others (*67*) report that the frequency of *P. hybrida* + *P. parodii* somatic hybrid plant formation was one out of 1 to 8×10^5 protoplasts. Their selection scheme was based on differential parental protoplast growth responses on Murashige and Skoog's medium containing actinomycin D. Melchers and Labib (*60*) indicate that 12 intraspecific somatic hybrids originated from 2.2×10^6 calli tested. In their experimental

design, complementing chlorophyll mutations in green hybrid callus were distinguished from the yellow parental calli. The selection scheme worked effectively only after reducing the organic constituents in the medium five- to tenfold. On the high organic medium, both calli of the parental chlorophyll mutants and somatic hybrids were deep green during exposure to high light intensities and could not be distinguished. Using auxin autotrophy of hybrid callus as the method for selecting somatic hybrids of *N. glauca* + *N. langsdorffii* (ch. 1), large numbers of hybrid plants were recovered (*72*). Kao (*48*) has established a number of *N. glauca* + soybean hybrid cell lines using another strategy. He physically isolated and cloned hybrid cells in small wells (Costar's Cuprak tissue culture dish, #3268), thus removing them from competition with parental cells, which grew faster.

Somatic plant hybrids have been recovered without selection (*30, 38*). Sexual hybrids are available for each combination so that the somatic hybrid could be recognized after differentiation. Belliard and associates (*4*) recovered many nuclear and cytoplasmic hybrids between a male sterile *N. tabacum* carrying *N. debneyi* cytoplasm + fertile *N. tabacum* without apparent selection. The nuclear hybrid had leaves that were intermediate between the parental sessile and petiolate types. Unique somatic mixing of the two cytoplasms was evidenced by unusual intermediate floral phenotypes and changes in fertility.

Potrykus and Lorz (*64*) attempted to repeat the successful somatic hybridization experiment of Melchers and Labib (*60*) based on complementing nuclear chlorophyll mutants by transferring nuclei from one haploid *N. tabacum* mutant line into protoplasts of the other mutant line. The green plants that were produced, however, did not appear truly complementary.

Cellular incompatibility between species, which do not form sexual hybrids, may not wholly account for the present lack of success in obtaining this kind of somatic hybrid. Constabel and co-workers (*19*) reported that the timing of first mitosis in intergeneric heterokaryons that were identified by the co-presence of chloroplasts of one parent (*N. langsdorffii, glauca, rustica* or *tabacum*) and leukoplasts of the other parent (soybean callus cells) was intermediate between the expected first mitosis of each parent after protoplast formation. Although they did not score fused hybrid nuclei, this observation suggests that lack of early and effective selection of this subpopulation of dividing cells, rather than their self-generated suicide caused by incompatibilities, may be the reason that few somatic hybrid cell lines have been recovered from populations known to include heterokaryons.

Biochemical Markers for Hybrid Cell Selection

An important area in plant cell genetics involves the selection and characterization of mutant cell lines (ch. 10). In a recent review, Maliga (*58*) cites plant cell lines that are resistant to various chemicals. However, drug resistant mutants and nutritional auxotrophs have not yet become standard methods for selecting hybrid plant cells as they have for animal cells. There is, however, a notable exception. Schieder (*70*) demonstrated that somatic hybrid plants could be recovered from complementing nicotinic acid and glucose auxotrophs in the liverwort *Sphaerocarpos donnellii* following protoplast fusion. Cell lines of *N. tabacum* resistant to amino acid analogs (aminoethyl-L-cysteine, *p*-fluorophenylalanine, hydroxylysine, methionine sulfoximine (MSO), 5-methyl tryptophan), purine and pyrimidine analogs (bromodeoxyuridine (BdUr), azaguanine), NaCl, $KClO_3$, streptomycin, and threonine have been isolated. *N. sylvestris* cell lines resistant to 8-azaguanine, NaCl, 2,4-dichlorophenoxyacetic acid, and low temperature are available.

Cell lines auxotrophic for hypoxanthine and lysine have also been selected from *N. tabacum* cultures. The verified gene mutants among these phenotypic variants are the MSO, BdUr, streptomycin, and 5-methyltryptophan resistant cell lines and hypoxanthine and lysine auxotrophs. All these cell lines are available as biochemical markers for somatic hybrid cell selection systems.

Because the ability to differentiate appears related to the time cells have been cultured, regeneration of somatic hybrid plants selected from complementing mutant cell lines may depend on the age of the cell lines used (*58*). Naturally existing differences in resistance to drugs among species bypasses this disadvantage because protoplasts can be prepared directly from leaves. Endogenous resistance to actinomycin D has been combined with differences in nutritional requirements of each species to select somatic hybrids of *P. parodii* + *P. hybrida* (*67*). Distinct hormone requirements also may have general application as a selection tool. Izhar and Power (*44*) have provided evidence that particular hormone dependencies segregate in backcrosses and may have a genetic basis. In addition, they showed that complementations occur between different hormone auxotrophs.

Animal cell genetic studies have made steady progress because of a number of general selection schemes, foremost of which is the HAT system (*59*). HAT refers to hypoxanthine, aminopterin and thymidine that are added to the selection medium. By specifically inhibiting dihydrofolate reductase, aminopterin prevents *de novo* synthesis of purines and pyrimidines. Purines and pyrimidines can be synthesized alternatively via a salvage pathway by hypoxanthine phosphoribosyltransferase (HPRT) and thymidine kinase (TK). Man and mouse mutant cell lines deficient in TK (TK^-) or HPRT ($HPRT^-$) cannot use thymidine or hypoxanthine as precursors in the salvage pathway. TK^-

and HPRT⁻ cells complement each other for the enzyme deficiencies and grow in HAT medium so that hybrid cells are enriched.

A general biochemical selection method similar to the HAT system has not yet been developed for plant cells. Continued success in producing cell hybrids can be expected with plant cells, nevertheless. Auxin autotrophy, complementing chlorophyll mutants, response to growth and differentiating media are distinctive botanical selection methods. Other particular selection devices may also work: for example complementing resistance to tobacco mosaic virus and tabtoxins in tobacco.

Methods

Protoplast Fusion

The extent of cell fusion and heterokaryocyte formation can be controlled in a number of ways (*8, 50, 51, 76*): (a) length of time in enzyme solution to produce protoplasts, (b) molecular weight and concentration of PEG, (c) duration and temperature of fusion treatment, (d) pH of fusion mixture, and (e) protoplast or cell density.

First method: For fusing protoplasts, Kao and coworkers (*48, 49, 50, 51*), used a method adapted for a variety of plant and animal cell systems (*3, 28, 46*). Separately prepare protoplasts from each source at the same time (ch. 8) (*76*). Filter the protoplasts together through a stainless steel screen (80 μm) into a centrifuge tube. Pre-moistened Miracloth is also a good filter. Centrifuge at 100 g (unit of gravity) for 3 min and resuspend protoplasts in wash solution (table 9-1). Repeat centrifugation step. Washing also can be done by allowing the protoplasts to settle in the tube, then drawing off the supernatant with a Pasteur pipet. Pipet 0.1 to 0.2 ml protoplast suspension onto a coverslip secured to a plastic Petri dish with a drop of silicone oil, or place protoplasts in glass Petri dish. Wait 5 min to allow cells to settle onto glass surface. While observing through the microscope, slowly add 0.2 ml PEG solution in small drops. The PEG goes through the protoplast suspension like a wave, forcefully agglutinating protoplasts at the moving front. The degree of agglutination can be controlled by adding more or less PEG solution (this solution also causes protoplasts to adhere to glass).

Incubate the protoplasts at room temperature for 15 to 40 min out of direct light. Slowly add 0.5 ml eluting solution. Wait 10 min. Add an additional 0.5 ml of eluting solution. Let stand 5 min.

Wash protoplasts free of PEG by the following dilution procedure: Add 0.3 ml protoplast culture medium (PCM) (table 9-2) to the protoplast suspension. Wait 5 min and remove 0.3 ml of liquid with a Pasteur pipet. Repeat 5 times. Always leave some medium covering the protoplasts while washing.

Culture the protoplasts in 0.2 to 0.4 ml PCM on the coverslip or glass dish. To keep the dish humid, add droplets of medium around the coverslip. Seal dish with Parafilm and incubate undisturbed in low light (50 lux). In 4 to 6 d division should occur. In 2 to 4 wk calli become visible. During this time add to the cultures increasing volumes of PCM in which the osmoticum (glucose) concentration is gradually decreased. When calli are 2 to 3 mm large, transfer them to differentiating medium.

TABLE 9-1.—*Solutions for protoplast fusion*

Wash Solution
0.5 *M* glucose
3.5 m*M* $CaCl_2 \cdot 2H_2O$
0.7 m*M* $KH_2PO_4 \cdot H_2O$
pH 5.7
PEG Solution
0.33 *M* PEG 1,540 or 0.09 *M* PEG 6,000
10.5 m*M* $CaCl_2 \cdot 2H_2O$
0.7 m*M* $KH_2PO_4 \cdot H_2O$
0.1 *M* glucose
pH 5.5
Prepare immediately before use
Eluting Solution
i
100 m*M* $CaCl_2 \cdot 2H_2O$
0.4 *M* glucose
ii
100 m*M* NaOH-glycine buffer, pH 10.5
0.4 *M* glucose
Prepare solutions *i* and *ii* separately and mix (1:1) just before use.

Second method:[2] Evans and coworkers (*32*) have a different method for fusing protoplasts from *N. tabacum* cv. Xanthi and *N. otophora*. This is a particularly useful model system for studying fusions with *N. otophora* because it possesses intranuclear heterochromic blocks that allow easy identification of heterokaryons (chs. 1, 2). Isolate protoplasts from leaves of each source using the high osmotic procedure detailed in chapter 8 (0.7 *M* mannitol as the osmoticum).

Sediment the protoplasts by centrifugation at 100 g for 5 min and resuspend them in a salts-sucrose solution (table 9-3). Centrifuge at 200 g for 5 min. The protoplasts should float to the surface while cells and debris sediment. Remove the protoplasts with a Pasteur pipet and transfer them to a solution containing the salts listed above but substituting 0.7 *M* mannitol for the sucrose.

Centrifuge the protoplasts at 100 g for 5 min and resuspend them in fresh salt-mannitol solution. Mix the two

[2]Prepared by T. A. Shalla, Department of Plant Pathology, University of California, Davis, Calif. 95616.

TABLE 9-2.—*Protoplast culture medium*[1]

A. Stock solution macro salts

	Grams per liter
KNO_3	25.0
NH_4NO_3	2.50
$CaCl_2 \cdot 2H_2O$	9.00
$MgSO_4 \cdot 7H_2O$	2.50
$CaH_2(PO_4)_2 \cdot H_2O$	.50
$(NH_4)_2SO_4$	1.34
$NaH_2PO_4 \cdot H_2O$	1.50

B. Stock Solution micro salts

	Milligrams per 100 milliliter
$MnSO_4 \cdot H_2O$	1000
H_3BO_3	300
$ZnSO_4 \cdot 7H_2O$	200
$Na_2MoO_4 \cdot 2H_2O$	25
$CuSO_4$	2.5
$CoCl_2 \cdot 6H_2O$	2.5

C. KI 75 mg/100 ml

D. Iron source

	Grams per liter
$FeSO_4 \cdot 7H_2O$	5.57
$Na_2 \cdot EDTA$	7.57

Aerate to dissolve

E. Vitamins

	Milligrams per 100 milliliter
Nicotinic acid	10
Thiamine·HCl	100
Pyridoxine·HCl	10
myo-Inositol	1000

F. 2,4-dichlorophenoxyacetic acid (2,4-D) 1 mg/ml

G. 6-benzyladenine (6-BAP) 1 mg/ml

H. α-naphthaleneacetic acid (NAA) 1 mg/ml

I. N^6-dimethylallyladenine (2iP) 0.01 mg/ml

To make 200 ml Protoplast Culture Medium, mix the above stock solutions at the volumes indicated.

A	milliliter	20.0
B	do	.2
C	do	.2
D	do	1.0
E	do	.2
F	do	.02
G	do	.02
H	do	.2
I	do	.2
Coconut milk (deproteinized)	do	4.0
N-Z amine Type A	milligrams	50.0
Xylose	do	50.0
Glucose	grams	13.7
H_2O	milliliter	to 200.0

Adjust pH to 5.7 with 0.1 *N* NaOH or 0.1 *N* HCl and filter sterilize through 0.22-μm filter.

[1]This medium promotes cell division and callus formation by protoplasts of *Nicotiana tabacum, glauca, langsdorffii, rustica, longiflora,* and *debneyi* as well as other Solanaceae and legumes (18, H. H. Smith, personal communication). It is essentially Kao's M-3 medium (*51*) but differs in iron source, addition of 2iP and elimination of MES buffer.

TABLE 9-3.—*Solution for protoplast purification*[1]

$CaCl_2 \cdot 2H_2O$	grams per liter	1.48
$MgSO_4 \cdot 7H_2O$	milligrams per liter	246
KNO_3	do	101
KH_2PO_4	do	27.2
KI	do	.16
$CuSO \cdot 5H_2O$	do	.025
Sucrose	grams per liter	210

Adjust pH to 5.8.

[1]Evans and others (*32*).

sources of protoplasts in a ratio of 1:1 and sediment by centrifugation at 100 g for 5 min.

Resuspend in F_5 culture medium (table 9-4) containing 40 percent PEG (mol. wt. 6,000) that had previously been heated to 37 C. Incubate for about 1 hr at 37 C. Remove a small sample and examine under the light microscope for the presence of fused protoplasts. If two or more protoplasts adhere to one another and subsequently round up into a sphere, fusion is judged to have occurred. When frequent fusions have occurred, remove the PEG by several washings with F_5 medium.

To detect heterokaryons, fix a small sample of the protoplast suspension overnight with 4 percent buffered formaldehyde, pH 6.8, containing 0.7 *M* mannitol. The remainder of the protoplasts may then be cultured in F_5 medium. Sediment the fixed protoplasts and place a drop of the sediment on a glass microscope slide together with a drop of carbol fuchsin stain (3 percent basic fuchsin in 70 percent

TABLE 9-4.—*F_5 culture medium for protoplast fusion*[1]

	Milligrams per liter
$CaCl_2 \cdot 2H_2O$	850
KNO_3	525
$MgSO_4 \cdot 7H_2O$	739
KH_2PO_4	353.6
$MnSO_4 \cdot 4H_2O$	11.15
H_3BO_3	3.1
$KnSO_4 \cdot 7H_2O$	4.3
$Na_2MoO_4 \cdot 2H_2O$	.125
KI	.408
$CoSO_4 \cdot 7H_2O$	.015
$CuSO_4 \cdot 5H_2O$	.025
$Na_2 \cdot EDTA$	18.75
$FeSO_4 \cdot 7H_2O$	13.9
NH_4NO_3	412.5
meso-Inositol	100
Glycine	1
Thiamine·HCl	1
Nicotinic acid	5
Pyridoxine·HCl	.5
Folic acid	.5
Biotin	.05
1-Naphthaleneacetic acid (NAA)	2
6-Benzylaminopurine (6-BAP)	1
Sucrose, weight/volume percent	1
Mannitol, weight/volume do	13

Adjust pH to 5.8.

[1]Frearson and others (*35*).

ethanol). Cover the sample with a cover slip and examine with bright field optics. Heterokaryons of *N. otophora* + *N. tabacum* can readily be detected in this manner because nuclei of the former species contain heterochromatin blocks that are absent in nuclei of the latter species.

Preparation and Transfer of Chloroplasts Into Protoplasts

A method of preparing clean chloroplasts from protoplasts of tobacco, as well as other species, has been devised by Edwards and colleagues (*43, 47, 68*). The method makes isolating physiologically active chloroplasts from tobacco a routine matter. Protoplasts are first purified by the aqueous liquid-liquid two phase method consisting of dextran and PEG containing sorbitol and salts. Chloroplasts are completely recovered from protoplasts following gentle rupture of the plasma membranes. Intact protoplasts collect at the interphase of the two phases. Debris from broken protoplasts remain suspended in the lower phase.

Protoplast purification by dextran-PEG two-phase method. Suspend washed protoplasts from 1 g of tissue in 0.6 ml solution I (table 9-5). Rathnam and Edwards (*68*) keep plants from which protoplasts are prepared for this use in the dark for 2 days and then illuminate them 30 min before collecting leaves. Thoroughly mix 5.4 ml Dextran-PEG solution in a 13- × 100-mm test tube by inversion. Gently add 0.6 ml protoplast suspension. Invert the test tube again to mix thoroughly. Centrifuge at 300 g for 6 min in a swinging bucket centrifuge. Intact protoplasts that collect at interphase tend to stick together. Transfer protoplasts with a Pasteur pipet into test tube.

Check protoplast preparation for purity and cellular integrity by mixing 0.1 ml of 2.5 percent Evan's Blue in 0.5 *M* sorbitol with 0.1 ml of the protoplast preparation on a microscope slide. Intact protoplasts exclude the dye and appear light against a blue background. If cellular debris is present, repeat protoplast purification procedure with Dextran-PEG solution and centrifugation.

Chloroplast isolation and transfer. If the protoplasts collected at the interphase are satisfactory, suspend them in equal volume of solution II (table 9-5) at 4 C. Attach a nylon net (20 µm mesh) over the tip of 1 ml disposable plastic syringe. To rupture protoplasts, suck them into the syringe through the net twice. Centrifuge the protoplast homogenate at 600 g for 90 sec and resuspend the sedimented chloroplasts in solution II (omit sodium pyrophosphate and BSA). The remaining steps are done at room temperature, 25 C.

In a 15-ml centrifuge tube, mix 1 ml of the chloroplast suspension (2×10^5/ml) with the recipient protoplasts (6×10^6) in 1 ml PCM. Slowly add 2 ml PEG solution to the chloroplast/protoplast mixture and gently but thoroughly mix. Wait 20 min. Gradually dilute the mixture by slowly adding 7 ml solution III (table 9-5) over 15 min.

TABLE 9-5.—*Solutions used to prepare and transfer chloroplasts in protoplasts*

Solution I

0.5 *M* Sorbitol
1 m*M* $MgCl_2$
1 m*M* KH_2PO_4
Adjust pH to 7.5

Dextran-PEG Solution

	Milliliter added	Final concentration
30 percent PEG 6000 (w/w)	1.1	5.5 percent
20 percent Dextran T_{40} (w/w)	3.0	10 percent
0.2 *M* Na_2HPO_4-NaH_2PO_4	0.3	10 m*M*
2.4 *M* Sorbitol	1.0	460 m*M*
Adjust buffer pH to 7.5		
cell suspension:	0.6	
5 m*M* $MgCl_2$		0.5 m*M*
50 m*M* Tricine[1]		5 m*M*
0.6 *M* Sorbitol		60 m*M*
Adjust buffer pH to 8		

Solution II

0.3 *M* Sorbitol
50 m*M* HEPES[2]
1 m*M* $MgCl_2$
2 m*M* KH_2PO_4
5 m*M* Dithiothreitol
5 m*M* Sodium pyrophosphate
0.1 percent Bovine serum albumin (BSA)
Adjust buffer pH to 7.6

PEG Solution

0.60 *M* aqueous PEG 1540
10.5 m*M* $CaCl_2 \cdot 2H_2O$
0.7 m*M* KH_2PO_4
0.1 *M* Glucose
Adjust pH to 5.5

Solution III

10 m*M* $CaCl_2$
0.1 *M* Glucose
0.7 m*M* $KH_2PO_4 \cdot H_2O$
Adjust pH to 5.7

[1]N-tris (hydroxymethyl)-methylglycine
[2]N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid

Allow the protoplasts to settle, pipet off solution III and resuspend them in 10 ml fresh solution III. Centrifuge at 100 g for 3 min.

To culture, resuspend the protoplasts in PCM at a concentration of 2×10^5/ml. Distribute 0.1 ml. droplets in 15- × 60-mm plastic Petri dishes and incubate in low light (200 lux) until cells begin to divide. Replace PCM as evaporation occurs.

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10

SELECTION OF BIOCHEMICAL VARIANTS FROM CELL CULTURE

G. W. Schaeffer[1]

Introduction

The major advances in understanding the nature of gene coding, the mechanisms of transcription, translation, and protein synthesis were made possible because of the availability of microbial mutants, which were easily identified and cultured. Plant scientists now need similar plant mutants to understand, define, and control plant growth and development. Selection of mutants from large numbers of individual or small aggregates of plant cells in liquid suspension or on agar should provide such tools. The rich reservoir of genetic variability present in plant cell cultures is strikingly demonstrated by the genetic mosaics found in sugarcane (*131*). In tobacco, a wide variety of morphological and anatomical types also may be recovered from *in vitro* cultures. The selection of variants at the biochemical levels should be equally rewarding.

Feedback Inhibition

The synthesis of many amino acids is controlled by feedback inhibition in which the end-product amino acid inhibits the first step in its own synthesis. Deregulation of the first-step enzyme permits the accumulation or overproduction of the end-product. This altered feedback is accomplished by selecting cells at inhibitory amino acid levels so that *only* deregulated cells grow rapidly. Thus, the nutritional value of plant tissue may be modified by the selection of deregulated, "feedback insensitive" cells. Also, analogs of specific amino acids can be used to apply specific selection pressures. Those that completely inhibit normal growth allow for the recovery of mutants that either metabolize the analog or have some by-pass mechanism to overcome the metabolic block. Therefore, nutritional cell types may be readily identified with growth inhibitor techniques. However, there are two major difficulties in the application of these techniques: Selection specificity is limited by the specificity of the analogs, and relatively few specific inhibitors are available.

Experimental Scheme

Tobacco selections: Haploid cells derived from anthers of *Nicotiana tabacum*, cv. Maryland *callerton*, have been selected against two amino acid analogs: aminoethyleysteine (AEC), an analog of lysine, and crotylglycine (CG), an analog of methionine. The steps in their culture and selection are illustrated and discussed below (fig. 10-1).

Cell Sources

Chop tissues (embryos and developing haploid seedlings) from anthers cultured *in vitro* on Nitsch and Nitsch's medium (*14*). Increase the tissue mass in Murashige and Skoog's medium (ch. 7) supplemented with either 1 mg/L indoleacetic acid (IAA) and kinetin or 2-4 dichlorophenoxyacetic acid (2,4-D) (*12*). The former medium yields partially differentiated stem-like structures in liquid culture, whereas the latter medium produces suspended cells and small cellular aggregates. Filter 2,4-D treated cells through an 18-mesh stainless steel sieve and collect on a 30 (50 μ) or 60 (230 μ) mesh sieve. Then mutagenize cells for 2 h with 0.2 percent ethylmethylsulfonate (EMS), wash with culture medium, and plate out on medium with

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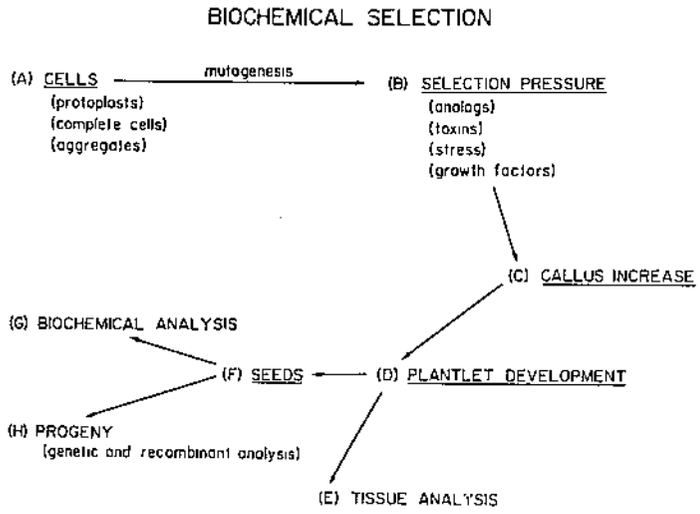

Figure 10-1.—Schematic for the selection of biochemical variants against inhibitors and the recovery of new cell types.

the selection pressure. Mutagenized cells are normally plated on 0.8 percent agar in 10-cm Petri dishes containing 40 ml of medium. However, when using expensive analogs, 5-cm dishes containing 4 to 5 ml are appropriate (Falcon makes a suitable dish with snap-on cover, appendix). Gamma irradiation of callus (6,500-10,000 rads) is a clean and effective method for inducing mutations as well. *N*-nitrosoquanidine (1 mg/ml) also may be used.

One of the major advantages of using embryonic tissues, such as those derived from anther culture, is that cells easily differentiate and form plantlets. Embryonic tissues maintain a strong morphogenetic potential, and cell selection against some analogs produces plantlets directly, for example, AEC with MS medium containing IAA and kinetin (1 mg/L).

Chemical mutagens require additional precautions. Horizontal, laminar flow, transfer hoods are designed to reduce contaminating particles but are not necessarily designed for user safety. Volatile mutagens may evaporate from open containers and other surfaces and create hazards. Therefore, maintain mutagens out of the air stream in plastic disposable glove boxes. Alternatively, use biohazard laminar flow hoods that keep samples aseptic and protect the operator as well (appendix). Sources of information on how to keep laboratory transfer conditions safe and aseptic include volume 41 of the Federal Register, which provides recombinant deoxyribonucleic acid (DNA) research guidelines along with environmental impact statements (*5*). An incineration device in the discharge duct may be desirable in some instances to prevent the contamination of environments if large quantities of mutagens are used. Ionizing radiation has advantages in terms of user safety. However, it produces albino plants more frequently than EMS or *N*-nitrosoquanidine, and this may be a disadvantage for some systems.

Selection Pressure

Biochemical selection pressure with amino acid analogs or analogs of other metabolites permits the selection of cells for which no obvious counterpart exists in nature and which could not be easily recognized at the whole plant level. Hypothetically, the analog may function in two ways.

First, as a feedback inhibitor in which it acts in the same manner as the natural amino acid inhibiting the first step in its synthesis. However, allosteric inhibition is often a precise and sensitively controlled regulation. Probably the analog could not control feedback inhibition as precisely as the natural amino acid. In the case of lysine synthesis, two separate and distinct pathways exist. In plants, this amino acid is apparently derived from pyruvate and aspartate forming α,ε-diaminopimelic acid, the immediate precursor of lysine. The initial reaction sequence, at which feedback inhibition occurs, involves an aldol condensation of pyruvate and aspartic-β-semialdehyde giving rise to the cyclic intermediate, 2,3-dihydropicolinic acid (*9*). AEC at m*M* concentrations should inhibit this enzyme to an undetermined degree much like lysine does. Thus, AEC and lysine at m*M* concentrations provide a selection pressure for lysine overproducers. Selection of cells insensitive to feedback inhibition can be accomplished with diploid cells because a change in any of the monomeric components should make the entire allosteric enzyme (frequently tetramers or multiples thereof) insensitive to feedback inhibition and allow the overproduction of the amino acids. Therefore, selecting amino acid overproducers from diploid cells is frequently possible (fig. 10-2).

The analog also may function as a true metabolic inhibitor in which it blocks a specific enzyme or reaction to cause a complete inhibition or death of all wild type cells. Only those cells resistant or with an alternative pathway for the synthesis of the product of the metabolic block would survive. Haploid cells and the use of mutagens enhance the probability of recovering true escapes or metabolic by-pass mutants.

Figure 10-3 illustrates the case for a complete inhibition between metabolic products 2 and 3. Only those cells with a by-pass mechanism shown by the sequence 2→ 2a→ 2b→ 3 would survive. The selection pressure may also expose variants with alternative pathways leading to product 4 (fig. 10-3) in which the selection is for cells with more than one pathway for the synthesis of the end product. Another possibility not illustrated exists in which

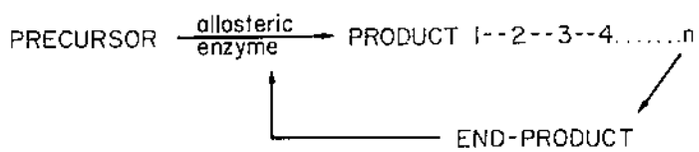

Figure 10-2.—Schematic showing allosteric inhibition of a metabolic sequence by the end products formed.

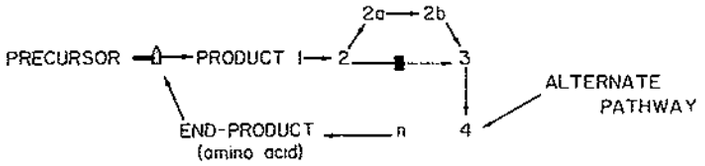

Figure 10-3.—Diagram for the selection of by-pass variants or alternate pathways.

cells are selected that inactivate or prevent the uptake of the inhibitor. This situation would be particularly useful for selecting cells against certain toxic substances such as herbicides and pesticides.

Additionally, a metabolic block by specific analogs in the pathways for amino acid synthesis may allow the accumulation of precursors required for secondary product synthesis. Thus increasing secondary product formation should be possible by introducing a metabolic block for amino acid synthesis, which at the same time provides the initial precursors for secondary production formation.

The more specific the inhibitor the more directed and predictable will be the phenotype of the recovered cells. Unfortunately, however, the mechanisms of inhibition are not precisely known for many analogs. In addition to the analogs of lysine [aminoethylcysteine (*3, 17, 20*), α-aminocaprylic acid (*10*), and ε-hydroxylysine (*20*)], other available analogs include a glutamic acid analog [methionine sulfoximine], analogs of methionine [methionine sulfoximine (*2*), ethionine (*21*), and crotyl-glycine (*8, 17*)], an analog of tryptophan [5-methyltryptophan (*19*)], and an analog of phenylalanine [*p*-fluorophenlyalanine (*16*)]. An analog and enzyme inhibitor of folic acid/dihydrofolate reductase [aminopterin (Mastrangelo, unpublished)], and an analog and enzyme inhibitor of thymidine/deoxthymidine monophosphate synthetase, [fluorodeoxyuridine (*15*)] have been tested (ch. 9). Another useful selection chemical is chlorate which selects for cells lacking nitrate reductase (*11*). Also, high levels of some amino acids such as threonine in tobacco and threonine plus lysine in rice inhibit cell growth through feedback inhibition (*4*, Schaeffer, unpublished). Threonine in tobacco cells prevents nitrate uptake, and resistant or escape lines have been recovered that can take up nitrate (*7*).

The compound 5-bromodeoxyuridine (BdUr) deserves special mention because of its usefulness. BdUr is incorporated into DNA during DNA synthesis. Cells with sufficient BdUr are killed when exposed to light, thus it becomes a powerful selection tool for the recovery of cells that did not go through DNA synthesis because they lacked specific metabolites (auxotrophs). These auxotrophic cells, which escaped death, can be recovered from BdUr-treated cells by adding the limiting growth factor after BdUr is removed from the medium. Thus, sycamore cells have been recovered that required auxin from mixtures of auxotrophic and autotrophic cells (*22*). Also, "leaky" cell types of tobacco have been selected, which respond to exogenous sources of hypoxanthine, biotin, *p*-aminobenzoic acid, arginine, and lysine (*1*). Additionally, Gavazzi and others (*6*) have reported a *Zea mays* cell type which requires proline.

Selection at the cell level is also useful for more general compounds or physical conditions as well. The selection pressures might be: (a) toxins, (b) high or low temperature, (c) high or low osmoticum, (d) pesticides, particularly herbicides, (e) environmental gases, (f) symbiotic and nonsymbiotic host-microbial associations, (g) hormones, (h) antibiotics, (i) specific inorganic salts including heavy metals, and (j) analogs of nucleic acid bases.

Systematic selection also requires optimizing the inhibition effects. The responses vary greatly when plant extracts, coconut milk, or casein hydrolyzates are included in the culture medium. Optimization requires an inhibitor concentration series for each selected medium. The cell selection process needs concentrations that inhibit the growth of wild type (normal) cells completely but avoid inhibitor concentrations beyond 2 to 5 times that required for 90 to 100 percent inhibition. A reasonable starting concentration for most amino acid analogs is 0.5 m*M*, and both higher and lower concentrations should be included for construction of the response curve. Methionine sulfoximine produces complete inhibition of tobacco cells on MS medium at 0.1 m*M*, for example. AEC requires higher concentrations for complete inhibition.

The size of the cell aggregates is critical in the selection process because the cell escape from inhibition is conditioned in part by the diameter of the aggregates. The greater the aggregation or differentiation, the greater will be the probability of obtaining transient escapes. This transient effect may be due to protection by a layer of dead cells or by transport effects within the callus that reduce the concentration of the inhibitor. Although this is not a problem with isolated protoplasts or tobacco cells grown in liquid suspension with 0.5-1 mg/L of 2,4-D, aggregation is a problem when macerated cells from compact callus are used as inoculum. Aggregation presents problems because (a) obtaining precise cell numbers is impossible and quantitation of the escape (mutant) events is imprecise, (b) autolysis of inhibited cells on the agar surface may provide growth factors for cells on the callus surface not in contact with agar, and (c) translocation of the inhibitor across aggregates may produce inhibitor gradients, it may be excluded by differentiated cells, or one might select for translocation and storage rather than for metabolic function. Recently a replica plating method was described in which the problems associated with aggregation are eliminated by spraying plant cells evenly on agar (*18*).

The preferred mode is to use cells from liquid suspension that have been sized from 2 to 200 cell aggregates by

fine sieve screening through 30- to 60-mesh screens (ch. 7). Alternatively, use callus macerated by mechanical chopping devices followed by size separations with cotton gauze, Miracloth, nylon, or Nitex screens of known size, or stainless steel sieve screens. Plate cells out on 0.8 percent agar in Petri dishes and tape the dishes to prevent dessication. Another effective method is to suspend the cells in the selection medium with 0.5 percent agar at 35 C and then pour this liquid agar containing inhibitor and cells as a thin layer over solidified inhibitor-containing medium made with 0.8-1 percent agar. This procedure has the advantage of keeping the cells or aggregates in intimate contact with the inhibitor.

Plantlet Development

Transfer genetic variants or putative mutants that escape the selection inhibitor three times to the same or higher inhibitor concentrations than was used during the initial selection to eliminate all cells sensitive to the inhibitor. After three transfer cycles on a nondifferentiating medium, the cells are ready for plantlet development or the initial biochemical analysis.

Callus Increase

Morphogenesis and differentiation of callus tissue has been extensively studied in *N. tabacum* and is induced by decreasing the auxin/cytokinin ratio (ch. 7). This is accomplished by reducing the auxin levels. Decreasing both auxin and cytokinins to one-tenth the level used for optimum cell division produces differentiated growth in tobacco. If 2,4-D is used in the cell increase phase, the auxin may be eliminated entirely (this is the case for some grasses as rice and sugarcane). Alternatively, decrease the ratio by increasing the cytokinin levels or by adding a more effective cytokinin. The key to differentiation in many systems appears to be the auxin or nutritional step-down, or both.

With the tobacco-AEC selection system, bud development occurs in the presence of the analog if macerated embryonic tissue is used as the source material. However, no roots occur in the presence of AEC, but they will develop when AEC-selected buds are transferred to MS medium containing IAA and kinetin (1 mg/L). All the major cytokinins including 6-benzylaminopurine (BA), 6-γ, γ-dimethylallylaminopurine (2iP), 6-trans-4-hydroxy-3-methyl-but-2-enylaminopurine (zeatin) function well in the culture of tobacco tissue; however, kinetin is the preferred cytokinin for inducing differentiation and morphogenesis (*4, 6*).

Tissue Analysis

Variants from amino acid analog selections should have alterations in the level of free or protein amino acids. In any case, the biochemical expression of the variant requires the separation and quantitation of the free and total amino acids. The separation, identification, and quantitation of the amino acid levels is frequently the most serious handicap because the methods are time-consuming and expensive.

Additionally, the biochemical expression of variants in the cell culture milieu may be different from leaf or seed tissue from the same cell line following regeneration and growth under greenhouse or field conditions. The expression of the variant in vegetative tissue may be different from that expressed in seed. For example, in leaf tissue, over 50 percent of the protein content may be Fraction I protein. Seed protein, on the other hand, has little Fraction I and a more diverse protein composition. Also some changes in free amino acid levels will alter the cell culture requirements for the regeneration of plants from callus.

Seeds

One obvious advantage of plant over animal cell cultures is the demonstrated expression of "totipoteney" in plants. Plant cultures permit the recovery of the cell-selected mutants as viable seeds. The fertile diploid plant (haploids must be doubled, ch. 5) may then be used for standard genetic analysis. The analysis of progeny is essential for the ultimate identification of true breeding mutants and for their use in plant breeding programs.

An additional advantage in having seeds from cells selected in tissue culture is that maintaining large numbers of cell lines in tissue culture is laborious; storing these same cell lines as seed is not. Also, cell lines maintained in culture over long periods may become genetically more diverse and undergo somatic selection (ch. 2). Instability is not a problem if the cell line can be regenerated and maintained through sexual reproduction and seed storage. On the other hand, somatic cultivation *in vitro* provides a potential reservoir of morphological and biochemical variants not easily found in seed. This potential is largely unexploited at the present.

Biochemical Analysis

Seed of 15 selfed lines of tobacco have been recovered using the *in vitro* selection techniques outlined above. Analysis of three cell isolates of tobacco, which had been induced to differentiate, showed large differences in the free amino acids but similar total amino acid content. Whether these differences are heritable or true breeding has not yet been determined. Four observations are relevant:

(a) Large carryover (epigenetic or metabolic) affects the rate and form of growth from the tissue culture environment to the greenhouse. However, the carryover effect can be minimized by standardizing the transfer techniques, such as choosing plantlets of similar size, age, leaf number, and degree of root development;

(b) The changes observed were not initially predicted due, in part, to a carryover effect from culture *in vitro;*

(c) An unidentified amino acid occurred in the selected cell type that was absent from the controls; and

(d) Seed from selected plants produced uniform progeny equal to or exceeding the growth rates of the controls. The selected lines appeared normal and vigorous in most respects.

However, the final conclusion pertaining to any selection product cannot be drawn until *progeny* have been analyzed biochemically.

Progeny Analysis

Inheritance and recombination patterns ultimately provide the most direct and true method for identifying selected mutants. However, other criteria may serve as early indicators of a mutation event. For example, mutations are rare events (about 1 in 10^5) and mutant lines will be stable even in the absence of the selection pressure. Additionally, the biochemical shift based on cell culture assays must agree with the change predicted.

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11

NICOTIANAS AS EXPERIMENTAL VIRUS HOSTS

R. W. Fulton[1]

Introduction

Many of the basic discoveries in plant virology have involved the genus *Nicotiana* and these were not merely coincidence. Such discoveries as virus transmissibility (*27*), filterability (*21*), aphid transmission (*1*), acquired immunity (*44*), local lesion infectivity assay (*17*), virus purification (*32*), virus mutability (*22*), infectivity of virus nucleic acid (*15*), and reconstitution of virus from nucleic acid and protein (*12*) have all used *Nicotianas* as experimental plants.

The advantages of tobacco mosaic virus (TMV) as an experimental object have often been pointed out. It is highly infective, stable, and highly concentrated in most tissues. Thus, many of the fundamental discoveries listed above have involved this virus and might have necessitated the use of *Nicotiana*. To some extent this is true, but a number of the qualities that made TMV a favorite object for research reflect the particular suitability of one or more *Nicotiana* spp. as experimental hosts.

The distinctive symptoms of TMV in *N. tabacum* undoubtedly played a part in drawing attention of early investigators to the disease. One of the qualities of some *Nicotiana* spp. that has made them particularly useful in plant virus research is the broad flat leaves on which various symptoms are particularly conspicuous.

Outlining some of the plant characteristics that have been exploited by virologists may be beneficial before discussing methods of using *Nicotiana* spp. in virus research. Particular species may be useful for one or several of the following distinct applications. The development of symptoms following inoculation may serve to *detect* the presence of virus in the inoculum. The type of symptom on one or more species may serve to *differentiate* the infecting virus from others, as least presumptively. The proportion of plants infected by a specific inoculum, or more accurately the numbers of primary (local) lesions produced, may serve as an *assay* of the amount of virus present in an inoculum. Species that contain a high virus concentration are desirable as a *source* of virus for purification and biochemical studies. One or more *Nicotiana* spp. are listed as a diagnostic, an assay, or a propagative host for 77 of the 170 plant viruses treated to date in the Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses (*16*).

Plant Characteristics Useful in Experimental Virology

Virus Susceptibility

Plant species differ markedly in degree of susceptibility to mechanical inoculation. To be useful as an experimental host, a plant should be readily infectible by a specific virus. *Nicotiana tabacum* was recorded by Thornberry (*40*) as the host having the greatest number of viruses, 115, and *N. glutinosa* as the host with the next greatest number, 78. Although these figures probably represent the popularity of these species as experimental plants as much as their susceptibility to viruses, they do reflect the general usefulness of members of the genus in plant virology.

Symptom Development

Susceptibility to viruses is not particularly useful unless infected plants develop unequivocal signs of infection.

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Rapid development of symptoms is also desirable. The broad, flat leaves of most of the *Nicotiana* spp. are particularly suitable for expressing a variety of symptom types, that is, mosaic patterns are readily apparent, as are patterns of ringspotting, etching, and local necrosis. The leaves of several species have been used extensively for comparing infectivity of virus preparations by comparing numbers of visible primary lesions. Many viruses produce discreet, necrotic spots within 3 to 5 d after inoculation. Lesions of some viruses, tobacco streak for example, may appear in less than 24 h.

Growth Habit

A species' growth habit is important in relation to specific experimental uses. Infectivity assays, for example, require many replicates of each of two or more virus preparations being compared. As pointed out by Samuel and Bald (*31*), the opposite halves of a leaf are similar in area and susceptibility and provide ideal areas for two replicates. Because susceptibility of individual leaves varies with their age, replicates must be distributed among leaves of each age. This is readily done on species such as *N. tabacum* or *N. glutinosa* with leaves produced successively on an apically dominant stem. Species producing a basal rosette of leaves, or species that branch, are less suitable for infectivity assay regardless of their susceptibility.

For many purposes using plants with leaves that are not so large requires less bench space. *N. glutinosa* is a conveniently sized plant. The *N. tabacum* × *N. glutinosa* F_1 hybrid was used as an assay plant for TMV for a number of years by Johnson (*23*) and others. However, the leaves were unnecessarily large. Thus, when the gene conditioning the necrotic response to local infection by TMV was incorporated in the *N. tabacum* genotype, varieties specifically adapted for experimental virology and infectivity assay of TMV were developed. Samsoun NN (*19*) and Xanth nc (*35*) are two such examples that combine the small, well-spaced, petiolate leaves typical of Turkish type tobacco with the necrotic response to TMV infection (ch. 3). These cultivars are useful for many viruses because accidental contamination by TMV, always a possibility, remains localized.

Plant size can also be controlled to some extent using various size pots, but well nourished, rapidly growing, young plants are most susceptible (ch. 4).

Wide, flat leaves on which virus symptoms appear are also well suited to mechanical inoculation. Inoculum can be applied more evenly, by any of several methods, if leaves are flat rather than rugose. The prominent midrib provides a ready method for differentiating half-leaf replicates in infectivity assays.

Availability of Genetically Uniform Seed

In experimental work, having large quantities of genetically uniform seed is a decided convenience. Because *N. tabacum* is cultivated in large amounts commercially, and for different purposes in different geographic areas, many types and varieties are available (appendix). Burley types, for example, develop much more prominent symptoms of potato virus Y than do many other varieties. Burley-type cultivars (or other types) differ among themselves in their reaction to certain viruses. Recently developed Burley cultivars may carry the N genes conditioning the necrotic response to TMV, which confers effective field resistance.

Variation Among Species and Varieties

Variation in reaction to specific viruses is characteristic of many *Nicotiana* spp. This provides a method for differentiating and, along with virus properties, to identifying viruses. Reactions of *Nicotiana* spp. to specific viruses have been characterized (*16*).

Variations exist among species and cultivars in the ease with which they can be infected by mechanical inoculation (*20, 42*). Variations in susceptibility are apparently not great enough to be exploited in disease control, although they are of interest in providing insights to the process of virus infection (*37, 38*).

Inoculating Methods in Experimental Virology

Mechanical Inoculation

Whole plants. The importance of *Nicotiana* spp. in experimental virology results in part from their suitability for mechanical inoculation. The infection of plants through slight wounds is not the natural means of dissemination of most viruses, although it is with TMV. Yet most of the advances in plant virology have depended on mechanical inoculation.

Wounding leaves by pricking or mutilating was a common, but inefficient, method of inoculation until Holmes (*17, 18*) pointed out the relation between numbers of local necrotic lesions on *Nicotiana* leaves and virus concentration. Numbers of local lesions also reflected the efficiency of inoculation. Gentle rubbing of leaf surfaces was found much more effective than pricking. Presumably, epidermal cells were wounded just enough to allow virus entrance, but not enough to kill them, which would prevent virus multiplication. However, in terms of particle numbers applied, mechanical inoculation is inefficient even with the best technique. Most estimates of the ratio of virus particles present to lesions produced are 50,000:1 or greater (*47*).

(a) Inoculum preparation:

Prepare inoculum by grinding tissue in a diluent with a mortar and pestle, rather than by simply applying sap from infected tissue. Undiluted sap allowed to dry

on succulent leaves may cause toxic reactions. Also, sap of many plant species contains substances that inactivate viruses. These are of various sorts chemically, either preformed in the tissue or arising from the oxidation of phenolic compounds after the tissue is ground. The inactivating effect is lessened by dilution so that moderately diluted inoculum may be more infectious than undiluted sap.

Prepare diluted inoculum by pipetting a small amount of sap, strained through cloth, into a larger volume of diluent and mixing. A more convenient method, and one just as reproducible, is to punch out discs of infected leaf tissue with a sharp cork-borer and grind these in the diluent. For greenhouse-grown tobacco, 100 discs of 8-mm diameter weigh about 1 g; one disc thoroughly ground in 0.99 ml of diluent thus represents a dilution of approximately 1:100. For routine virus transmission, precise dilution is not necessary; a square inch or so of tissue ground per 1- to 2-ml of diluent will suffice.

(b) The phosphate effect:

The most commonly used diluent in preparing inoculum is phosphate buffer. Thornberry (*39*) found that TMV was more infectious when applied in this buffer at pH 8 than when applied in water. This effect of phosphate has been repeatedly confirmed for a range of viruses (*47*) although the mechanism of the effect remains obscure. It occurs with relatively low concentrations (0.02 to 0.05 *M*) and under conditions where the buffering capacity is not sufficient to maintain the pH around 8.

Many other substances may be included in the buffer for specific purposes when preparing inoculum. Certain viruses such as tomato spotted wilt or tobacco streak are sensitive to oxidized phenolic compounds that form quickly after tissue is ground. To avoid their formation, a variety of antioxidants (.01 to .02 *M*) such as ascorbic acid, cysteine hydrochloride, 2-mercaptoethanol, sodium thioglycolate, or sodium diethyldithiocarbamate may be included in the inoculum. Several of these alter pH, and this effect must be compensated. Use only freshly prepared solutions because most of these compounds combine readily with atmospheric oxygen and become ineffective.

(c) Abrasives:

An important refinement in inoculating technique was described in 1934 by Rawlins and Tompkins (*29*), who dusted leaves with 600-mesh Carborundum (silicon carbide) before applying inoculum. Other abrasives had been used previously (*11, 43*), but they had been used rather harshly to produce visible injury to the leaves and, consequently, were less efficient.

Various finely divided abrasives are commonly used and appear to be about equally effective. Carborundum is a blue-grey powder; corundum (aluminum oxide) is white to pink; Celite (diatomaceous earth) is a fluffy white powder. The first two of these are easily dusted on leaves from a shaker with fine holes. Takahashi (*34*) recommended applying abrasive by sieving it through fine mesh nylon taffeta. Celite is difficult to sprinkle evenly on leaves, so it is usually added to the inoculum. Some grades of these abrasives contain small amounts of such deleterious impurities as oil, alkali (sufficient to alter pH), or iron.

The particle size usually used is 600 mesh, perhaps because this was the size originally used by Rawlins and Tompkins (*29*). Other sizes are also effective. The number of particles, rather than their size, is important (*7, 26*). Mesh size is not a particularly accurate way of designating particle size, which may be expressed directly in microns. Abrasives having an average diameter of 20 to 30 microns seem to approximate the size of 600 mesh.

(d) Methods of applying inoculum:

Inoculum may be applied effectively in many ways. The method of choice may depend on efficiency, convenience, the necessity to conserve small amounts of inoculum, and the necessity to avoid oxidation in extracts. Noordam (*28*) has described methods in detail.

One of the more common methods is to dip a small square of folded cheesecloth in the inoculum and then gently wipe the surface of leaves being inoculated. The method is advantageous if a considerable area of leaf is to be inoculated, as the cloth pad holds enough liquid so that it does not need to be constantly dipped in the inoculum. Also, the cloth surface is slightly rough and is uniform with different samples. One disadvantage is that a certain minimum volume of inoculum is required to saturate the cloth. The cloth may also pick up detrimental material from leaf surfaces, which reduces inoculation efficiency if the same cloth pad is used for many plants (*28*). Another disadvantage is that hands need to be decontaminated by thorough washing in soap and water between different inoculations.

Various devices have been used to apply inoculum to avoid repeated handwashing. Medium-stiff poster brushes may be used (*34*), as well as pipe cleaners (*24*), Q-tips, or simply the end of the pestle used to grind tissue. With all such devices, the hand that supports the leaf must be protected from becoming contaminated with inoculum. Paper towels are useful for this.

Another device that is particularly useful is a small glass spatula made by heating one end of a glass rod, flattening it at an angle while molten, and then grinding the surface flat with abrasive cloth. Small pieces of tissue can be homogenized quickly between two spatulas in a drop of buffer and applied to leaves with virtually no loss.

Air brushes, such as types used by artists, have been used with some success to apply inoculum (*25, 41*). A disadvantage of this method is that it produces an aerosol of virus particles that may be a source of contamination with stable viruses.

Inoculation by pinpricks has almost entirely been super-

ceded by more efficient methods. However, pricking is effective in some situations. Beet curly top virus is apparently confined to the phloem of its hosts so that no infection is obtained by applying virus to leaf surfaces. Pin pricks through droplets of juice placed in the axils of tobacco leaves have resulted in a fair amount of infection (*13*).

(e) Leaf rinsing:

Some early data (*18*) indicated that rinsing leaves with water immediately after inoculation increased the amount of infection. This was before phosphate buffer or abrasives were used and when more concentrated crude inoculum was necessary. Plant sap allowed to dry on leaf surfaces may be toxic and reduce infection by killing or damaging epidermal cells. Rinsing may then increase infection.

With inoculum prepared in phosphate buffer, rinsing leaves with water usually decreases infection (*45*). This response is probably also involved in the effect of quickly drying, inoculated, leaf surfaces. Yarwood (*46*) showed that this increased the amount of infection. Drying rate will be markedly affected, of course, by relative humidity, as well as by air movement. Do not apply so much inoculum to leaves that they remain wet for more than a few minutes.

Although the surface of inoculated leaves should be dried quickly, factors which promote this may result in a damaging loss of water from these leaves. Even the lightest rubbing of leaf surfaces with abrasive seems to enhance leaf desiccation. Leaf damage can be prevented by covering plants with damp (not wet) paper for several hours after inoculation or by shading them.

Do not inoculate the youngest leaves of a plant. They are much less susceptible than nearly mature leaves and are more easily damaged. Also these young leaves will develop systematic symptoms later, and these will be more apparent if the leaves have not been damaged by rubbing.

(f) Altering susceptibility:

The susceptibility of individual leaves and individual plants varies daily and seasonally. This may be demonstrated by using as inoculum a virus such as TMV that maintains its infectivity for considerable periods. Bawden and Roberts (*3, 4*) pointed out that succulent, shade-grown plants were more susceptible than those grown in full sunlight. This is readily demonstrable with field-grown tobacco, which usually requires about 10 times as much virus to obtain the same number of lesions as when using greenhouse-grown tobacco. Bawden and Roberts (*4*) also showed that darkening tobacco for one to several days before inoculation increased susceptibility.

This response to light is of some interest because of its bearing on mechanisms of infection. Troutman and Fulton (*42*) found that tobacco varieties varied in response to darkening. A variety innately susceptible did not increase in susceptibility when darkened, but a variety much less susceptible increased markedly in susceptibility when darkened.

These changes in susceptibility were correlated by Thomas and Fulton (*37, 38*) with changes in microscopic appearance of ectodesmata. The numbers of stainable ectodesmata increased when the resistant variety was darkened, but darkening did not affect the more numerous ectodesmata of the susceptible variety. This suggests that an early stage in the infection process involves movement of an infective principle from the surface of the epidermal cell, beneath the ruptured cuticle, through ectodesmata into the protoplast.

Emphasis should be given to the necessity for keeping everything virus-free that touches, or may touch, experimental plants. Tobacco mosaic virus is stable and may remain infective for years on benches and equipment. Any equipment used in making inoculations should be made virus-free by autoclaving, boiling, or baking with dry heat. Door handles and drawer pulls are often contaminated with virus. After hands are thoroughly washed, nothing should be touched before making inoculations except equipment known to be virus-free.

Protoplasts.[2] Replication studies of plant viruses have been hampered until recently by an incapability to synchronously infect plant cells. If a leaf, for example, that has been manually inoculated with a virus one day before, some of the cells will be in advanced stages of infection whereas others will only recently have become infected. Such a mixture of cells in various stages of infection does not provide a suitable system for analyzing the sequential, step-by-step events that occur during infection and viral replication. This problem has been largely overcome through the use of isolated plant protoplasts. In this approach, which was originally pioneered by Cocking and Pojnar (*10*) and greatly refined by Takebe and Otsuki (*36*) and Aoki and Takebe (*2*), cell walls are enzymatically removed from cells in the presence of an osmoticum, thereby releasing naked, intact protoplasts. When a suspension of these protoplasts is mixed with virus under suitable conditions, infection will occur simultaneously in a large proportion of them.

Subsequent events in infection and virus replication will take place synchronously in all of the protoplasts that become infected at the moment of inoculation. When properly incubated, the protoplasts will live and support virus synthesis for several days. If they are sampled at various intervals after inoculation and subjected to biochemical and microscopic analyses, determining the sequence of viral multiplication and pathogenesis is possible. The initial, exploratory work with this method was performed with TMV. Since then, protoplasts have been found to be suitable hosts for other plant viruses such as potato virus

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X, cucumber mosaic virus, and cowpea chlorotic mottle virus.

The following is a detailed description of the procedure for infecting isolated protoplasts of tobacco with TMV. Any of several cultivars of *N. tabacum* may be used. Among those that have been infected with TMV are White Burley, Bright Yellow, Xanthi, Xanthi nc, and Samsoun. Use plants 50 to 70 d old, about 30 to 40 cm, and growing vigorously. Maintain them in a greenhouse at 20 to 28 C. Select leaves that are deep green, tender, but almost fully expanded (15 to 20 cm). The procedure for isolating protoplasts to be inoculated with viruses is the two-step, digestion method of Takebe and Otsuki (*36*). Isolate protoplasts from tobacco leaf mesophyll under high osmotic conditions using mannitol (ch. 8). They should be inoculated immediately after isolation.

Inoculation is the most critical step in the procedure. Slight variations in the concentration of the components, pH, or timing can easily result in unsuccessful inoculation or toxicity to the protoplasts. To 35 ml of distilled water add 5 ml of 0.2 *M* potassium citrate buffer, pH 5.2, and 6.3 g mannitol. Make to 50 ml with distilled water. Add 0.1 ml poly-L-ornithine (mol weight $\simeq$ 120,000, 1 mg/ml in distilled water) and 50 µl of purified TMV (2 mg/ml water). Incubate 10 min at 25 C. Immediately before inoculation, centrifuge the protoplast suspension and resuspend in a volume of 0.7 *M* mannitol sufficient to give a concentration of 3-10 $\times$ 10^5 protoplasts per milliliter. Determine protoplast concentration with a hemocytometer. To the protoplast suspension, add an equal volume of the TMV-poly-L-ornithine mixture to give a final TMV concentration of 1 µg/ml. Incubate for 10 min with occasional swirling. This 10-min incubation must include the 2 min of centrifugation used for the first of the following washes. Wash three times by centrifugation at 100 g and resuspend in 0.7 *M* mannitol and 0.1 m*M* $CaCl_2$.

Resuspend the washed, inoculated protoplasts in 50 to 100 ml of the incubation medium (table 11-1). To prepare this medium, mix all of these components except the antibiotics (loridine and rimocidin) in 100 ml distilled water. Adjust pH to 5.4. Autoclave and make back to 100 ml with sterile, distilled water. Allow to cool and add the antibiotics.

TABLE 11-1.—*Protoplast incubation medium*

	Concentration
Mannitol	.8 *M*
$CaCl_2$	10 m*M*
KNO_3	1 m*M*
$MgSO_4$	1 m*M*
KH_2PO_4	0.2 m*M*
KI	1 µ*M*
$CuSO_4 \cdot 5H_2O$	0.01 µ*M*
Loridine	300 mg/liter
Rimocidin	10 mg/liter
Adjust pH to 5.4 with 0.1 *N* KOH	

Incubate the protoplasts in an Erlenmeyer flask stoppered with aluminum foil at 25 C under continuous, white fluorescent light (intensity not critical).

To evaluate the progress and degree of infection, protoplasts may be sampled at various intervals after inoculation for infectivity measurements, staining with fluorescent antibodies, or electron microscopy (ch. 8). To test infectivity, swirl the incubation mixture until the protoplasts are dispersed throughout the incubation medium. Remove 10 ml and centrifuge at 100 g for 2 min. Disperse the pelleted protoplasts in 3 ml of 0.05 *M* phosphate buffer, pH 7.0, and freeze. After thawing, grind the preparation with a ground glass homogenizer. Use the homogenate to inoculate leaves of a suitable local lesion host such as *N. glutinosa*. With TMV, a maximum rise in infectivity should be reached 24 to 48 h after inoculation of the protoplasts.

Fluorescent antibody staining is the most rapid means of assessing the degree of infection attained. Remove a 1 ml sample from the incubation mixture and centrifuge at 100 g for 2 min. Resuspend the protoplasts in two drops of 0.7 *M* mannitol. Place two drops of the thick protoplast suspension on an albumin-coated glass slide (Meyer's albumin—egg white:water:glycerol, 1:1:2). Quickly dry the drop in a stream of warm air (a hair dryer is suitable) and place the slide in acetone for at least 30 min. Wash the slide in several changes of 0.05 *M* phosphate buffer (PBS), pH 7.0, containing 0.85 percent NaCl for 2 h. Drain the slide and cover the protoplasts with several drops of fluorescent antibody solution. Place the slide in a moist chamber for 1 h. Rinse in two changes of PBS for 1 h. Mount the protoplasts under a cover slip in PBS-glycerol (1:1). Controls should be prepared using inoculated protoplasts stained with conjugated normal serum and noninoculated protoplasts stained with conjugated antiserum. Examine the preparation using an ultraviolet microscope with barrier filters for observing fluorescence in the wavelength range of 400 to 650 nm. Specific staining will appear as a bright, apple-green fluorescence. Protoplasts also may be embedded and sectioned for electron microscopy (ch. 8). Infection is indicated by the presence of virus particles or inclusion body components, or both.

Insect Transmission

Tobacco has been used repeatedly in research involving insect transmission of virus, particularly by aphids. Experimental procedures involving insects require (a) cages to confine insects to particular plants or plant parts, (b) a method of transferring insects from one plant to another, and (c) a method of removing insects at the end of an experiment. Detailed descriptions of methods are given by Swenson (*33*) and Noordam (*28*).

Aphids are usually handled in cages containing one or

several plants. Cages may be devised to fit the upper part of a pot or may be larger containers with hinged doors. Aphid transmission often involves timed feedings of short duration. For such experiments no cage is needed; individual aphids are watched during their feeding and moved to healthy plants at appropriate intervals.

Small, plastic "pill-box" cages, consisting of a shallow, plastic cylinder with one or both ends covered with fine mesh net may be clamped on individual leaves. Leafhoppers feed readily through the mesh and the cages are easily transferred. Such cages damage *Nicotiana* leaves, and they are not generally suitable for aphids.

Aphids may be transferred in groups from one plant to another by detaching a leaf or part of a leaf on which they are feeding and placing it on another plant. If the detached leaf contains virus, place it on a piece of clean paper on the recipient plant to avoid contact transmission of the virus. As the leaf dries, aphids leave it and crawl to the recipient plant.

Large numbers of aphids can be transferred by holding the plant on which they are feeding horizontally over another plant and tapping the stem of the first plant to dislodge the insects. They are dislodged from glaucous plants more readily than from hairy ones. Collect aphids on a large sheet of paper and, by continued tapping, transfer them to vials for experimental starvation periods.

Individual aphids are most conveniently transferred with a small, camel-hair brush that has slightly moistened bristles. To move aphids that are feeding, first induce them to withdraw their stylets from the leaf by gentle prodding; to brush an aphid from the leaf surface will often injure the stylets and prevent further feeding. When depositing an aphid on a healthy plant, first place it on a piece of paper and allow it to crawl from that to minimize the possibility of virus transmission from the brush.

Aphids are usually removed from plants at the end of an experiment by spraying or fumigating them with a suitable insecticide. When only a few aphids have been transferred, do not attempt to remove and destroy them individually because viviparously produced offspring may be overlooked and left behind.

Contamination of experimental plants with unwanted aphids is not uncommon. For this reason, culture aphids away from experimental plants and make aphid transfers in a room or chamber isolated from experimental plants. Use regular control measures to eliminate stray aphids in greenhouses or chambers reserved for incubation of plants.

Graft Transmission

Most viruses infecting tobacco can be transmitted mechanically; a few, however, have not yet been so transmitted. For these and for special experiments, grafting is a means of transmission in the absence of a vector.

Nicotiana spp. are readily intergraftable. As far as has been determined, *Nicotiana* can be intergrafted with most other herbaceous, solanaceous plants. The types of grafts commonly used are wedge, tongue, or approach grafts, and tissue implantation (ch. 4). A detailed discussion of grafting has been presented by Bos (*8*).

With wedge grafts, trim the base of a growing stem terminal to a wedge shape after removing most of the leaves. Make a cut part way through the stem of the stock plant, insert the wedge, and bind it in place with budding rubber, grafting tape, or self-stricking latex tape. Accurate juxtaposition of the cambial layers of scion and stock is not essential with young stem material. Virus present in the scion (or stock) will move into the other member of the pair after a certain degree of healing. Bennett (*5*) demonstrated differences among several viruses in the time required to move across a graft union as it healed.

With wedge grafts, or any graft involving scion tissue separated from its own roots, protection from desiccation is essential until vascular elements are formed at the union. Large numbers of plants may require a chamber with controlled, high humidity. For single plants, polyethylene bags are useful. Plants inside the bags must be protected from direct sunlight.

To make tongue grafts, prepare a scion with a diagonal cut across a young stem. Make a similar cut to remove the top of the stem of the stock. Make shorter cuts lengthwise from the cut ends of the stock and scion stems. Fit these together so that there is maximum contact of cut surfaces, bound and protected with plastic bags. With such grafts, the diameter of the scion should not be greater than the diameter of the stock.

To make a graft rapidly, that will transmit virus, cut a vertical slit completely through a young stem. Into this fit a slice of stem tissue from an infected plant and trim it to a double wedge shape. Bind this with grafting tape; virus implanted tissue will move into the stock when the tissues heal together.

Dodder Transmission

Virus transmission by dodder, a leafless parasitic vine, has been reviewed by Bennett (*6*), and the techniques involved have been described by Noordam (*28*). Many *Cuscuta* spp. have been used; some parasitize certain seed plant groups readily and will not grow on others. *C. campestris* and *C. subinclusa* are two commonly used species. A table of *Cuscuta* spp. and the viruses reported to have been transmitted has been compiled (*14*).

Usually one starts dodder from seed and maintains it vegetatively by transferring shoots to new host plants. Make such transfers at intervals of several weeks because, when the dodder begins to flower, vegetative growth may soon cease.

Sow dodder seed with seed of a host such as clover. As both germinate, the dodder parasitizes the clover seedlings.

A more certain method is to germinate the dodder seed on filter paper in a Petri dish until it is 2 to 3 cm. Lightly tie the tip against the stem of a host, such as tomato or beet, and immerse the "root" end or peg of the dodder in a small tube of water to prevent desiccation until the haustoria contact the vascular system of the host. Young dodder seedlings seem to attach to nonhairy stems more quickly than to hairy ones.

The basis for virus transmission by dodder is that it forms a bridging, vascular connection between an infected and a healthy plant. This connection can be made between plants that cannot be intergrafted. Cochran (*9*) demonstrated that virus passes between dodder-connected plants more quickly when the recipient plant is darkened while the donor plant is exposed to light.

Many viruses do not infect the dodder but simply move through the vascular system. To free dodder of such viruses, grow it on a host not susceptible to the virus. Some viruses, on the other hand, infect the dodder. With these a bridge need not be formed between infected and healthy plants. Transfer of a dodder stem tip to a healthy susceptible plant will transmit virus.

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12

DISEASE RESISTANCE

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Introduction

Tobacco has been an important crop in the Eastern United States for more than 350 years. Soon after its cultivation began, the colonists concluded that tobacco "wore out the soil," necessitating continual movement to virgin soils and long rotations (*137*). Pathogen buildup in the soil was undoubtedly a major factor in this situation (*86*). During the past 60 years, great progress has been made in breeding disease resistant tobacco. Available resistant cultivars as well as government acreage and poundage control programs have allowed and encouraged production to become stabilized in specific areas of the country.

The scientific effort to breed disease resistant tobacco in the United States was initiated by J. Johnson at Wisconsin about 1912. His first release, Havana 142, a black root rot resistant cultivar, was introduced in 1922 (*88, 90*). In 1919, W. D. Valleau began breeding disease resistant tobacco in Kentucky (*39, 195*), and in 1931, E. E. Clayton began research in this area with the USDA (*122*). Much of the disease resistance now available in tobacco cultivars came from the pioneering work of these three men, each of whom spent more than 35 years in this research area. Valleau (*194*), Clayton (*26*), and Graham and Burk (*56*) have published reviews on breeding tobacco for disease resistance.

Van Der Plank (200) used the terms "vertical" and "horizontal" to indicate the likelihood that a particular source of resistance will be effective against all biotypes of a pathogen (horizontal) or only one or a few biotypes (vertical). Generally, vertical resistance, usually controlled by a single gene pair, is less desirable than the usually polygenic horizontal type. However, economic necessity and expediency can rule out strict adherence to this principal. Relative availability of the kinds of resistance, the degree of pathogen variability, and ease of pathogen dispersal must temper such judgments. Both types of resistance have been effective in reducing tobacco disease losses. Since the late 1930's, much effort has been directed towards transfer of apparently vertical resistance from wild *Nicotiana* spp. into *N. tabacum*, and some of these efforts have been successful in reducing losses to tobacco planters.

Diseases and Their Relative Importance

Pathogens that cause the most destructive tobacco diseases and the common names for the diseases are listed in table 12-1. Among these pathogens, the viruses, *Peronospora tabacina, Erysiphe cichoracearum,* and the nematodes are obligate parasites, multiplying only in living, infected host tissues. Among the viruses, tobacco mosaic virus (TMV) is easily transmitted mechanically (ch. 11). Cucumber mosaic (CMV), tobacco etch (TEV), potato virus Y (PVY), and tobacco vein mottle (TVMV) viruses are transmitted by aphids. Leaf fungal pathogens that produce abundant airborne spores in the field, *P. tabacina, E. cichoracearum, Alternaria alternata, Cercospora nicotianae,* and *Colletotrichum destructivum*, are usually more easily and rapidly dispersed than soilborne pathogens. Environmental factors, such as moisture and temperature, are critical limiting factors for infection, disease development, and pathogen reproduction for all the pathogens (*83, 106, 210*).

Disease loss estimates by the Tobacco Disease Loss Evaluation Committee (*186, 187*) indicate that the most serious losses in the United States and Canada are caused

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by black root rot and black shank. They totaled $55 and $60 million, respectively, from 1974 to 1976 (*186, 187*). Diseases that have caused losses of $10 to $40 million in the same period are, in decreasing order: nematodes, barn rot, mosaic, weather fleck, brown spot, and etch. Losses of $2 to $5 million were caused by frogeye, *Pythium* rot, sore shin, blue mold, angular leaf spot, vein banding, bacterial wilt, and damping off. Other diseases that caused serious losses in localized areas include *Fusarium* wilt and wildfire. Vein mottle is becoming increasingly important since it was first described in the United States in 1972 (*52*).

Many diseases occur in only certain geographic areas. For instance, the major nematode pathogens, *Meloidogyne* spp., are not serious north of Maryland and Kentucky, while black root rot is not as serious in the Florida-South Carolina area as it is farther north. In Europe, the Near East, and Australia, blue mold has been severe in recent years, but it has not yet appeared in southern Africa. Black root rot has long been a serious European problem. White mold has long been serious in Europe, Africa, and Asia, but it does not occur in North America. Cucumber mosaic virus and bacterial wilt cause much greater losses in the Far East than in the United States (*106*).

Symptoms

Laboratory and greenhouse studies are essential for conclusively identifying pathogens. Symptoms can be variable, so assuming solely from symptoms that a particular pathogen is responsible for a disease may be dangerous. Nevertheless, each disease has certain characteristic symptoms that can greatly aid identification and which can be expected to appear following inoculation. The following summarizes the symptoms of the most common diseases. In addition, books by Lucas (*106*), Hopkins (*83*), and Wolf (*210*) contain detailed descriptions and many pictures.

Weather fleck. This disease usually develops on recently mature leaves. The characteristic symptoms are mono- or bifacial, round or angular, necrotic flecks 0.5 to 6 mm but usually 0.5 to 1 mm diameter. The lesions are dark for about the first day, then bleach to a light color (*66, 112*).

Mosaic. These symptoms are commonly caused by TMV, however CMV causes indistinguishable symptoms on tobacco leaves. The characteristic symptom is an extremely mottled, blotched pattern of light and dark green areas. The virus moves systemically, producing these symptoms in immature inoculated leaves and subsequently produced leaves. On older leaves, large necrotic areas can develop, producing the so called mosaic burn (*106, 210*).

Etch. The tobacco etch virus occurs as several distinct strains and produces leaf symptoms that vary with tobacco type and environment. Veinclearing is usually the initial symptom. Mottling resembling that caused by TMV and CMV can occur, but it does not extend into the tip leaves like that caused by the mosaic viruses. Chlorotic spots, the most common symptom, usually develop into necrotic lesions (*106*). Sometimes the necrotic lesions resemble large weather fleck lesions. Veinal necrosis and browning can occur on older leaves.

Vein banding. Vein banding symptoms show considerable variability on different cultivars under different environmental conditions. Yellowing and bleaching of the interveinal leaf tissue, leaving a band of dark green in and along the veins, are the most common symptoms. Mild strains of the virus cause a faint, mosaic mottle in young leaves. In severe cases, the veins and midribs darken, becoming necrotic, and turn brown to black. In these severe reactions, the dark necrosis may even extend into the central stem. Light, necrotic spots, resembling those caused by TEV, can develop in advanced infections (*106*).

Vein mottle. These symptoms include intermittent bands or blotches of dark green tissue along the leaf veins without chlorotic spotting and crinkling. In field plants, severe systemic necrotic spotting can develop, resulting in the loss of affected leaves (*106, 128*).

Bacterial wilt. This disease was first recognized as causing serious losses in tobacco in Granville County, N.C., giving rise to another name for it, Granville Wilt (ch. 13). Being a disease favored by high temperatures, the symptoms first appear on seedlings in the tropics, often killing them. In temperate climates, symptoms appear 2 to 4 wk after transplanting to the field, as a wilting of one or more leaves. Characteristically, half of the leaf wilts first. Later the leaves become light green, gradually yellow, then brown. The stalk first yellows in the xylem and soon darkens to brown or black, which spreads into the cortex. Infection usually occurs through the roots. At first, only one or a few roots are darkened, but eventually most of the roots decay. A pearly, grey, slimy, viscous, bacterial ooze that can be squeezed from cut vascular elements of affected stems is diagnostic (*83, 106, 210*).

Wildfire. On both seedlings and field plants, this disease first appears as circular, yellow leaf spots. Smaller, brown, dead areas quickly develop in the lesion centers. Diffusable toxins produced by the bacteria are responsible for a yellow halo. With time, the lesions increase, often run together, and can result in irregular lesions covering much of the leaf. The leaves may become distorted and ragged looking. Small lesions appear in the capsules, and the seed can become infected. The extent of the halo varies with the weather. In wet weather it may extend little beyond the necrotic area caused by rapid bacterial invasion of the tissue (*83, 106, 210*). On greenhouse-inoculated plants, halos develop within 4 to 7 d.

TABLE 12-1.—*Major tobacco diseases and causal pathogens*

Disease	Pathogen
	Air Pollutant:
Weather fleck	Ozone
	Viral:
Mosaic	Tobacco Mosaic Virus (TMV)
Mosaic	Cucumber Mosaic Virus (CMV)
Etch	Tobacco Etch Virus (TEV)
Vein banding	Potato Virus Y (PVY)
Vein mottle	Tobacco Vein Mottle Virus (TVMV)
	Bacterial:
Bacterial wilt	*Pseudomonas solanacearum* E. F. Sm.
Wildfire	*Pseudomonas tabaci* (Wolf & Foster) Stevens
Angular leaf spot	*Pseudomonas angulata* (Fromme & Murray) Holland
	Fungal:
Damping off and stem rot	*Pythium* spp.
Black shank	*Phytophthora parasitica* var. *nicotianae* (Breda de Haan) Tucker
Blue mold	*Peronospora tabacina* Adam
White mold or powdery mildew	*Erysiphe cichoracearum* DC
Barn rots	Several
Black root rot	*Thielaviopsis basicola* (Berk. & Br.) Ferraris
Brown spot	*Alternaria alternata* (Fr.) Keissl.
Frogeye leaf spot	*Cercospora nicotianae* Ell. & Ev.
Fusarium wilt	*Fusarium oxysporum* (Schlecht) Wr. f. sp. *nicotianae* Johnson
Anthracnose	*Colletotrichum destructivum* O'Gara
Sore shin	*Thanatephorus cucumeris* (Frank) Donk
	Nematode:
Root knot	*Meloidogyne* spp.
Brown root rot	*Pratylenchus* spp.
Cyst nematodes	*Heterodera* spp.

Angular leaf spot. The major difference between the bacterium causing this disease and that causing wildfire is the absence of toxin produced by the angular leaf spot organism so that there is no halo. Otherwise, the symptoms are similar except that the necrotic area is usually darker and more angular than that caused by the wildfire bacterium (*83, 106, 210*).

Damping off and stem rot. These are diseases of seedlings and young plants. Initially a brown, watery, soft rot is visible at or near the soil line, girdling the hypocotyls, and causing the plants to fall over. The roots may or may not be infected. With the rate dependent upon abundance of moisture, the plants rot and die leaving a mass of dried, shriveled, light tan remains. Affected seedlings often are in circular patches surrounded by healthy plants (*83, 106, 210*).

Black shank. As indicated by the name, this disease affects the base or shank of the stalk. Infection is usually through the roots, which are seriously affected. In young plants, the stem becomes brown to black and girdled at the soil line, and the plant damps off. The darkening can extend up the stalk several centimeters and down into the root system. When older plants become infected, the leaves suddenly wilt and turn yellow while portions of the root system blacken and die. The stalk becomes girdled with a black lesion running for 30 cm or more above the ground. If the diseased stalk is split longitudinally, diagnostic, horizontal, plate-like discs are usually visible in the dried, blackened pith. Large, circular, concentrically zonate lesions, up to 8 cm diameter, can occur in the basal leaves following wet weather (*83, 106, 210*). This disease can be devastating to tobacco in the greenhouse as well as in the field.

Blue mold. This disease derives its name from the bluish or grayish, downy, fungal growth that commonly occurs on the lower surface of infected leaves. These leaves often become twisted downward, cupped, and distorted. The upper surface of affected leaves may be normal appearing or yellowish green. Circular, yellow spots or blotches commonly occur on the upper surface. The affected leaf area becomes necrotic and light brown. Stem infection occurs when inoculum is abundant. Young plants are more susceptible than older ones. Blue mold is a common seedling problem in the United States, uncommon in the field. In Europe it causes serious field losses (*106*). Blue mold can cause extensive damage to greenhouse tobacco, especially if the temperature is cool.

White mold or powdery mildew. This disease also derives its name from the appearance of the fungus on the leaf surface. Small, white patches of powdery, sporulating, fungal growth appear and spread over the leaf and stem surfaces. With time, tiny spherical, black, fruiting bodies appear in this white fungal material (*83, 106*).

Barn rots. Into this general grouping fall a number of diseases of harvested, curing tobacco, including house burn, pole rot, sweat, web rot, butt rot, freckle rot, shed burn, stem rot, stalk rot, and others. Because little or nothing has been done on resistance to these disorders, their symptoms are not described here. Lucas (*106*) gives good descriptions of the symptoms.

Black root rot. Blackening and decay of the root system are the main symptoms of this disease. The pathogen can cause damping off of small seedlings when infection occurs at the soil line. On roots of older plants, the fungus causes black, rough lesions that result in severe root pruning. In greenhouse plants, the leaves often become dark green as the infected plants become stunted and wilted. In severely affected field plants, the leaves turn pale green to yellow and stunting is severe. Leaves of infected field plants wilt on hot, sunny days, but with sustained hot weather, many plants recover, make new growth, and mature a crop (*83, 106, 210*).

Brown spot. Characteristic symptoms of this disease are necrotic, brown, roughly circular lesions 0.5 to 5 cm diameter that contain concentric zonations and are often surrounded by a halo of yellow tissue. These symptoms are

found on older, mature leaves (*83, 106, 210*). When inoculum is plentiful, pinpoint necrotic lesions occur on the young, expanding leaves (*172*) and larger, sunken, dark brown, necrotic lesions occur on the veins, midribs, stalks, and seed capsules. Brown spot is a common field disease in the warmer, humid areas, but chance infections are rare in the greenhouse (*83, 106, 210*).

Frogeye leaf spot. Typical frogeye lesions are necrotic, roughly circular, 2 to 15 mm diameter and have thin, light tan, gray, or almost white centers surrounded by a narrow dark-brown margin. These lesions are nearly identical to the lesions produced by TMV in cultivars having the *N. glutinosa* factor for mosaic resistance. The main difference is the presence of minute, black dots that are fruiting structures of *C. nicotianae* in frogeye lesions. Mature leaves are affected in both greenhouse and field plants when the environment is hot and humid. Natural infections are sometimes a greenhouse problem in warmer climates.

Fusarium wilt. When leaves of field plants gradually yellow, wilt, and dry on one side of the stalk, *Fusarium* wilt is the likely cause. While this one-sided wilt is symptomatic, the entire plant can be affected. When the infected stalk or root is sliced, the interior is stained brown to nearly black, without the wet ooze present with bacterial wilt. Otherwise, the symptoms of the two diseases are similar. The one-sided effect occurs when infection occurs through one to several roots on one side of the plant (*83, 106, 210*).

Anthracnose. Anthracnose is mainly a disease of seedling leaves, but it can also spread from transplants into field plants during wet weather. Small, sunken, dull green spots first become noticeable on older leaves. These spots enlarge to about 3 mm diameter. They are dry, become thin, and develop a grayish-white center with a raised, brownish, watersoaked border. The pathogen also causes oblong, reddish-brown lesions on the midribs and stalks. Tiny, sterile, black structures and pink spores are produced by the fungus in the lesion centers (*83, 106, 210*).

Sore shin. The major symptom of this disease is darkening and decay near the soil line on the stems of seedlings and older plants. Girdling of the stem can cause it to break so that the plant falls over. The roots usually remain healthy until the plant dies. The pith in the infected area becomes dry and brown and interspersed with gray areas of fungal tissue. Being favored by cool, wet weather, the disease occurs in plant beds and in the field shortly after transplanting. Occasionally, the symptoms extend up the stalk and into the bases of the bottom leaves, causing them to rot and fall off (*83, 106, 210*).

Root knot. As indicated by the name, the characteristic symptom of this disease is knotting or galling of the roots. These galls range from scarcely noticeable to 3 cm or more in diameter. They are typically spherical, but larger galls are often irregularly shaped. When close together, continuous, elongated swellings are formed. Large galls usually contain many of the minute, rounded, white adult females, whereas small galls may contain only one. In the absence of other pathogens and with favorable rainfall, the disease has little effect upon the aboveground plant parts. But under stress, infected plants show drought injury and stunting. Such plants become light green to almost yellow, and the leaves become yellow and necrotic around the margins (*106, 210*).

Brown root rot. This disease is characterized by root lesions ranging from yellow through brown to almost black that girdle the smaller roots, often break open, and cause the outer layer to slough off. The resultant pruning of the smaller roots becomes severe enough by midsummer that the affected plants easily can be pulled from the soil. Often clusters of newer, healthier roots develop near the soil surface. The effect on aboveground plant parts is similar to that of root knot (*106, 210*).

Cyst nematodes. Diagnostic symptoms are the dark brown, oval cysts, approximately 0.5 mm diameter that are attached anywhere on the root system. Stunting, wilting, and reduction in the number of roots are also consequences of infection (*106*).

Determining Disease Resistance

Inoculation Techniques

To determine the relative resistance or susceptibility of *Nicotiana* spp. to tobacco pathogens, specific inoculation techniques have been developed for most of the important ones. The novice should start on a small scale with a few susceptible plants before launching into large scale, definitive tests. Proper techniques for growing the plants, including watering, fertilizing, insect control, and control of unwanted diseases are essential (ch. 4).

Weather fleck. The disease inciting agent in this case is not an organism, but gaseous air pollutants, the principal one being ozone. In Menser and Heggestad's facility (*113*) for ozone fumigation of tobacco, 0.06 to 1 ppm of ozone is generated by passage of a controlled flow of gaseous oxygen through dual corona discharge ozonizers to which a controlled voltage is applied. The generated ozone is passed into a lighted, plant growth chamber equipped with filters on the ambient air intake to eliminate ozone from external sources. Both ozone concentration and exposure time control host response. Commonly employed dosages are 0.15 to 0.60 ppm ozone for 1 to 6 h (*117*). The plants are placed into an area of the chamber that is equipped for relatively quiet air movement. Chamber humidifying equipment should be adjusted to provide at least 70 percent relative humidity (RH) to induce rapid and consistent sto-

matal opening. Before fumigation is started, the plants should be in the lighted chamber, with humidity and temperature adjusted, for at least 1 h.

Plants to be fumigated must be grown uniformly. Nitrogen nutrition should be at an intermediate level (*116*), and the plants should not be kept under excessively long photoperiods before fumigation (*115*). Rapidly growing tobacco plants, 8 to 9 wk old and grown without moisture stress, are ideal. Recently mature leaves are most susceptible (*114*). The symptoms develop within 24 h after fumigation.

Tobacco viruses. See chapter 11.

Bacterial wilt. The bacterium causing this disease, *Pseudomonas solanacearum*, can quickly lose virulence in culture from mutation and selection. Mutants differing from the wild type in virulence are readily identified by streaking bacterial suspensions on a medium containing 1 percent peptone (wt/vol), 0.1 percent casein hydrolysate, or 0.033 percent casamino acids (Difco), 0.5 to 1 percent glucose, 1.7 percent agar, and 0.005 percent triphenyl tetrazolium chloride in distilled water. Prepare the triphenyl tetrazolium chloride as a sterile, 1 percent aqueous solution and add the proper amount aseptically to the melted, sterile, agar medium before pouring dishes. Keep the streaked dishes at 32 C for 36 h before inverting and examining under a dissecting microscope with obliquely transmitted light. Mutant, avirulent, or weakly virulent colonies are deep red with a narrow, light bluish border, and the virulent colonies are white or white with a light red center (*63, 91*). Maintain virulence by covering colonies, growing on solid media, with sterile mineral oil (*92*), or place 3 to 5 loopfuls of bacteria into 5 ml of sterile, distilled water in a capped test tube (*93*).

For resistance evaluation (*209*), produce inoculum on potato dextrose agar (PDA) (*92*), a nutrient agar containing 1 percent tryptone, 0.1 percent yeast extract, 0.5 percent glucose, and 1.7 percent agar (*91*); nutrient broth incubated on a shaker; or other appropriate media. Obtain bacterial suspensions from agar media by pouring sterile water over the cultures and gently agitating them, or from broth by transferring a small amount into water. Use a spectrophotometer to determine the bacterial concentration in the inoculum. Set the spectrophotometer at 600 nm with 100 percent transmittance using a water blank. For greenhouse inoculations, Gwynn (*63*) uses inoculum with 75 percent transmittance and for field inoculations, 90 percent.

Winstead and Kelman (*209*) developed two techniques for inoculation of greenhouse tobacco plants. In the first, place a drop of bacterial suspension, containing about 7.6×10^8 cells per milliliter (*59*), in the axil of the second or third fully expanded leaf below the apex. Use a needle or other sharp object to puncture the stem through the drop. In the second inoculation method for potted plants, cut the lateral roots along one side of the plant about 2.5 cm from the stalk with a scalpel to a depth of approx 4 cm and pour 10 ml of bacterial suspension over the severed roots. Maintain adequate soil moisture and temperatures at or above 30 C during the day and 20 C at night. During the winter, use artificial light to increase the photoperiod to 15 h. Symptoms develop by 15 d after inoculation. Both techniques give good indices of degree of resistance, but the latter technique gives wider differentiation between resistant and susceptible plants.

The method of Moore and others (*121*) is used to inoculate field plants. Prepare a large volume of the virulent bacterial cells in water to give 8×10^6 cells per milliliter. Supply about 50 ml of this suspension per plant when transplanting by hand. For machine transplanting, dilute the inoculum to 6×10^6 cells per milliliter and supply about 85 ml per plant (*63*). When plants for the field are drawn from the plant beds, considerable root wounding occurs. Use of bacteria in the transplant water was found to be more effective than wounding and inoculating established field plants (*121*).

Wildfire. To maintain virulence, store *Pseudomonas tabaci* in sterile water at about 5 C (*197*). Beef-peptone broth (8 g/L Difco Bacto nutrient broth) is also satisfactory for maintaining virulence for a considerable time at 5 C and, with addition of 8 to 10 g glucose per liter, is an excellent medium for increasing inoculum at room temperature. Wooley and others (*211*) have developed a synthetic culture medium for *P. tabaci* that favors toxin production. If necessary, the active filtrate can be purified (*181*). Prepare inoculum for assessing the resistance of large plant populations by making a 1:100 dilution in water of a bacterial suspension from 16 to 24 h nutrient dextrose broth cultures kept at 20 to 24 C (*152*). The inoculum concentration should be less than 5×10^6 cells per milliliter if wounding is used in inoculation (*124*).

In nature, *P. tabaci* invades mainly through wounds or stomata of leaves that are water soaked as a result of rain storms. Some invasion occurs through stomata without watersoaking, but the effect is much less severe (*21*). When the stomata are open, artificially water soak the leaves by spraying the lower surface with water under pressure. If a bacterial suspension is then sprayed or poured over the leaf of susceptible cultivars, abundant infection occurs. Pressurized spraying of an inoculum suspension gives water soaking and inoculation concomitantly (*197*). Use spray inoculation for tobacco plant beds and field plants.

Klement (*94*) developed an inoculation technique for identifying phytopathogenic *Pseudomonas* spp. and for physiological studies using tobacco. This technique is described in chapter 13.

The simplest and speediest method for inoculating large numbers of individual, greenhouse plants was developed by Silber (*150*). He fabricated a self-feeding, inoculation device by repeatedly driving a sharp nail through the in-

side of the metal cap of a small, screw cap container. The metallic protrusions (about 0.5 mm diameter) on the outside of the cap act as cutting edges to wound the leaf. Place the inoculum suspension into the vial and screw on the cap. Then place a rigid, flat object wrapped in cheesecloth under a young leaf, invert the vial, and press the cap into an intervenial area on the leaf surface to induce wounding and introduce the inoculum. Do not inoculate or crush the midrib and main side veins as the bacteria may grow in the crushed tissue and produce symptoms on resistant plants (*152*). Include plants of at least one known resistant and one known susceptible tobacco cultivar or breeding line as controls in all inoculations.

Angular leaf spot. *P. angulata* differs little from *P. tabaci*. The two organisms are maintained and cultured similarly. The relation of wounding and water soaking to infection is the same for both (*22*). *Nicotiana* material resistant to one is usually resistant to the other. Because *P. angulata* is of less economic importance than *P. tabaci*, little specific information has been published on inoculation methods for *P. angulata*. However, inoculation methods used for *P. tabaci* are satisfactory (*21, 22, 197*). The following method has been successfully used. First, dust a young leaf with carborundum. Then dip a cheesecloth pad into an inoculum suspension (a 16 to 24 h broth culture diluted 1:100 with water). Wipe it over the leaf to gently wound it, and introduce the inoculum. In the Kentucky method (*101, 197*), which also works well, the broth culture is diluted 1:250 with water and sprayed under pressure onto the lower surface of young leaves. Extensive necrosis develops in 2 to 3 d if water soaking has been used. If the leaves are not water soaked, necrotic lesions less than 2 mm diameter, and having little or no halo, develop about 6 d after inoculation.

Damping off and stem rot. For these diseases little work has been done to develop specific inoculation techniques for *Nicotiana*.

Black shank. As is true with many fungal plant pathogens, isolates of *Phytophthora parasitica* var. *nicotianae*, the black shank pathogen, show a wide variation in virulence (*4*), which may decrease or be lost after prolonged maintenance in culture (*106, 208*). The organism can be cultured on several agar media. Use oatmeal agar (dehydrated form from Difco) or prepare it by soaking 50 g of Quaker Oats in distilled water for several hours, blending it, and adding 15 g agar and distilled water to 1 L (*4, 69, 103, 175*). Potato dextrose broth also is sometimes used for increasing inoculum (*192, 208*). Stock cultures remain viable when stored at 15 to 20 C (*4*).

A useful medium (table 12-2) for isolation of *P. parasitica* from infested soils was developed by Flowers and Hendrix (*44*). Make up the PCNB, penicillin G, and nystatin as separate solutions and add after autoclaving. Pour the medium into Petri dishes, allow it to gel, and chill to 3 C before use. Seed the dishes with 1 ml of a suspension of infested soil in 0.5 percent agar (1:50), incubate in the dark at 24 C for 36 h, and then gently wash off the soil with tap water leaving white, dense, easily identifiable colonies of *Phytophthora* (about 1 mm diameter). These are readily distinguishable from the larger, less compact, *Pythium* colonies that also are usually present.

TABLE 12-2.—*Medium for isolation of* Phytophthora parasitica *var.* nicotianae[1]

$NaNO_3$	grams per liter	2
$MgSO_4 \cdot 7H_2O$	do.	.5
KH_2PO_4	do.	1
Yeast extract	do.	.5
Sucrose	do.	30
Thiamine·HCl	milligrams per liter	2
Gallic acid	do.	425
Rose bengal	do.	.5
Pentachloronitrobenzene (PCNB)	do.	25
Penicillin G	units	80,000
Nystatin (mycostatin)	do.	100,000
Agar	grams per liter	20

[1]Flowers and Hendrix (*44*).

Several greenhouse inoculation methods have been published for *Phytophthora parasitica* var. *nicotianae*. They are of two general types, stem and root. Hendrix and Apple (*69*) developed a stem inoculation technique that gives good differentiation between plants having high monogenic resistance to race 0 of the pathogen and those lacking this resistance. However, this technique is not sufficiently precise to differentiate levels of horizontal resistance. Wills and Moore (*208*) modified the technique to obtain differentiation in average lesion size among cultivars in replicated tests. They concluded that their method also was too variable to identify individual, horizontally resistant plants in segregating populations. To use this technique, deeply wound the cortex of vigorously growing, nonflowering plants having seven or more leaves by drilling a hole (4 mm diameter) with a bit or cork borer. Prepare an aqueous, mycelial suspension by blending with water the mycelium from a 10-day-old, potato dextrose broth culture that has been incubated at room temperature. Place a portion of the suspension in a small piece of cheesecloth. Secure this into the wound with tape and cover with a plastic film for 72 h.

Root inoculation methods for the black shank pathogen have been described and refined by several workers (*4, 43, 103, 175, 192*). In Apple's method (*4*), grow nine seedlings in methyl bromide-treated soil in 20 by 20 by 5-cm aluminum trays. When they reach the five-leaf stage, inoculate by flooding with 100 ml of mycelial suspension. Prepare the suspension by blending the contents of a 2-week-old oatmeal agar plate with 200 ml of water. Disease symptoms appear in 2 to 3 d. The method of Fan (*43, 175*), as modified by Litton and others (*103*), is more laborious but probably more precise. Sow about 10 to 15 seeds per line on the surface of moist vermiculite in a 50-ml plastic

tube having a hole (6 mm) cut into the base for entry of water, nutrients, and inoculum. Place the tubes in the holes, cut to the appropriate size, of metal supporting racks. Before germination, supply moisture by lowering the racks into water; after germination use one-third strength Hoagland's solution. Inoculate plants about 25 d after seeding by submerging the tubes to within 1 cm of their top in a suspension containing 1,000 *P. parasitica* var. *nicotianae* zoospores per milliliter of Hoagland's solution.

Position the racks so that the bases of the tubes are about 2.5 cm above the surface of water circulating in a temperature-controlled tank. Milk coolers, without lids, can be equipped as water tanks for this use by attaching a small-bladed, electric stirer for water circulation and an immersion-type, heating coil with thermostatic control. The water temperature is maintained to give 31 to 32 C in the root zone. Use of this or some other technique to obtain uniform and optimal moisture and temperature in the root zone considerably improves the precision of the method (*103*).

Produce inoculum in 125-ml flasks containing 40 ml of sterile oatmeal agar. Keep cultures at 23 to 24 C for about 25 d. Then scrape the mycelial mat from the agar surface and incubate for 48 h in a Petri dish containing 5 ml of one-third strength Hoagland's solution. Sporangia will form in the mycelia. Then, to induce release of zoospores, chill the dish for 30 min at 10 C. Two mycelial mats per liter of one-third Hogland's solution give an approximate concentration of 1,000 zoospores per milliliter along with mycelial fragments (*103, 175*).

Susceptible plants wilt 3 d after inoculation and die in 10 to 14 d. Wash the root systems and score the disease severity using a scale ranging from 0 to 5. Assign a rating of 0 if symptoms are absent and 5 if the roots are dead and discolored. Score individual plants in segregating populations or compute an average for the plants from each tube. The symptoms are more severe than occur with field infestation (*103*).

A large amount of inoculum is needed to infest field plots with *P. parasitica* var. *nicotianae*. To produce it (*58*), pour clean oats into a 1.9-L (½-gal) mason jar up to ½ to ¾ capacity, add 300 to 400 ml of water, secure aluminum foil over the mouth, and autoclave for 2 to 3 h. Aseptically place a small plug of the fungus, growing on an agar medium, next to an oat kernel having a broken seed coat, and allow the fungus to grow 4 to 12 wk in the dark at room temperature. Mix the infested oats from three such jars with an equal volume of moist sand and evenly distribute for 30 m into a 5 to 10 cm furrow in moist soil in the field. Then furrow (bed) up the soil over the inoculum and set the tobacco plants into this furrow within 1 d. Plant a susceptible cultivar in alternate rows to maintain a high inoculum level for the next year and to serve as a control.

Blue mold. Because the blue mold fungus, *Peronospora tabacina*, will not grow on artificial media, it must be maintained on living tobacco plants or in cold storage. If the conidia are stored attached to the conidiophores on tobacco leaves at 5 C and 30 to 40 percent RH, germination will decrease to 15 percent in 90 d and to less than 1 percent in 131 d (*70*). However, viability can be maintained for at least 25 mo by storage in liquid nitrogen (*7*). To do this, wash conidia from the leaves into distilled water, concentrate on a filter by mild vacuum filtration, and immediately suspend them in 15 percent dimethyl sulfoxide (DMSO) v/v (volume ratio). Pipet 0.5 ml of the DMSO suspension into a small ampoule, seal, and put it at −20 C for 1 to 2 h. Finally, quickly transfer the ampoule to a liquid nitrogen refrigerator.

To prepare the frozen, stored conidia for use, rapidly plunge the ampoule into a 40 C water bath for about 45 sec or until the ice melts, remove and open the ampoule, and dilute the contents in water. Use the water suspension to inoculate several tobacco plants on which sufficient inoculum will be produced to inoculate many plants (*7*).

Apply inoculum by atomizing a water suspension of conidia onto the aerial surfaces of the plants. Inoculum concentration is usually less critical than plant age, atmospheric temperature, or humidity. Conidial suspensions as dilute as five per milliliter will result in some infection if other conditions are suitable, although much higher concentrations are usually employed. However, low concentrations could be useful in selection for low levels of resistance (field resistance) (*71*). Use plants of resistant and susceptible cultivars as checks.

Plants of susceptible tobacco cultivars can be infected by *P. tabacina* at any age, but as they become older disease severity decreases (*23, 71*). Clayton (*23*) found that 6- to 8-week-old plants growing in 5-cm clay pots are ideal in age and size for screening for resistance. Leaf wetness or RH approaching 100 percent continuously for 8 h or more after inoculation is essential for rapid and severe blue mold development; some will develop with as little as 2 to 4 h of high humidity after inoculation (*71*). Regardless of other conditions, no blue mold will develop if the plants are kept at 30 to 32 C or above (*30, 73*). Hill and Green (*73*) found that the disease spreads most rapidly at night temperatures of 20 to 24 C, regardless of day temperatures. Clayton and Gaines (*30*) concluded that 24 C d and 16 C night temperatures with 96 h at or near 100 percent RH are optimal. Under these conditions the fungus will sporulate 6 to 7 d after inoculation. Blue mold can be controlled in greenhouses by raising the temperature to 43.5 C for 4 consecutive hours per wk (*30*).

White mold or powdery mildew. *Erysiphe cichoracearum*, the white mold pathogen, like *P. tabacina*, must be maintained on tobacco leaves.

Inoculation with *E. cichoracearum* is relatively simple. The fungal spores (conidia) are produced abundantly on the surfaces of infected leaves. Before use of these leaves

as an inoculum source, dust or blow off the bulk of the conidia present so that the inoculum will consist of newly produced, highly viable conidia. About 24 h later the new crop of dry conidia are dusted, shaken, or blown from the infected leaves onto the leaves of the plants being inoculated.

Alternatively, prepare water suspensions having known concentrations of conidia by washing them off with a jet of water, centrifuge (3 000 g for 6 min) the resultant suspension, measure the conidial concentration with a haemocytometer, and make an appropriate dilution. A concentration of approximately 350,000 condia per milliliter of glass-distilled water gives about the same degree of infection as dusting with dry conidia (*34*). Minimum, optimum, and maximum temperatures for infection of tobacco by *E. cichoraceurum* are 5, 23.5, and 25 C, respectively. The disease fails to develop if the average maximum-minimum temperature is 25 C or higher (*138*). This sensitivity to high temperature is probably one of the reasons why the disease does not occur on tobacco in the United States.

Relative humidity has less effect on disease development than temperature. Conidial germination occurs over a wide range of RH, but it is highest at RH's near saturation. The subsequent growth of the fungus on tobacco leaves is favored by drier conditions, saturation deficits of 7 to 9 millibars being optimal (*34*).

Barn rots. Because relatively little research has been done on these disorders, no inoculation methods are given here.

Black root rot. The fungus causing black root rot, *Thielaviopsis basicola*, has a wide host range affecting many plants in addition to tobacco (*106*).

T. basicola can be easily isolated from infested soil by spreading some of the soil over the surface of dises (5-mm thick) of carrot (*214*). Atomize the soil with water until it is quite wet. Then transfer the dises and soil to a Petri dish. After 3 d incubation at room temperature, wash the soil from the dises and return them to the dish. In 4 to 6 d white colonies of *T. basicola* develop on which black masses of macroconidia ultimately appear. Macerated, infected root tissue can be substituted for infested soil (*176*). If dry, soak the roots in water before macerating them with a mortar and pestle. Smear the macerated roots over the surface of potato tuber dises. Carrot and potato dises can be used interchangeably for the two methods. A grayish-white layer of endoconidia develops on the surface of the dises in 48 to 72 h. Using a dissecting microscope, transfer bits of aerial mycelium or fungal mat to acidified potato dextrose agar (PDA). After about 48 h of growth, make transfers from the outer edges of apparently pure colonies to pH 6 PDA, the conventional medium for growing *T. basicola* in pure culture.

A useful medium (table 12-3) for isolation of *T. basicola* from soil in dilution plates was developed by Papavizas (*125*).

TABLE 12-3.—*Medium for isolating* Thielaviopsis basicola *from soil*[1]

$CaCO_3$	grams per liter	1
Glucose	do.	2
Yeast extract	do.	2
Agar	do.	20
V-8 juice	milliliters per liter	200
Sterilize by autoclaving, then add to cooled, liquid medium:		
Pentachloronitrobenzene (PCNB)	grams per liter	.5
Oxgall	do.	1
Nystatin	milligrams per liter	30
Streptomycin sulfate	do.	100
Chlortetracycline·HCl	do.	2

[1]Papavizas (*125*).

T. basicola is a highly variable fungus, readily losing virulence in culture (*176, 177*). Biotypes differing in pathogenicity are common, so inoculum should be made from several isolates if it is to be used in screening for resistance in segregating host populations. The pathogen exists in two distinct forms in nature, having either brown or gray pigmentation on PDA (*176*). Gray isolates are often used, but they are less pathogenic and endure dormancy less well than brown isolates. Addition of methyl green (1:100,000) or malachite green (1:400,000) to PDA or other media preferentially inhibits growth of the gray type. Hence, these dyes can be used to facilitate selection of highly pathogenic brown isolates (*177*). Brown isolates occasionally mutate to gray on culture media, in soil, or in infected plants. Gray isolates also mutate to brown in infected plants. Both types produce mycelial and other mutant types on media that support abundant growth, such as PDA.

Maintain virulent isolates by storing infected roots in envelopes and reisolating pure cultures as needed (*176*). *T. basicola* withstands dessication well. Another maintenance method involves pouring small aliquots of a fungal suspension into tubes of sterile soil or sand and storing them. Isolates can be kept at room temperature for 3 yr or longer by either method without losing virulence. Troutman (*190*) transferred isolates to carrot decoction agar, allowed time for chlamydospores to be produced, and then froze them for long-term storage.

For many years the conventional inoculation method for *T. basicola* has been to transplant 4-week-old seedlings to infested soil in 5-cm clay pots (*28*). The infested soil is obtained from fields known to have severe black root rot. Alternatively, susceptible plants can be planted in large containers containing sterilized soil, artificially inoculated by pouring a fungal suspension into the root zone, given several months for disease development, and then using the soil from the root zone as a source of infested soil. The latter source avoids contamination with *Phytophthora parasitica* var. *nicotianae* or other pathogens.

Thoroughly mix the stock infested soil from either source with an equal amount of sterilized soil as it is used. Use susceptible and resistant cultivars as checks. If pre-

cise comparisons are desirable, pot half of the plants in comparable sterilized soil. After 8 to 10 wk growth in the infested soil, wash the soil from the roots and estimate the percentage of damage to the root system. The weight of plants from infested soil can be compared with that of those from sterilized soil.

Do greenhouse studies on black root rot in the fall, winter, or early spring, when maintaining sufficiently low temperatures (17 to 23 C) is easier and disease development is favored (*28*). Disease severity is greatly reduced at 26 C and is negligible at soil temperatures above 30 C (*89*). Soil acidity is also important. The minimum pH should be about 6.0 (*41*) as the disease fails to develop at pH 5.6 or below. With proper pH, temperature control and sufficient infestation, the conventional inoculation method works well.

A quicker and more precise inoculation method was developed by Troutman (*190*). When using this method, sow the seed on vermiculite in bottom-watered, perforated, aluminum loafpans. Transplant each plant at the four-leaf stage to artificially infested vermiculite held in a depression of a 12-cup, aluminum muffin pan. Modify the pans by drilling five 32-mm ($1\frac{1}{4}$-in) holes in the base of each cup to allow introduction of water, nutrients, and inoculum. Place the muffin pan, with plants, into an appropriate sized cakepan.

Inoculum can be grown on several media, but Troutman recommended a carrot-glucose broth, made by steaming 200 g of diced carrots in 500 ml of water for 1 h, straining this through cheese cloth, and adding 20 g of glucose and water to 1 L. Place a 100 ml aliquot of the broth into a 200-ml prescription bottle, inoculate using a frozen culture, and allow it to grow for 10 to 12 d at room temperature.

Prepare the inoculum suspension by diluting 200 ml from carrot broth cultures to 5:1 with a proper nutrient solution for tobacco. Troutman used the nutrient solution of Steinberg and others (*174*), but other nutrient solutions with a buffered pH close to 7 should be satisfactory. At this pH higher temperatures are somewhat less inhibitory to disease development than at lower pH (*41*, *190*), but even at pH 7 little or no black root rot will develop at 30 C (*41*). The amount of nutrient solution used for diluent can be varied if there is a desire to adjust the inoculum concentration to a constant level. Pour the inoculum suspension into the cakepan, lower the muffin pan containing the vermiculite into the pan and keep it there until the vermiculite is saturated. (A vermiculite-filled, 12-cup muffin pan absorbs about 700 ml.) Then remove the muffin pan, allow to drain, transplant tobacco seedlings into the pan, and set it back into an empty cakepan. Add a fresh supply of nutrient solution to cakepan. When necessary, replenish moisture with water in the same way. Allow the plants to grow 3 to 5 wk in the muffin pans before rating root damage or weighing the plants.

Gayed (*49*) developed the following procedure: inoculate wounded leaf discs by moistening them with a standardized suspension of *T. basicola* endoconidia. Float the discs on tap water in covered Petri dishes. Necrotic lesions will develop in 3 to 4 d. Count the lesions or rate disease severity on a 0 to 10 scale. Lesions develop on all tobacco cultivars, including those deriving black root rot resistance from *N. debneyi*. However, the average severity is much less on resistant cultivars. Relative resistance of moderately resistant cultivars cannot be differentiated by this method.

In New Zealand, breeding lines have been indexed for black root rot resistance outdoors in plant beds (*212*). Produce inoculum of *T. basicola* by first flooding an endoconidial suspension onto a sterilized sand-oatmeal medium (dry sand:water:oatmeal, 1:1:5). Grow the inoculum for 5 wk in the dark at room temperature. Seive the inoculum, mix it with sterilized dry sand, evenly spread over the seed bed, and incorporate it into the top 5 cm of soil. Keep the beds well watered for 4 wk before seeding. Eight weeks after sowing pull and subjectively rate 30 plants of each line for root rot severity. This method gives excellent statistical differentiation of degrees of horizontal resistance, but probably does not work well for resistance derived from *N. debneyi*.

Field screening for resistance has been done for many years by transplanting the test lines and cultivars with known degrees of resistance to a field that has had a history of severe black root rot infestation. Transplant as early as possible to maximize the chances for cool temperatures during plant development. The amount of aerial growth and severity of root lesions give the best index of relative resistance (*131*). With these scores, a discriminant function can be used to aid in ranking resistance.

Brown spot. The fungus causing brown spot, *Alternaria alternata*, can be isolated readily from brown spot lesions (J. R. Stavely, unpublished). Surface sterilize a small portion of leaf tissue involving the edge of a lesion by immersion in 0.6 percent sodium hypochlorite (Clorox:water, 1:8) for 12 min, rinse in sterile water and aseptically place on sterile V-8 juice agar in a Petri dish. Within a few days, *A. alternata* can be identified. Transfer from pure isolates to V-8 agar slants.

A. alternata grows and sporulates well on V-8 juice agar with or without pH adjustment (Stavely, unpublished). Prepare the unadjusted medium by autoclaving 200 ml of V-8 vegetable juice with 300 ml of water. In a separate flask autoclave 16 to 20 g of agar with 500 ml of water. If 3 g per liter of $CaCO_3$ is added before autoclaving, the pH is elevated sufficiently so that separate autoclaving of the V-8 juice and agar is not necessary (at the pH of V-8 juice the gelling properties of the agar are destroyed by autoclaving). Abundant, dark *A. alternata* conidia will form after 3 to 4 d. Flood Petri dishes containing V-8 agar with a conidial suspension and incubate the dishes under constant fluorescent light (3 by 10^3 ergs/cm^2-sec) at about 27 C.

A. alternata is a ubiquitous fungus and nonpathogenic or weakly pathogenic isolates are commonly isolated. Therefore, the pathogenicity of new isolates should be determined by inoculating leaves or plants as given below. Loss of pathogenicity in culture can occur over months or years. This can be avoided by pouring sterile mineral oil over V-8 agar slant cultures and storing them in a refrigerator at about 2 to 5 C.

Inoculum suspensions are prepared by agitating or scraping the conidia from the surface of the V-8 agar into water containing about 0.01 percent Triton B-1956 or another suitable wetting agent (*168*). Determine the conidial concentration with a haemocytometer and dilute to the desired level. Concentrations of 30,000 to 120,000 conidia per milliliter give moderate to extreme brown spot severity under proper temperature and humidity conditions (*168, 172*). On leaf discs, 10,000 conidia per milliliter are sufficient (*163*).

If potted plants are used, they should be about 2 to 3 mo old, vigorous, and growing in 10- or 15-cm pots (ch. 4) (*168, 172*). Plants of this age have sufficient mature leaf area for a good assessment of relative resistance. Brown spot lesions produced on mature leaves are quite different from those on immature leaves (*172*) to the extent that resistance or susceptibility of immature leaves is often unrelated to the reaction of mature leaves (*171*). Uniformly spray the conidial suspension to runoff onto all mature, aboveground plant surfaces. Allow plants to dry and then place them in controlled environment chambers.

Postinoculation temperature and humidity are critical for successful infection. The optimum temperature is close to 20 C (*168*). Keep the inoculated leaves wet for 48 to 72 h following inoculation (*173*). The total period of wetness is critical; disease severity increases proportionately as the time is increased up to 96 h, but the results are the same, whether the period of wetness is continuous or broken by dry intervals. Excellent differentiation of levels of resistance occurs if the inoculated plants are exposed to 72 h of wetness at 20 C with a 12-h photoperiod. Place the plants on a greenhouse bench for about 2 wk to allow the lesions to develop. Then subjectively rate the degree of resistance or susceptibility based upon the percent of leaf area damaged. For precise comparisons on a cultivar of known reaction, take results by counting the number of lesions per plant.

Fukuda (*46*) developed and Spurr (*163*) refined an inoculation technique that uses leaf discs rather than whole plants. Cut discs (9 cm diameter) from the center of vigorous, unwilted mature leaves of equivalent age and place them bottom side up in a moderate-sized, enclosed humidity chamber for 2 h. Inoculate the discs by spacing 12 drops (0.01 ml per drop) of the conidial suspension (10,000 conidia per milliliter) on the lower surface of the lamina. After the drops have dried, place the lid on the humidity chamber and incubate the discs for 5 to 10 d at 21 C and an 8-h photoperiod. When lesion development is completed, rate the lesions on a subjective scale. This method is more efficient for testing pathogenicity of *A. alternata* isolates and efficacy of fungicides and for some other kinds of research than the whole plant method. The latter method is more desirable for assessing resistance.

Do field inoculation with *A. alternata* by spraying a conidial suspension or by dusting coarsely ground, dried infected leaves from the previous crop onto plants 3 to 4 wk after transplanting (Stavely, unpublished). If you use a conidial suspension, it should have time to dry before the leaves are wetted by dew or rain. If you use dried, infected leaves, take care not to overinoculate. Not more than 5 ml of dried leaf should be used per plant. Symptoms will appear in about 2 wk subsequent to several nights of ample dew or a period of wet weather. Make subjective ratings, based upon the percent of leaf area affected, late in the growing season on the green leaf, or rate the leaves after they have been harvested and cured (*107*).

In all inoculations with *A. alternata* include resistant and susceptible cultivars as checks.

Frogeye leaf spot. The frogeye leaf spot pathogen, *Cercospora nicotianae,* can be isolated from lesions cut out of diseased leaves. Place these in a Petri dish on moist filter paper in the light at room temperature until the hyaline conidia are produced on the prominent, dark conidiophores. With the aid of a dissecting microscope and a sterile needle moistened in sterile V-8 or other agar, pick off the conidia and transfer them to V-8 juice agar (pH unadjusted) slants. By this procedure a high percentage of pure isolates are obtained. After one more transfer, store the slant cultures with a covering of mineral oil in a refrigerator. These will last for years without loss of pathogenicity.

Produce inoculum by flooding V-8 agar plates with a mycelial and conidial suspension. This is obtained by lightly scraping the surface of young, freshly darkened colonies with a sterile needle (*169*). The fungus produces conidia in 7 d if the V-8 agar plates are incubated in constant fluorescent light (same intensity as for *A. alternata*) at 18 C (*170*). Blend the cultures with water and filter the suspension through cheesecloth. Determine the inoculum concentration with a haemocytometer and adjust it to about 400,000 mycelial fragments and conidia per milliliter (*164*). This fungus does not sporulate abundantly even under optimal conditions (*169, 170*), so inoculum is usually about 90 percent mycelial fragments and 10 percent conidia.

Plants 6 to 7 wk old are large enough to test for *C. nicotianae* reaction, although 3-month-old plants give more precise results. Spray the leaves with inoculum to run off and allow them to dry before placing them in controlled-environment chambers. Best disease development occurs if incubation is for 5 d at about 28 C

with sufficient humidity to keep the leaves wet (Stavely, unpublished). Some lesions are produced with a much shorter period of wetness. Light is supplied for 12 h per day and may be essential for infection to occur. Lesions begin to appear 10 d after inoculation. Take results, based on a subjective scale of percent leaf injury or lesion numbers, 12 to 16 d after inoculation.

Do field inoculations by spraying a fungal suspension, similar to that used in the greenhouse, or by dusting roughly ground, dried, infected leaves from the previous crop over the plants (*164*). Apply the inoculum at any time after vigorous growth has gotten started, but about 4 to 6 wk after transplanting is usually the best time. Since moisture is so critical for infection, field inoculations may need to be repeated several times in a dry year.

Include resistant and susceptible checks in inoculations of breeding material.

***Fusarium* wilt.** The fungus causing *Fusarium* wilt, *F. oxysporum* f. sp. *nicotianae*, is easily isolated from infected tobacco stems. Remove the outer stem tissue with a sterilized knife blade, cut out pieces of the clean, inner tissue, rinse the pieces in sterile water and place them on sterile water agar or PDA. Pure cultures are obtained from most stem pieces plated in this way (*87*). The fungus grows well on several media, such as PDA and oatmeal agar, but the former is probably the most commonly used medium (*42*, *87*). Isolates pathogenic on tobacco have been maintained on PDA slants in a refrigerator with a single transfer to fresh PDA each year for 15 yr or longer without loss in pathogenicity (*152*).

Prepare an inoculum suspension by first adding water to the surface of 7-day-old cultures that have been incubated at 27 C, then scrape the spores and mycelium into the water (*42*). Johnson (*87*) produced large volumes of inoculum by incubating the fungus for 4 to 5 wk at 25 to 30 C on a sterile medium containing 100 g sand, 10 g corn meal, 1 g glucose and 50 ml water in mason jars or other large containers. These constituents are mixed, cooked 1 h in an autoclave, stirred, again autoclaved for sterilization and inoculated.

The two inoculation techniques given here are those of Johnson (*87*), which is still satisfactory, and the more precise technique of Everette (*42*).

In Johnson's (*87*) technique, pulverize the dry medium and thoroughly mix it with soil at the rate of one mason jar of inoculum per flat (41 by 61 by 7.6 cm) of soil. Transplant the plants into this soil and allow them to grow for about a month at about 30 C. Rate disease severity on each plant, using a 1 to 4 scale. More uniform results are obtained if one intentionally wounds the roots or bases of the stem of each plant at transplanting.

Using Everette's (*42*) method, uproot 4-week-old tobacco seedlings and wash the roots. Then dip them into a suspension of spores and mycelium. Take no particular care to avoid root injury. Plant the seedlings in soil:sand mixture (3:1) in aluminum pans and water lightly. Place the pans in a thermostatically controlled water tank, similar to that described for black shank, at about 33 C. After 10 to 15 d make a visual rating of leaf symptoms and split the stem to determine whether or not vascular discoloration is present. Use the severity of leaf symptoms and presence or absence of vascular discoloration to assign disease indices. For field tests, uproot the plants from the plant bed, wash the roots, immediately dip them in inoculum, and machine set the plants as soon as possible.

Anthracnose. The anthracnose pathogen, *Colletotrichum destructivum*, sporulates well on Czapek's agar (*110*), PDA (*149*), and other media. Obtain a conidial suspension by rinsing the surface of agar cultures with a stream of water. With the aid of a haemocytometer, determine the conidial concentration and then adjust it to 250,000 per milliliter for use as inoculum (*149*).

Because young plants are susceptible to anthracnose, use 45-day-old seedlings, growing several per 10-cm pot, for inoculation (*149*). Wetting of the leaves is recommended (*149*) before atomizing or spraying them with the inoculum (*110*, *149*). High humidity is essential for successful infection. Incubate the inoculated plants in a moist room or chamber for approximately 75 h (*149*). Temperatures from 13 to 32 C have been used successfully during this incubation period (*110*, *149*). Light is not critical (*149*). Move the plants from the moist chamber to a greenhouse bench after the incubation period. Symptoms appear in 2 d. Make disease ratings 5 to 15 d after inoculation (*149*).

Sore shin. The fungus causing sore shin, *Thanetephorus cucumeris*, commonly found in the *Rhizoctonia solani* imperfect stage, has a wide host range (*106*). Little or no work has been done to breed resistant tobaccos. Inoculation techniques developed for other crops should work for tobacco. Generally, dry inoculum can be mixed from pure cultures with moistened soil. Then sow a known quantity of seed in the soil. After 2 to 3 wk record the percent germination and infection severity on the seedlings (*126*).

Root knot. Root knot nematodes infecting tobacco include *Meloidogyne arenaria*, *hapla*, *incognita acrita*, *incognita incognita*, and *javanica*, of which the two subspecies of *incognita* and *javanica* are the most common pathogens (*106*). Because all are obligate parasites, inoculum must be increased on susceptible plants, usually using tomato, *Lycopersicon esculentum* cv. 'Rutgers'.

Take care to avoid mixing inoculum. Because the characters used to differentiate species and varieties of *Meloidogyne* are minute, the novice should work with a nematologist to ensure that the isolates are properly identified and are not mixtures.

Inoculation methods are similar for all *Meloidogyne* spp. The commonly used inoculation method for tobacco involves mixing chopped, knotted roots, having egg-producing females, with sterilized soil into which month-old

plants are transplanted (*31*). Fewer of the plants die if transplanting and inoculation are not done at the same time. Transplant the month-old plants to sterilized soil in 6.6 cm-pots and then about 3 wk later, transplant them without loss of soil to 8.5 cm-pots. Fill the additional volume of the larger pots with soil containing chopped, diseased roots (*157*).

An inoculation method that gives more uniform results and fewer problems from contaminating fungal pathogens, employs *Meloidogyne* larvae rather than knotted roots for inoculum. To obtain clean larvae, use a forceps or needle to carefully pick off individual, young, coherent egg masses, which are produced on the surface of infected roots. Place these in 0.1 percent cetavlon (cetyl trimethylammonium bromide), decant the liquid after 5 min, and rinse the egg masses with distilled water. Then immerse the egg masses in 0.5 percent hibitane diacetate (bis[*p*-chlorophenyl-diguanido]-hexane-diacetate), and rinse this away with distilled water after 15 min. The surface sterilized, unhatched eggs begin hatching as soon as they are placed in water (*127*). Use the clean larvae that are produced to inoculate tomatoes growing in sterilized soil. Before planting, surface sterilize the seed with hibitane diacetate or some other appropriate sterilant. In about 6 to 8 wk abundant eggs and larvae will be present on the tomato roots.

Obtain larvae for inoculum by the sifting-gravity and funnel method of Christie and Perry (*18*). Place about 1 kg of knotted roots and surrounding soil in a large pan or pail, cover with water, manually stir and work to break lumps, and allow the debris to settle for 10 sec. Pour this muddy suspension twice through a 60-mesh (250-μm) screen. Dip the screen into the water several times before discarding the debris retained on it. Pour the remaining suspension through a 400-mesh (38-μm) screen and rinse the screen with water until the retained residue is clear. Wash the larvae and small bits of organic material that are retained on the 400-mesh screen into a 250-ml beaker.

Next, attach a piece of rubber tubing to the stem of a 15-cm funnel and clamp it, making what is known as a Baermann funnel. Partially fill the funnel with water. Cover the beaker of larvae with a piece of moderately close-mesh, damp muslin, and secure it with a rubber band. Then invert the beaker in the funnel, being sure to submerge the mouth. The larvae work through the mesh and settle to the bottom of the tube. After a few hours open the clamp and release 2 to 4 ml of water with larvae into a large watch glass or similar container. Determine the number of larvae per milliliter with a dissecting microscope. Adjust the larval concentration to about 375 per milliliter and pipet 2 ml into the soil around a plant growing in a 6.6- or 8.5-cm pot (*157*). Water the plants gently with a fine mist for the first few days after inoculation. Maintain soil temperatures at 25 to 29 C.

In Rhodesia, eggs alone, as well as larvae, have been used successfully to inoculate greenhouse plants (*144, 147*).

About 8 wk after inoculation wash the soil from the roots, estimate the infected percent of the total root area, and use this data to calculate a disease index (*31, 157*).

For field tests, pour 100 ml of sand or soil containing chopped, knotted roots into the bottom of each hole before setting the tobacco plants (*31, 54*).

Brown root rot. Several species of migratory, root invading nematodes of the genus *Pratylenchus*, including *P. brachyurus, zeae, penetrans,* and *neglectus* are tobacco pathogens. The first two species, of which *P. brachyurus* is most common, occur in southeastern United States, whereas the last two are most common in the Connecticut Valley and Canada (*106*). They are commonly called root lesion nematodes. Being obligate parasites, inoculum is increased on susceptible plants, such as cowpea (*57, 106*).

Graham and Ford (*57*) compared two methods for inoculating tobacco with *P. brachyurus*. For both methods, transplant the plants into 10-cm pots at the four-leaf stage and then inoculate. Place the pots in a sand-filled greenhouse bench equipped with soil-heating cables or maintained in some other way to keep the soil temperature at 32 to 35 C.

In the first method, wash infected cowpea, *Vigna sinensis*, roots, then chop these in a blender and place the tissue in wet-strength facial tissues on a Baermann funnel for 48 to 72 h. Draw the nematodes from the funnel, allow them to settle, and concentrate by decanting the excess water. Use a hypodermic syringe to suspend the nematodes and deliver aliquots containing about 100 larvae and adults to each pot.

In the second method, thoroughly chop infected cowpea roots and mix them with the soil in which they were grown. Place a tobacco seedling and 50 ml of this mixture, containing about 50 larvae and adults, in the center of each pot. The chopped root-soil inoculum gives better results than the extracted nematodes with the above concentrations and procedures.

After about 60 d, regardless of method, remove the plants from the pots and wash the roots thoroughly. Weigh the tops and roots separately, visually rate the root symptoms, and determine nematode populations. This determination is done by the following procedure: Cut the washed roots into approx. 5-mm lengths, blend for 15 sec, wash with a gentle spray over a 350-mesh (40-μm) screen to remove debris, and pour into a Baermann funnel fitted with wet-strength facial tissue. After 48 h release 10 ml of water containing the nematodes from the funnel into a counting tray. Count the number of nematodes in 10 fields at 80X magnification to obtain an estimate of the population per sample.

Use chopped roots and soil from greenhouse cultures to inoculate field plots (*53*).

Increased top growth is often observed after inoculation

with *Pratylenchus* spp. This is usually associated with a low inoculum concentration, certain host species or cultivars, or the early stages of disease development (*57*).

Cyst nematodes. The complex of cyst nematodes infecting tobacco includes three named species: *Heterodera tabacum*, the tobacco cyst nematode; *H. solanacearum*, the Osborne cyst nematode; and *H. virginiae*, the horsenettle cyst nematode (*106, 130*). The first two species are difficult to distinguish morphologically but differ in pathogenicity on *N. longiflora* (*5, 64, 118*). The first species occurs in the Connecticut River Valley and the last two in separate areas of southern Virginia (*130*).

Egg-containing cysts (*64, 105*), crushed cysts with eggs and larvae (*118*), eggs and larvae (*5*), and only eggs (*45*) have been used successfully for inoculum. Inoculum concentration can be more accurately standardized if eggs or larvae are used due to the variability in the number of eggs and larvae/cyst.

Cut the cysts and squeeze the eggs into water. Then make a count. Fox and Spasoff (*45*) recommend inoculating seedlings, freshly transplanted to 5-cm pots, with 20,000 eggs per pot. Keep the inoculated plants in a greenhouse for 2 to 3 mo maintaining a soil temperature of about 24 C (*45, 64*). Carefully wash the roots and soil from each plant to remove the females and cysts for counting. Obtain a fresh weight for each plant. The ability of tobacco lines to inhibit nematode development (resistance) is independent of their ability to grow well in the presence of the nematodes (tolerance), thus fresh weight is as important as nematode population in assessing host reaction (*45*).

Resistance Sources

Table 12-4 lists the major tobacco pathogens and the *N. tabacum* accessions and *Nicotiana* spp. that have been reported to have resistance to each pathogen. Only basic sources of resistance are included; commercial cultivars to which resistance has been transferred are listed by Lucas (*106*).

The U.S. Department of Agriculture maintains *Nicotiana* germplasm at the locations given in the appendix. This germplasm is grouped into three categories: tobacco cultivars, Tobacco Introductions (TI's), and *Nicotiana* spp. The cultivar collection contains 462 accessions, including 169 flue-cured, 74 burley, 61 cigar-wrapper, 51 miscellaneous, 26 dark, 25 cigar-filler, 22 Maryland, 18 Turkish, and 16 cigar-binder accessions. The TI collection consists largely of accessions collected by plant explorers in Latin America in the 1930's as well as those collected from all over the world before and since then. Most foreign cultivars are in the TI collection. This collection has over 1,100 accessions, with assigned TI numbers ranging from 4 to 1620. The *Nicotiana* spp. collection contains 63 of the 66 known species.

Many examples of accessions exist that have been reported to be resistant by one author but less resistant or even susceptible by another author. In this case, a reference is given to the initial report of resistance for each accession listed in table 12-4. Generally, accessions reported to have slight resistance have been omitted.

Inoculation method, pathogen isolate, and seed source can all influence results in testing for resistance. The tobacco cultivars, TI's, and *Nicotiana* spp. in the seed collections have, in most cases, resulted from many generations of selfing and selection for certain attributes. Disease resistance has rarely been a factor in selection of the plants from which seed was saved. In cases where resistance is controlled by multiple genes, assume that, if resistance had not been considered in selecting seed plants, seed was often saved from less resistant individuals. Because of the large quantity of seed produced by single plants, convenience usually resulted in seed being saved from two, three, or at best several plants at every 8- to 10-year interval when the seed was increased. Thus, genetic drift in seed stocks is an important factor in changing the responses of cultivars, TI's, or species to pathogens. For example, *N. tabacum* cultivars Big Cuba and Florida 301 had high levels of black shank resistance in 1930 (*185*) and TI 706 had considerable root knot resistance in the 1930's (*31*), but neither is true today (*104, 157*).

Examples of pathogenic races occurring that overcome monogenic resistance are known for several tobacco pathogens. Perhaps even more significant is Valleau's (*194*) evidence for increased pathogenicity of *T. basicola* against polygenic *N. tabacum* resistance. Burley cultivars Ky 16 and Ky 41A, once highly resistant in the field, have become only moderately resistant, perhaps because of changes in the pathogen population. However, environmental influences cannot be eliminated as causal factors in this phenomenon.

Mosaic. Among the accessions listed as resistant to TMV (table 12-4) there are two kinds of resistance: (a) a symptomless reaction in which there is usually some multiplication of the virus, and (b) a hypersensitive local lesion reaction (*12, 40, 75, 79, 194*). In the latter type of resistance, the leaf tissue surrounding the point of invasion dies, restricting spread of the virus. Among the resistant species, only *N. benavidesii, glauca, tomentosiformis,* and *wigandioides* have the symptomless type of resistance; the remainder have local lesion resistance. However, Ambalema (*196*) and all of the other resistant TI's have symptomless resistance (*12, 29*). At least 11 TI's not listed in table 12-4, having TI numbers above 1400 and accessioned since 1961, have local lesion resistance that probably originated from *N. glutinosa* (*12*).

Intensive efforts to transfer TMV resistance from Ambalema, which is governed by two independent, recessive genes, to commercial cultivars were initiated in the 1930's (*26, 194*) but were finally abandoned in the 1950's because of an adverse linkage.

N. glutinosa is the source of resistance in all TMV resis-

TABLE 12-4.—*Sources of resistance in* Nicotiana *to the major tobacco pathogens*

Pathogen[1]	Sources of resistance[2]
TMV	*N. tabacum* cv. Ambalema (TI 1560) (*123*); TI's 203, 407, 468, 1203, 25, 383, 384, 410, 411, 412, 413, 431, 436, 437, 438, 439, 448, 448A, 449, 450, 465, 470, 471, 692, 1467 (*12*); *N. acuminata* (*1, 79*); *N. goodspeedii* (*1*); *N. glauca* (*2, 40, 79*); *N. glutinosa* (*2, 75*); *N. langsdorffii, N. repanda, N. rustica, N. wigandioides* (*79*), *N. benevidesii, N. gossei, N. nesophila, N, stocktonii, N. suaveolens, N. undulata, N. velutina* (*40*); *N. sanderae, N. tomentosiformis* (*194*).
PVY	*N. tabacum* cv. Virgin A Mutant (TI 1406) (*95, 96, 97, 148*); cv. Enshu (TI 1586) (*140*); *N. benavidesii, N. glauca, N. knightiana, N. miersii, N. noctiflora, N. otophora, N. raimondii, N. thyrsiflora, N. tomentosa, N. tomentosiformis, N. wigandioides* (*148*).
CMV	*N. tabacum* cv. GAT (*17, 82, 202, 203, 213*); TI 245 (*47, 191*).
TEV	*N. tabacum* cv. Havana 307 (*153*); cv. V20 (TI 1440) (*19, 142*); cv. Virgin A Mutant (TI 1406) (*51*); *N. arentsii, N. glauca, N. knightiana, N. otophora, N. paniculata, N. raimondii, N. setchellii, N. tomentosa, N. tomentosiformis, N. undulata* (*102*).
TVMV	*N. tabacum* cv. Havana 425 (*129*); cv. Virgin A Mutant (TI 1406) (*101*).
Pseudomonas solanacearum	*N. tabacum* cv. Xanthi, TI's 79A, 448A (*32*); cv. Awa, cv. Kokubu (*109*); cv. Enshu (*189*).
P. tabaci — Race 0[3]	*N. alata, N. attenuata, N. bigelovii, N. longiflora, N. nudicaulis, N. repanda, N. rustica, N. suaveolens* (*3*); *N. arentsii, N. bonariensis* (*40*); *N. debneyi, N. glauca* (*29*); *N. plumbaginifolia* (*40, 143*); *N. langsdorffii, N. trigonophylla* (*134*); *N. acaulis, N. cavicola, N. fragrans, N. undulata, N. wigandioides* (*8*).
Race 1[3]	*N. nudicaulis, N. repanda, N. rustica, N. undulata* (*154*).
P. angulata[4]	*N. alata, N. occidentalis, N. sylvestris* (*134*).
Phytophthora parasitica — Race 0	*N. tabacum* cv. Florida 301, cv. Big Cuba (TI 1565), cv. Little Cuba (*185*); cv. Beinhart 1000-1 (TI 1561), cv. Beinhart 1000 (TI 1562) (*68*); cv. Amarillo Parado (TI 1583) (*6*); *N. longiflora, N. nudicaulis, N. plumbaginifolia, N. repanda* (*40*); *N. rustica pumila* (*100*); *N. stocktonii* (*104*).
Phytophthora parasitica—Continued — Race 1	*N. tabacum* cv. Beinhart 1000-1 (TI 1561), *N. longiflora, N. nesophila, N. repanda, N. rustica* cv. Ky. 31, *N. stocktonii* (*104*).
Race 2	*N. tabacum* cv. A22, cv. A23, cv. Delerest 202 (TI 1608), cv. Hicks 21 (*98, 99*).
Race 3	*N. tabacum* cv. Consolidated I, cv. Consolidated L, cv. Beinhart 1000-1 (TI 1561), cv. Amarillo Parado (TI 1583), cv. NC 1071, *N. nesophila* (*179*).
Peronospora tabacina[5]	*N. tabacum* cv. Chileno Correntino (TI 157) (*23*); TI's 575, 657, 722W, 748, 805, 845, 1461, 1462, 1463, 1466 (GA955), 1506 (Chemical Mutant), 1550 (*61*); *N. debneyi, N. excelsior, N. exigua, N. goodspeedii, N. maritima, N. velutina* (*160*); *N. longiflora, N. megalosiphon, N. plumbaginifolia, N. rotundifolia* (*23*); *N. cavicola, N. occidentalis, N. rosulata, N. simulans* (*74*); *N. amplexicaulis, N. gossei, N. hesperis, N. ingulba, N. simulans, N. suaveolens, N. umbratica* (*205*).
Races APT-1 and 2	*N. amplexicaulis, N. cavicola, N. debneyi, N. eastii, N. exigua, N. ingulba, N. megalosiphon, N. occidentalis, N. rosulata, N. rotundifolia, N. simulans, N. suaveolens, N. velutina* (*72*).
Race APT-3	*N. acuminata acuminata, N. acuminata multiflora, N. attenuata, N. bigelovii, N. bigelovii quadrivalvis, N. cavicola, N. clevelandii, N. debneyi, N. eastii, N. exigua, N. ingulba, N. knightiana, N. langsdorffii, N. megalosiphon, N. occidentalis, N. paniculata, N. rotundifolia, N. simulans, N. suaveolens, N. trigonophylla* (*72*).
Race PT-2	*N. exigua, N. megalosiphon* (*85*).
Erysiphe cichoracearum	*N. tabacum* cv. Turkish Samsun; cv. Harmanliska Basma; cv. Bursa (*110*); cv. Kuo-fan (*8*); *N. acuminata, N. alata, N. bigelovii, N. fragrans, N. glauca, N. glutinosa, N. langsdorffii, N. longiflora, N. noctiflora, N. nudicaulis, N. paniculata, N. plumbaginifolia, N. repanda, N. rustica, N. sanderae, N. suaveolens, N. sylvestris* (*182*); *N. tomentosa* (*108*); *N. attenuata, N. benthamiana, N. debneyi* (*139*); *N. excelsior, N. exigua, N. goodspeedii, N. gossei, N. knightiana, N. megalosiphon, N. occidentalis, N. palmeri, N. paniculata, N. raimondii, N. trigonophylla, N. velutina* (*133*).

See footnotes at end of table.

TABLE 12-4.—*Sources of resistance in* Nicotiana *to the major tobacco pathogens*—Continued

Pathogen[1]	Sources of resistance[2]
Thielaviopsis basicola	*N. tabacum* cv. Brasile, cv. Halladay Havana, cv. Little Dutch, cv. Page's Comstock, cv. Shade-Grown Cuban, cv. Xanthi (*88*); cv. Harrow Velvet (*65*); cv. Kozarsko #541 (TI 1379), cv. Kulsko (TI 1380), cv. Mandrensko (TI 1381), cv. Nevrokop #5 (TI 1382), cv. Rila #9 (TI 1383), cv. Sandanski #144 (TI 1384), cv. Sekirka (TI 1385), cv. Stanimashka Basma #536 (TI 1386), cv. Sultansko (TI 1387), cv. Wisoka Mrrulea, (TI 1389), cv. Dubek 7 (TI 1410), cv. Alma-Alta 315 (TI 1412), cv. PRZ65 (TI 1462), cv. Forchiem F.O. (TI 1486), cv. Djebel 174 (TI 1492), cv. Djebel 359 (TI 1493), cv. Dubek 566 (TI 1567), TI's 381, 1458 (*60*); TI 89 (*28*); TI's 87, 88 (*194*); *N. debneyi, N. glauca, N. repanda* (*29*); *N. acuminata, N. alata, N. attenuata, N. benthamiana, N. exigua, N. gossei, N. longiflora, N. maritima* smooth leaf, *N. megalosiphon, N. nesophila, N. nudicaulis, N. paniculata, N. plumbaginifolia, N. rustica, N. stocktonii, N. undulata, N. wigandioides* (*28*).
Alternaria alternata	*N. tabacum* cv. Beinhart 1000-1 (TI 1561) (*13*); cv. Beinhart 1000 (TI 1562), cv. Ambalema (TI 1560) (*167*); cv. Amarillo Parado (TI 1583) (*6*); TI's 505, 804, 820, 995, 1043, 1138, 1211, 1467 (*167*); *N. suaveolens* (*135, 167*), *N. acaulis, N. bonariensis, N. debneyi, N. forgetiana, N. goodspeedii, N. hesperis, N. longiflora, N. nesophila, N. noctiflora, N. occidentalis, N. repanda, N. stocktonii, N. wigandioides* (*171*).
Cercospora nicotianae	*N. debneyi, N. nudicaulis, N. repanda* (*135, 166*); *N. amplexicaulis, N. knightiana, N. noctiflora, N. stocktonii, N. wigandioides* (*166*).
Fusarium oxysporum	*N. tabacum* cv. Connecticut Havana, cv. Texas Sumatra, cv. Shade-Grown Cuban, cv. Pennsylvania Broadleaf, cv. Narrow Leaf Orinoco (*87*); cv. Gertz; cv. Ward, cv. Catterton, cv. Thompson (*152*); cv. Chileno Correntino (TI 57) (*194*); TI 448A (*120*); TI's 552, 566 (*188*).
Colletotrichum destructivum	*N. alata, N. debneyi, N. langsdorffii, N. longiflora, N. nudicaulis, N. sylvestris, N. trigonophylla* (*110*); *N. glauca, N. sanderae* (*132*); *N. bonariensis, N. forgetiana, N. fragrans, N. hesperis, N. noctiflora, N. paniculata* (*140*).
Meloidogyne incognita	*N. tabacum* cv. Faucett Special, TI's 419, 422, 517, 706, *N. arentsii, N. glauca, N. longiflora, N. megalosiphon, N. plumbaginifolia, N. repanda* (*31*); *N. knightiana, N. otophora, N. paniculata* (*67*); *N. nudicaulis* (*15*).
M. arenaria	*N. longiflora, N. megalosiphon* (*53*); *N. arentsii, N. repanda* (*155*).
M. incognita acrita	*N. megalosiphon, N. sylvestris* (*53*); *N. arentsii, N. repanda, N. tomentosa* (*156*).
M. incognita acrita C[6]	*N. otophora* (*157*); *N. glauca, N. nudicaulis, N. paniculata, N. repanda* (*155*).
M. incognita incognita	*N. megalosiphon* (*53*); *N. arentsii, N. thyrsiflora, N. tomentosa* (*156*).
M. javanica	*N. tabacum* cv. Nyoka strain 256 (*144, 145*); *N. megalosiphon, N. sylvestris* (*53*); *N. longiflora, N. repanda* (*147*); *N. otophora* (*157*).
Pratylenchus brachyurus	*N. tabacum*, some plants in many cultivars (*161*); *N. glauca* (*55*).
Heterodera tabacum	*N. acuminata* (*64*), *N. forgetiana, N. noctiflora, N. plumbaginifolia* (*118*).
H. solanacearum	*N. glutinosa, N. longiflora, N. paniculata, N. plumbaginifolia* (*5*).

[1]TMV = Tobacco Mosaic Virus, PVY = Potato Virus Y, CMV = Cucumber Mosaic Virus, TEV = Tobacco Etch Virus, TVMV = Tobacco Vein Mottle Virus.

[2]TI = Tobacco Introduction.

[3]Race 0 is the long known biotype; Race 1 is a biotype recently isolated from Wisconsin tobacco.

[4]*P. angulata* resistance has been assumed or proven to occur in species resistant to *P. tabaci*.

[5]Those sources of resistance listed here were identified using inoculum for which the race was not identified.

[6]A new isolate of *M. incognita acrita* identified by T. W. Graham that differs from the original isolate in pathogenicity and may differ morphologically.

tant commercial tobacco cultivars (*8, 26, 106, 194*). The mosaic virus includes many races or strains differing in pathogenicity and symptoms produced (*106, 194, 196*). Nevertheless, a strain pathogenic to *N. glutinosa* or to cultivars with *N. glutinosa* resistance has not been found, although mosaic-resistant tobacco cultivars have been grown commercially for 35 yr (*106, 194*). This resistance is controlled by a monogenic, dominant factor (*76, 77, 78*). A tetraploid *N. glutinosa-N. tabacum* hybrid, *N. digluta*, was obtained by Clausen and Goodspeed (*20*) in the early 1920's. Holmes, in the 1930's, transferred the monogenic resistance governed by the N factor from *N. glutinosa* to the Turkish cv. Samsoun (*78*). By the early 1940's Valleau was able to release burley cultivars having the N factor (*194, 195*). Gerstel (*50*) has shown that the N factor was transferred from a *N. glutinosa* chromosome to the H chromosome of *N. tabacum*. However, the N factor has been difficult to transfer to flue-cured cultivars caused by the associated quality defects in this type (*14*). These problems in the flue-cured type and the stability of the resistance to the many strains of the virus suggest that the N factor is transmitted as a complex locus.

Other viruses. Although table 12-4 presents a number of sources of resistance to PVY, CMV, TEV, and TVMV, none of this resistance is yet available in commercial cultivars. In the case of PVY, the principal effort has involved TI 1406; for CMV, GAT; for TEV, Havana 307, TI 1406, and TI 1440; and for TVMV, Havana 425 and TI 1406. Radiation of seed of tobacco cv. Virgin A gave rise to the PVY-resistant Virgin A mutant (TI 1406). This mutant is susceptible to blue mold whereas Virgin A is somesomewhat resistant (*95, 96, 97*). Subsequently, TI 1406 was found to be resistant to TEV and TVMV (*51, 101*). The PVY resistance in TI 1406 was thought to be controlled by a single recessive factor (*95*) that is located in chromosome E (*62*). However, the genetics of this resistance may be more complex. Unfortunately, although TI 1406 is resistant to some common strains of PVY, an Argentine strain has been found to which it is susceptible (*51*). Enshu (TI 1586) is resistant to a mild strain but not to the severe strain of PVY (*51*). Fortunately, some commercial tobacco cultivars have considerable tolerance to TEV and TVMV. Burley 21 and Kentucky 12 and 14 tolerate some or most strains of TEV (*51*), and Havana 307 is highly tolerant (*153*). Kentucky 10, 12, 14, and 41A tolerate TVMV (*128*). When lightly inoculated, TI 245 is resistant to most tobacco viruses (*47, 191*). Holmes (*82*) combined *N. glutinosa*, TI 245, and Ambalema (TI 1560) resistance into a stabilized line (*81, 82*), GAT, that Taiwanese workers are using in attempts to develop CMV-resistant commercial cultivars (*17, 202, 203, 204, 213*).

Bacterial wilt. From 1934 to 1941, the available *Nicotiana* spp. and over 1,000 TI's were tested for resistance to *Pseudomonas solanacearum* (*32*). The most desirable source discovered for plant type and resistance was TI 448A. Many cultivars with resistance from TI 448A have been released since the mid-1940's (*106*). This resistance is governed by polygenic recessive factors (*109, 159*) and has reduced wilt from a major to a relatively minor disease in the Southeastern United States.

Wildfire and angular leaf spot. Clayton's (*24*) successful cross of *N. longiflora* with *N. tabacum* resulted in the production of wildfire-resistant TI 106 in 1942. The resistance is controlled by a monogenic dominant factor. Plants of TI 106 are also resistant to *P. angulata*. The gene from TI 106 has been transferred to many cultivars through the efforts of Clayton and others (*25, 106*). All presently available wildfire resistant cultivars derive their resistance from this source (*106*). Biochemical mutants of the wildfire bacterium were reported in 1958 (*48*). Valleau and others (*198*) reported the natural occurrence in Kentucky of resistance breaking races of both bacteria in 1962, just 7 years after release of the first resistant commercial cultivar. Since then, similar virulent races have been reported from Rhodesia and Wisconsin (*134, 154*). *N. rustica* and *N. repanda*, species resistant to the virulent Wisconsin race, have been crossed with tobacco and stabilized resistant tobacco lines have recently been obtained from the *N. rustica* derived material (Stavely and Skoog, unpublished). Use of methionine sulfoximine in place of the tabtoxins in an artificial medium has resulted in production of toxin-resistant plants from tobacco cells cultured *in vitro*. However, they do not resist the entire disease syndrome (*9*).

Black shank. Nearly all American commercial cultivars resistant to black shank derive their resistance from Florida 301 (*106*). As reviewed by Chaplin (*11*), the evidence suggests that this resistance is inherited quantitatively. It is effective against at least the two races, 0 and 1, that are encountered in the Southeastern United States. In cultivars with resistance from *N. longiflora* and *N. plumbaginifolia*, the resistance is governed by a single partially dominant or dominant factor (*11, 35*). The genetic locus for black shank resistance is identical in these two species (*36*). Chaplin (*10*) transferred resistance from *N. plumbaginifolia* to a stable, flue-cured breeding line, PD 468, in 1961.

Valleau and others (*199*) transferred resistance from *N. longiflora* to the burley cultivar L8 in the late 1950's. However, a race of *Phytophthora parasitica* var. *nicotianae* pathogenic on L8 and *N. plumbaginifolia* was discovered before the release of L8 (*199*). *N. longiflora* and *N. plumbaginifolia* are practically immune to the original race 0, but *N. longiflora* develops slight symptoms and *N. plumbaginifolia* is moderately to highly susceptible to race 1 (*104*). Other species that give strong differential reactions to the two races include the Kentucky 31 accession of *N. rustica*, *nesophila*, and *nudicaulis*.

Race 2 of *P. parasitica* var. *nicotianae* was identified in

South Africa in the early 1970's. It is differentiated by the reaction of tobacco cv. Delcrest 202, which is resistant to race 2 but susceptible to races 0 and 1 (*98, 99*). The resistance of Delcrest 202 to race 3 is controlled by a single dominant factor. A South African tobacco line, H21, has a second, independent single dominant resistance factor. Race 2 has not been found in the United States. In 1973, black shank was first found in Connecticut (*201*). The Connecticut isolate is the fourth, distinct, pathogenic race (*111, 179*). It is differentiated from the other races by the reactions of tobacco cultivars A 23, L 8, and NC 1071 and *N. nesophila* (*179*). Race 3 appears to be unique in its tolerance to cold temperatures (*180*). Luckily, Consolidated Cigar Corporation's cultivars L and I have good field resistance to race 3 (*179*).

In the Dominican Republic, where *P. parasitica* var. *nicotianae* has been endemic for many years, several local cultivars have moderate to high black shank resistance (*6*). Of the latter, Beinhart 1000 and Beinhart 1000-1 (TI's 1562 and 1561) and Amarilla Parado (TI 1583) have the greatest resistance. These Dominican cultivars have not been tested with race 2, but they are resistant to all three other races (*11, 104, 151, 179*). This resistance is probably controlled by multiple factors, but the evidence is incomplete (*11*). Resistance from these TI's has been successfully transferred to cigar tobacco breeding lines but not to cigarette tobaccos.

Blue mold. Clayton (*27*) transferred blue mold resistance from *N. debneyi* to *N. tabacum* and obtained in the late 1950's, 20 yr later, the first commercially acceptable, F_1 hybrid tobacco cultivar. He reported that this resistance is controlled by three dominant factors (*27*), but the resistance of the parental *N. debneyi* may involve additional factors (*205*). Wark and coworkers (*205*) have bred for blue mold resistance in Australia for many years. In *N. goodspeedii*, they found that resistance is controlled by a single dominant factor that they transferred to tobacco (*205*). Lucas (*106*) lists the cultivars of tobacco that derive blue mold resistance from *N. debneyi* and *goodspeedii*. Wark and others (*207*) have obtained tobacco breeding lines with resistance from *N. excelsior* (GA 955), *velutina*, and several other species.

Breeding for blue mold resistance became more complex about 1960 when three pathogenic races of *Peronospora tabacina* were recognized in Australia (*206*). They have been named Australian *Peronospora tabacina* (APT) races 1, 2, and 3. *Nicotiana* spp. that differentiate APT 3 from APT 1 and 2 are listed in table 12-4. More than 30 Australian tobacco breeding lines, deriving blue mold resistance from *N. amplexicaulis*, *excelsior*, *goodspeedii*, and *knightiana* are highly resistant to APT 1 but susceptible to APT 2 and APT 3 (*72*).

After the pathogen was introduced into Europe in 1958, the losses were so severe that American and Australian resistant germplasm was immediately introduced and used to produce resistant F_1 hybrid cultivars (*106*). Several European breeders began work on blue mold resistance. In 1966 a new pathogenic race was reported from Hungary (*193*). In 1971, a race pathogenic on tobacco with resistance from *N. debneyi*, designated race PT-2, was reported from Poland (*84, 85*). Such resistance breaking races have now been found in several European countries (*37, 141*). Collaborative plantings of selected resistant and susceptible germplasm are now made yearly in 13 European countries, Turkey, and Morocco to monitor the changing virulence of *P. tabacina* (*38, 141*).

The resistance recently identified in TI's 575, 657, 722W, 748, 805, and 845 could be of great value, particularly if conditioned by multiple genes that could be less vulnerable to changes in pathogen virulence (*61*). The resistance in the TI's numbered above 1400 had been identified previously.

White mold or powdery mildew. The only sources of resistance to *E. cichoraceārum* that have been used in cultivar development are *N. glutinosa* and *tabacum* cv. Kuo-fan (table 12-4). Ternovsky (*183*) transferred dominant resistance from *N. glutinosa* through *N. digluta* to Russian cultivars nearly 40 yr ago. However, this resistance is not effective against a Rhodesian race of this fungus (*133*). Kuo-fan is now being used in breeding programs in the Eastern Hemisphere. The resistance of Kuo-fan is controlled by two recessive factors (*8*).

Like *P. tabacina*, *E. cichoraceārum* has great potential to produce new pathogenic races. Thus, collaborative plantings of appropriate cultivars and breeding lines, with the same objectives as for blue mold, have recently been initiated in Europe and the Near East.

Moderate, polygenic resistance may have to be used for *E. cichoraceārum* for the resistance to remain stable against such a variable and easily dispersed pathogen.

Black root rot. Resistance to black root rot has been a major objective of American and European tobacco breeding programs since early in the twentieth century. Numerous resistant cultivars have been released, most of which are listed by Lucas (*106*). In Kentucky, black root rot resistance has given farmers of that state an estimated sufficient additional income to pay for the operation of the State agricultural experiment station for the rest of this century (*39*). In spite of this fact, losses to this disease are still high (*186, 187*). Furthermore, cultivars with *N. tabacum* resistance may suffer considerable undetected loss. Better deployment of presently available resistance, as well as utilization of additional sources of resistance, could result in substantial further loss reduction.

N. tabacum cultivars and selections have been the major source of resistance for the newer improved resistant cultivars (*65, 88, 194*). Valleau (*194*) reviewed the breeding work up to 1952. Among older as well as newer culti-

vars, there is a wide range of reaction to *T. basicola*, varying from those that make practically no growth when infected to those that show no obvious symptoms of injury. Johnson (*88*) reported the polygenic nature of this type of resistance. From resistant Canadian selections, ev. Little Dutch, and other sources, he developed several resistant breeding lines and important cultivars (*88, 194*). Resistant TI's 87, 88, and 89 received much attention in breeding efforts from the 1930's to the 1950's (*28, 194*).

Clayton (*28*) identified numerous immune or highly resistant *Nicotiana* spp. (table 12-4). He obtained root-rot-immune allotetraploid *N. debneyi* × *N. tabacum* in the late 1930's and embarked upon an effort to transfer the immunity of *N. debneyi* to *tabacum*. When transferred to *N. tabacum*, immunity was inherited as a single dominant factor. The first cultivars with this immunity were released in the mid-1960's (*106*).

Although variability in *T. basicola* is well documented (*194*), a race of the fungus pathogenic on *N. debneyi* and tobacco cultivars with *N. debneyi* immunity has not been reported. However, cultivars with the *N. debneyi* immunity have not yet been widely grown due to the difficulty in combining desirable yield and quality with immunity. This difficulty and the stability of the resistance suggested that immune tobaccos contain a considerable segment of the pertinent *N. debneyi* chromosome.

The wealth of resistance to *T. basicola* in *N. tabacum* and other species offers great potential for further advances in black root rot control through host resistance.

Brown Spot. Tobacco cultivars and TI's vary in reaction to *A. alternata* from resistant to highly susceptible (*106, 167*). An immune *N. tabacum* has never been reported. All the tobacco cultivars and TI's listed in table 12-4 are considerably more resistant than any of the presently grown cultivars, which range from moderately resistant or tolerant to highly susceptible (*106, 167*). The control of resistance in TI's 505, 820, 995, 1043, 1138, 1467, 1561, and 1562, the only ones for which the genetics of resistance have been studied, is polygenic (*165*). Considerable breeding work has been done with resistant TI 1561 in the United States (*13*), Latin America (*6*), and Rhodesia. In the Dominican Republic TI 1583 is a leading cigar filler cultivar (*6*).

The *Nicotiana* spp. most resistant to brown spot are *N. bonariensis, debneyi, longiflora, noctiflora, repanda, suaveolens,* and *wigandioides* (*171*), all of which are more resistant than any *N. tabacum* accession. The F_1's from crosses of *N. bonariensis, longiflora, noctiflora,* and *repanda* with *N. tabacum* are susceptible. However, resistant F_1 plants and resistant lines from subsequent backcross generations have been obtained from sesquidiploid *N. suaveolens* × *N. tabacum* (Stavely, unpublished).

Frogeye leaf spot. Tobacco cultivars vary in degree of susceptibility to *C. nicotianae*. Generally highly susceptible burley cultivars are more susceptible than flue-cured or cigar cultivars. None of the tobacco introductions have much resistance, although some are considerably less susceptible than the commercial cultivars (*164*). The *Nicotiana* species *amplexicaulis, debneyi,* and *repanda* are immune (*166*). These species have considerable potential as species from which the resistance can be transferred into tobacco.

***Fusarium* wilt.** The tobacco cultivars listed in table 12-4 all have a high level of resistance to *F. oxysporum* f. sp. *nicotianae*. Much of the Fusarium wilt resistance in flue-cured cultivars originated in TI 448A (*120*). This resistance was transferred along with bacterial wilt resistance from this TI to flue-cured cultivars. The resistance is polygenic. Many commercial cultivars have *Fusarium* wilt resistance of this type (106).

Anthracnose. None of the tobacco cultivars and none of the TI's are resistant to anthracnose (*110, 136, 149*). Of the *Nicotiana* species, *N. fragrans* and *nudicaulis* are immune (*149*), as is *N. debneyi* in the Eastern hemisphere (*132, 136*) but not in the United States (*149*). This discrepancy for *N. debneyi* is probably caused by testing of different accessions of the species, although pathogenic races are a possibility, as such races occur in other phytopathogenic *Colletotrichum* spp. The allotetraploid *N. tabacum* × *N. nudicaulis* is highly resistant (*149*). All three immune species have considerable potential as sources of resistance for tobacco.

Root knot. Clayton and others (*31*) identified sources of resistance to *M. incognita* in the 1930's and embarked upon a breeding program that led to the release of a succession of resistant cultivars (*106*). The initial source of resistance was TI 706, in which resistance was apparently under polygenic control (*31*). By 1950, breeding lines had been obtained that had good resistance but small leaves and poor yields. In attempting to break the apparent linkage between resistance and small leaves, Clayton crossed a resistant line, RK 42, with a hybrid from Kostoff that was thought to be allopolyploid *N. sylvestris* × *N. tomentosiformis*. As a result of this cross, the problems in RK 42 were overcome beyond expectation. Not only was leaf size increased, but also the resistance was subsequently controlled by a single dominant factor. Resistant cultivars began to be released in 1960 (*106*). Clayton and others (*31*) theorized that the cross with Kostoff's hybrid has enabled elimination of resistance genes of lesser importance, leaving only a major single dominant factor from TI 706.

Cultivars having resistance from the program of Clayton and others (*31*) have been successful in reducing losses from root knot in the flue-cured region of the United States (*106*). However, this resistance is not effective against *M. arenaria* and *M. javanica*. The former species has caused only minor losses, but the latter is a significant

cause of losses in the southernmost part of the United States and the more tropical areas of the world. As reviewed by Slana and others (*157*), new attention was focused upon the root knot problem in 1969 when Graham reported a new strain of *Meloidogyne* in South Carolina. It is pathogenic on all cultivars having the monogenic dominant resistance apparently derived from TI 706.

Research was then undertaken to identify sources of resistance to *M. arenaria* and *M. javanica,* as well as to learn more about the resistance apparently derived from TI 706 (*157*). *N. tomentosa, N. tomentosiformis,* TI 706, and other pertinent tobaccos and *Nicotiana* spp. were inoculated with each of the five *Meloidogyne* spp. and subspecies pathogenic on tobacco. The results indicate that the resistance in tobacco did not come from TI 706 but from *N. tomentosa* or possibly an unidentified resistant selection of *N. tomentosiformis.* The Kostoff hybrid used in the 1950 cross is the apparent source of this resistance. That hybrid must have had either *N. tomentosa* or a resistant *N. tomentosiformis* as a parent. The resistance of *N. tomentosa* is identical to that of the resistant tobacco cultivars against the *Meloidogyne* isolates (*157*) and is controlled by a single dominant gene that is probably identical to that in the resistant tobaccos (Slana and Stavely, unpublished).

Several *Nicotiana* spp. exist that have high levels of resistance to *M. arenaria,* the virulent *Meloidogyne* from South Carolina, and *M. javanica* (table 12-4).

Schweppenhauser (*144, 145, 146*) has made significant progress towards transferring resistance to *M. javanica* from strain 256 of *N. tabacum* cv. Nyoka and N. *longiflora* into agronomically acceptable Rhodesian tobacco breeding lines. In lines with resistance from either of these sources, the resistance is controlled by a major, monogenic, dominant factor, the effect of which is enhanced by one or more modifier genes. Undesirable linkages to the resistance from strain 256 were broken by a cross with alloploid *N. sylvestris* × *N. tomentosiformis* (*145*). Resistant substitution lines of *N. tabacum* have been obtained having a segment of the *N. longiflora* chromosome conferring resistance (*144, 146*).

Brown root rot. Southards and Nusbaum (*161*) reported that individual plants of cultivars and breeding lines of *N. tabacum* vary in their response to *Protylenclus brachyurus* from high tolerance, in which host growth is enhanced, to susceptible, in which growth is severely inhibited by infection. By selecting and selfing tolerant plants, their percentage was increased in the succeeding generation. These results suggest that recurrent use of selection and selfing would eventually produce highly tolerant lines. Indeed this method has been used successfully to produce weather fleck tolerant cigar wrapper tobacco cultivars in Connecticut (*6*).

Graham and Burk (*55*) found that all 21 of the *Nicotiana* spp. they tested were less damaged by *P. brachyurus* than *N. tabacum; N. glauca* had the lowest disease index.

Cyst nematodes. As indicated in table 12-4, several *Nicotiana* spp. are resistant to *H. tabacum* and *H. solanacearum.* Among those resistant to *H. solanacearum* is *N. longiflora* (*5*). As discussed above, this species is resistant to race 0 of the wildfire bacterium and is the source of wildfire resistance in a number of tobacco cultivars. In certain of these wildfire resistant cultivars and breeding lines, the wildfire resistance was discovered to be linked to *H. solanacearum* resistance (*162*). However, the linkage has been broken rather easily, and *H. solanacearum* resistance has been shown to be polygenically controlled (*162*).

Discussion

As much or more has been accomplished in tobacco towards controlling diseases through breeding as in any other crop. Each of the disease resistances bred into tobacco cultivars has saved growers from millions of dollars of crop losses. Even in cases where new pathogenic races have overcome resistance, it still has been of great economic value.

The philosophy on sources and genetic control of resistance in tobacco has gone through an interesting evolution. Before the 1940's, interspecific transfer of resistance was impossible because of the agronomic and quality problems, as well as the genetic barriers. All resistance was thought to have to come from within *N. tabacum* and preferably from within commercial cultivars. The work of Clayton and Valleau brought about an almost complete reversal in this philosophy so that monogenic dominant resistance from the wild species, particularly those more distantly related to *N. tabacum,* was considered the best possibility. Today, limitations of this kind of resistance are well recognized. Hopefully, we now realize the strengths and weaknesses of both extremes, and consider both as being among the usable alternatives.

As more is learned about the physiology of pathogenic processes responsible for disease, cell selection techniques, using tissue and cell culture technology developed with tobacco, could become a viable alternative. However, these techniques may also have certain weaknesses. Application of the anther culture technology to breeding for disease resistance also promises to be a valuable tool (ch. 5). Disease losses can be further reduced through resistance, and new resistances and better management of presently available resistances are going to be needed in the years to come to maintain our defences against this wide, and ever variable, group of tobacco pathogens.

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13

BACTERIAL HYPERSENSITIVITY

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Introduction

For many years, the hypersensitive reaction (HR) of tobacco to viral infection has been extremely useful for determining infectivity and in the study of physical and chemical properties of many plant viruses. Until fairly recently, similar reactions against bacterial plant pathogens did not seem to occur, although, in retrospect, evidence for rapid lesion formation in leaves inoculated with high populations of incompatible bacteria had been available for many years (*2*). The development of simple, effective techniques to introduce bacteria into the intercellular spaces of *Nicotiana tabacum* leaves allowed a clear-cut demonstration of the bacterially induced HR (*15, 20*). This discovery opened up innumerable avenues for investigation of host-parasite interactions involving plant-pathogenic bacteria and facilitated the study of recognition phenomena in compatible and incompatible systems. The reaction also has been useful in taxonomic studies involving the fluorescent plant pathogenic pseudomonads (*21*).

As in the case of local lesion formation by viruses, questions have been raised concerning the existence of the bacterially induced HR under natural conditions. Because a visible HR is obtained only after infiltrating with high inoculum concentrations, which are unlikely to occur in nature during the infection process, a possibility exists that the reaction is an artifact of no particular significance in the normal host-parasite interaction. Whether or not the reaction ultimately will be shown to occur under natural conditions, it is of inestimable value to scientists whose primary interests are in the response of plant host cells to potential pathogens. Because tobacco leaf cells are extremely sensitive to the presence of bacteria and react in a rapid and predictable fashion, the tobacco plant has been the host of choice in physiological investigations of the HR. The ease with which the intercellular spaces in tobacco mesophyll tissues can be infiltrated also has been a major factor in the widespread use of the tobacco plant for the study of hypersensitivity.

This chapter will describe (a) how the bacterially induced HR is obtained in tobacco leaves, (b) what factors affect the reaction, and (c) what are some of the major applications of the reaction in the study of host-parasite interactions.

Methods Used To Induce HR

In its simplest form, as originally described by Klement (*15*), the method to induce the HR requires a minimum of equipment and facilities. Tobacco leaves are infiltrated on the abaxial side with a bacterial suspension of at least 5×10^6 bacteria of the appropriate strain per milliliter. A hypodermic syringe fitted with a 30-gage needle is used to inject the bacteria under the epidermis and into the mesophyll tissue (fig. 13-1). Although apparently simple in

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Figure 13-1.—Tobacco leaf just after infiltration with a bacterial suspension. Note the water-soaked appearance of the treated, intercostal areas.

theory, considerable experience is required to distribute the bacteria evenly in the leaf tissue without excessive puncturing of the epidermis and other damage to the leaf tissue. In addition, other factors are important for obtaining proper results.

Detailed instructions for the procedure are as follows: Use fully developed, but not senescent tobacco leaves for infiltration. Young leaves have closely packed cells and small intercellular spaces so that considerable skill and a steady hand are required to infiltrate small areas. With expanded leaves, on the other hand, a single puncture along a lateral vein or midrib will allow infiltration of the laminar area between two lateral veins (panel) if sufficient pressure is steady and continuous. Injections along the lateral veins can be controlled more easily, and damage to the tissue prevented more readily, than when they are made in the middle of the panel. If infiltration in the middle is necessary, however, support the tissue directly underneath the injection point with the other hand. Use only sharp needles and introduce them with the bevelled side down and at an angle such that lifting of the epidermis is accomplished without perforating through the leaf.

Most cultivars of *N. tabacum* appear to react in the same manner to infiltration with incompatible or avirulent bacteria, although occasionally differences exist between varieties in their response to certain isolates. The period necessary for complete collapse of the leaf cells may vary depending on the environmental conditions provided. The cultivar 'Bottom Special' (a flue-cured variety developed in North Carolina and no longer in commercial use) is one of the most convenient to use because it has large and succulent leaves, regularly reaching 30 cm by 20 cm under growth room conditions. The leaves are flexible and can be bent easily to reach the lower epidermis for injection. This cultivar grows well at 20 C in sand culture but requires high light intensity (2,000 ft-c) and a long photoperiod (12 h) in the growth room. A combination of cool white and Gro-Lux fluorescent tubes and tungsten incandescent bulbs provides adequate light conditions. Under normal circumstances, plants are ready for infiltration at approximately 1 mo from transplanting.

Use bacterial cultures at the logarithmic growth phase and prepare suspensions in 0.1*M* phosphate buffer, pH 7.0, saline, or in other appropriate fluids designed to prevent bursting of the cells. Wash the cells several times by centrifuging at 10,000 g for 30 min and resuspending the pellet in buffer. Check populations carefully by dilution plating immediately before infiltrating the leaves.

The speed and intensity of the HR depend on the number of bacteria in the inoculum and on the nature of the strain used. With strain B-1 of *Pseudomonas solanacearum*, an avirulent variant, a concentration of at least 5×10^6 bacteria per milliliter is necessary to induce the HR at 28 C. The most rapid response is obtained with an initial inoculum of 10^9 cells per milliliter and this is the concentration used in most assays. The latter corresponds to an optical density of approximately 0.5 at 600 nm.

For assay purposes, infiltrate an area, 3 to 5 cm^2, in each of four to six intercostal areas on one half of each leaf with the suspension. For control, infiltrate similar areas with buffer (or other solutions used to suspend the bacteria) on the opposite side of the leaf. Under normal environmental conditions, the initial watersoaking caused by infiltration will disappear within 15 to 20 min. By 6 h, the infiltrated area will appear darker than normal, and small water-soaked spots will become evident. These

spots become coalescent, and water is lost rapidly, leading to total collapse of the infiltrated area. This area becomes translucent by 12 h after infiltration and is sharply delimited by the healthy tissues surrounding it. By 24 h, the affected tissues appear bleached and become progressively thin and brittle (fig. 13-2). In contrast, control infiltrated areas remain turgid throughout this period and, except for the puncture caused by injection, they appear normal.

If compatible, rather than incompatible, strains of *P. solanacearum* are used, no symptoms are evident until 36 h after infiltration, when a dark, necrotic reaction appears. A distinct yellow margin is formed at the spreading edge of the lesion, and by 60 h after infiltration the bacterium spreads to the tissues adjoining the infiltrated area. The bacterium eventually invades the veins and veinlets, which turn dark brown, becomes systemic throughout the plant, and causes wilting.

Factors That Affect HR

Induction Period

HR induction is a time-dependent process apparently initiated by a recognition phenomenon between cell wall components of both bacterium and host cell. Once the process is initiated, it is irreversible and proceeds to completion in a highly predictable fashion. Initial estimates for the contact period between host and pathogen necessary to initiate the reaction, based on experiments in which streptomycin was infiltrated at various times after infiltration, were as short as 20 min (*18*). More recent evidence, based on the use of rifampicin, which selectively inhibits bacterial RNA synthesis, suggests that 3 h is a more reasonable estimate necessary for induction of the HR (*37*).

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Figure 13-2.—Appearance of the hypersensitive reaction 24 h after infiltration. Intercostal areas on the left side were treated with a suspension containing 10^8 cells per milliliter of *Pseudomonas solanacearum*; those on the right side were treated with a buffer.

The 3-h induction corresponds to that necessary for envelopment of the bacteria following initial attachment to the cell wall (*42*). Although the initial recognition between bacterial and host cell wall components must occur quickly, the reaction that is set in motion for envelopment of the bacterium probably is dependent on increased synthesis of protein and of cell wall components, a process that requires both time and energy. Similarly, induction of the HR apparently is dependent on the metabolic activity of the trapped bacterium. Time is required for the production and release of a putative inducer that affects the integrity of the plasmalemma of the host cell. Presumably, this occurs during the latent period between induction and the appearance of symptoms.

Inoculum Concentration

HR induction and the rate of appearance of the visible confluent collapse of the host cells are directly related to the number of cells in the inoculum. With the B-1 strain of *P. solanacearum* at 10^7 to 10^9 cells per milliliter, the threshold level is reached and the reaction proceeds to completion. At 10^9 cells per milliliter, the reaction is evident within 12 h; however at 10^7 cells per milliliter, the HR is not induced until the threshold level is reached at approximately 36 h after infiltration (*37*).

Inoculation with 10^6 cells per milliliter and below does not lead to a visible HR, nor to the rapid population reduction of the bacterium, which is normally associated with the HR. These results are in conflict with those reported by Turner and Novacky (*45*) for tobacco leaves inoculated with *P. pisi*. They concluded that at inoculum levels below those necessary to cause confluent, visible lesions, the HR still occurred at scattered locations, causing individual

cell collapse, which could be detected by staining with Evans blue. By extrapolation, a 1:1 ratio was shown between number of bacteria and number of dead host cells. Although no histological observations of leaves inoculated with B-1 cells have been made, the fact that bacterial populations do not decline when the initial inoculum is low suggests that death of the bacterial cells is not necessarily associated with death of the host cell.

For confluent lesion development, the ratio between tobacco leaf cells and *P. cichorii* cells must be near 100 (*43*). at inoculum levels below 10^6 cells/ml, the ratio drops below one bacterium per host cell, and confluent collapse of the host cells is not obtained. These data are in agreement with the concept that in heterologous combinations, symptom production results from joint, rather than independent, action of the bacteria on the host (*4*).

Age of Cultures

Age of the bacterial culture used to prepare the inoculum for infiltration of tobacco leaves appears to affect HR development (*17, 44*). When 1-, 10- and 20-day-old *P. phaseolicola* cultures were used, the reaction appeared 8 to 9, 10 to 11, and 12 to 13 h after infiltration, respectively. The affect of culture age apparently is on the initial period of induction, for the length of the latent period between induction and cell collapse was not affected by culture age. Attachment of bacteria to host cell walls, a process which appears necessary for induction of the HR, may be inhibited by the increasing amounts of extracellular polysaccharide produced in aging cultures.

Temperature

As is characteristic of all biological processes, development of the HR is affected by temperature. With the B-1 strain of *P. solanacearum*, Lozano (*28*) reported a progressive delay of the HR in tobacco leaves of plants exposed to a temperature range from 28 C to 16 C. At 28 C, the reaction was essentially complete by 12 h after infiltration; at 16 C, the reaction was not complete until after 72 h, even at inoculum concentrations as high as 3.5×10^9 cells per milliliter. At high temperatures, however, the reaction is suppressed. No HR develops in tobacco plants maintained at 37 C for at least 5 h after infiltration with *P. phaseolicola* (*17, 19, 44*). Whether the effect of high temperature is on the bacterium or on the host cell has not been established; Klement (*17*) believes that a thermosensitive period exists in host tissues immediately following induction. Possibly, envelopment of the bacterium is the process that is suppressed at high temperatures, but this has not been determined with certainty.

Light

Although the HR will reach completion in infiltrated leaves placed in the dark for short periods, long exposure to darkness will allow multiplication of the bacteria and movement into adjoining tissues. Lozano and Sequeira (*30*) have shown that as the period of darkness increases, the reaction shifts from a hypersensitive one to the slow, necrotic type caused by compatible strains of *P. solanacearum*. In some experiments, tobacco leaves were infiltrated with isolate S 210 (incompatible) and immediately covered with aluminum foil for 6 to 48 h. With periods of darkness up to 12 h, the HR proceeded normally. Bacteria could not be recovered from the lesions after 48 h. As the darkness increased to 18 h and beyond, bacterial populations also increased within and outside the infiltrated area.

When the procedure described above was reversed, that is, when the leaves were exposed to light first, then to darkness, the results led to the same conclusion: long periods of darkness cause the reaction to shift from the hypersensitive to the compatible type.

The effect of light on the HR, as described for *P. solanacearum* strains, may not be characteristic of the reaction obtained with other bacteria. Klement and Goodman (*19*) indicate, for instance, that the HR appeared in plants kept either in the light or in the dark. However, the length of the light and dark periods or the bacterial strain used in these experiments was not indicated.

Relative Humidity

No detailed studies have been done of the relation of the development of the HR to the relative humidity (RH) of the environment in which the plants are kept. Because the HR causes cell collapse, at low RH values water loss occurs more rapidly than under water-saturated conditions. Thus, under high RH, the HR onset may be delayed, and bleaching of the tissues may not become evident. In pepper inoculated with *Xanthomonas vesicatoria*, the HR may be prevented completely if the leaves are kept water-soaked continuously (*1*). On the other hand, with the tobacco-*P. solanacearum* system, incubation of inoculated tissues at either 87 to 92 percent or 50 percent RH did not visibly affect either the rapidity of induction or the appearance of the lesions (*28*).

Age of Inoculated Leaf

Klement and Goodman (*19*) reported that in Xanthi tobacco inoculation of young leaves with *P. syringae* resulted in lesions developing 1 to 1.5 h earlier than on old leaves. Observations generally have shown that old, senescent leaves do not respond to inoculation as rapidly as younger ones. In practice, however, only fully expanded leaves are inoculated because infiltrating young leaves that have tightly packed cells and small intercellular spaces is extremely difficult. Young leaves are used mostly for fine structure studies of the HR because the cells have small vacuoles.

Applications of the Bacterially Induced HR

The discovery that the HR was induced by infiltrating bacteria into tobacco leaves marked a significant technological advance. It allowed examination of the host-pathogen interaction from both structural and physiological standpoints. In addition, the reaction has been of considerable value for taxonomic purposes and in the study of mechanisms of induced resistance.

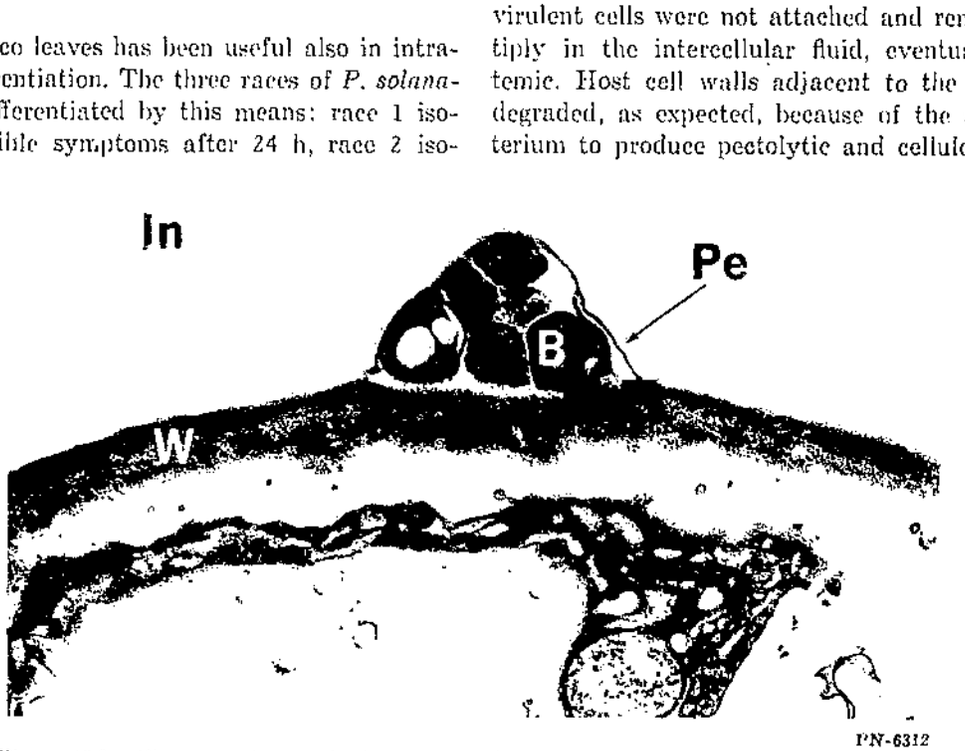
Taxonomic Implications

The initial work of Klement and co-workers (*15*, *20*) provided a clear indication that the HR was induced by pathogenic but not by saprophytic pseudomonads; further, the reaction was characteristic only of heterologous combinations. Thus, with this technique saprophytic pseudomonads could be distinguished from pathogenic pseudomonads, as well as those that were specifically pathogenic on tobacco. The usefulness of this concept is evident from the extensive studies of Lelliott and others (*21*) in which determinative schemes for the plant-pathogenic pseudomonads are based on five tests: production of levan, oxidase reaction, soft-rotting of potato, presence of arginine dihydrolase, and the HR on tobacco. These criteria are widely used to differentiate plant-pathogenic nomenspecies from each other and from the saprophytes. Although some saprophytes, such as *P. fluorescens*, cause necrosis similar to that associated with the HR, they can do so only in plants previously exposed to continuous darkness for 3 d (*24*).

The HR on tobacco leaves has been useful also in intraspecific, strain differentiation. The three races of *P. solanacearum* could be differentiated by this means: race 1 isolates caused no visible symptoms after 24 h, race 2 isolates caused the HR by 10 to 12 h after infiltration, and race 3 isolates caused only a yellowish discoloration of the infiltrated area by 48 h (*29*). Although this is a reasonably consistent pattern, the reaction cannot be relied upon entirely. Granada & Sequeira (*11*) reported that five race 1 isolates induced the HR on cv. 'Cuba 12'; four of these were avirulent to tobacco, as expected, but one (S 123) was highly virulent. This isolate multiplied rapidly before the HR was induced, unlike incompatible strains, which generally do not multiply during the first few hours after inoculation at high population levels. These results show that the HR can be used as preliminary evidence for the lack of pathogenicity of most isolates, but this must be confirmed by direct pathogenicity tests.

Fine Structure

Attention has been given to the ultrastructural details of the initial interaction between tobacco mesophyll cell walls and compatible or incompatible forms of *P. solanacearum* (*37*, *42*). Specimens examined under the electron microscope at 3 h after infiltration, at the time when the HR becomes irreversible, showed that only the avirulent cells were surrounded by granular and fibrillar material. A thin, fibrillar pellicle enveloped the bacteria completely by 7 h after infiltration, and granular material accumulated in the space between the pellicle and the cell wall (fig. 13-3). Attached bacteria did not divide. In contrast, virulent cells were not attached and remained free to multiply in the intercellular fluid, eventually becoming systemic. Host cell walls adjacent to the virulent cells were degraded, as expected, because of the ability of the bacterium to produce pectolytic and cellulolytic enzymes.



PN-6312

Figure 13.3.—Electron micrograph showing avirulent cells of *P. solanacearum* (B) enveloped by a fibrillar pellicle (Pe). The cells are lying in an intercellular space (In) next to a cell wall (W), magnification 25,000×.

Attachment and envelopment of bacteria by mesophyll cells have been reported also for tobacco infiltrated with water suspensions of *P. pisi* (*9*). This process is a general phenomenon and may be a prerequisite for the HR initiation. However, the HR does not proceed if the bacterium is not metabolically active, as demonstrated in experiments in which heat-killed bacteria were used (*42*). These cells are attached and enveloped in the same fashion as are live ones, but the violent host response associated with the HR is not induced.

Marked changes occur in the host plasmalemma immediately under, and extending for several micrometers on either side of, the place of attachment of the live bacterium on the cell wall. Membrane-bound vesicles accumulate in the space between the plasmalemma and the host cell wall; the material that accumulates around the bacterium seems to originate at the plasmalemma and perhaps is transported across the cell wall, but this is difficult to ascertain. Within 7 h after infiltration, organelles are deranged; mitochondria, in particular, do not have well-defined cristae (*8, 42*). As the HR proceeds to completion, disruption of all cellular organelles is evident. The plasmalemma, tonoplast, and the bounding and internal membranes of chloroplasts and mitochondria lose their integrity and water is lost rapidly (*8*). The chloroplast lamellar structure becomes disorientated, and chloroplast membrane proteins undergo changes in such physicochemical properties as solubility and lecithin-binding capacity (*13*). Permeability changes appear to be correlated with specific changes in the chloroplast thylakoid membrane protein (*14*).

Bacterial Multiplication

The infiltration technique to induce the HR in tobacco leaves allows accurate measurement of the changes in numbers of bacteria that occur before and during development of the reaction. Analysis of these population changes yields meaningful information regarding threshold levels for induction of the HR and on the suitability of the intercellular fluid as a medium for bacterial growth.

Bacterial populations in infiltrated tissues can be determined by standard dilution plating techniques. Take samples immediately after infiltration and at suitable intervals thereafter until collapse of the tissues occurs. For this purpose, cut leaf disks with a cork borer from the center of the infiltrated area and comminute them with a TenBroek tissue grinder in 1 ml of distilled water or appropriate buffer. Dilute the resulting suspension with water or buffer in a standard logarithmic series and plate out appropriate dilutions on a suitable medium. Because variability is usually high, the number of samples removed at each sampling time must be adequate for meaningful, statistical analysis. Calculations can be made simple if the leaf disk sample is exactly 1 cm^2 of leaf surface; thus, the populations per milliliter will represent those per square centimeter. The intercellular space of a leaf disk of this size will vary from 0.01 to 0.1 ml, depending on the age and condition of the leaf.

The general growth pattern obtained when low inoculum levels (10^5 cells per milliliter or below) are used indicates substantial differences between compatible and incompatible bacteria (*40*). The typical growth curve for compatible *P. solanacearum* strains consists of a short (8 h) lag period followed by rapid exponential increase to approximately 10^9 cells per milliliter at about 45 h after inoculation. With incompatible strains, populations also increase after the initial lag but remain below the threshold level, that is, below 5×10^6 cells per milliliter, for induction of the HR and then decline slowly. At higher inoculum levels (10^7 cells per milliliter), populations may increase steadily until the threshold level is reached 36 h later and then decline rapidly. At still higher inoculum levels (10^9 cells per milliliter), populations remain steady for 6 h and then decline precipitously as the HR is induced (*37, 40*).

Bacterial multiplication is not essential for HR induction. If the inoculum is introduced at levels higher than the threshold, bacterial populations remain steady for about 6 h and then begin to decrease. At this time, electrolyte leakage first becomes evident. Populations drop precipitously before complete collapse, and dessication of the host cells occurs. This is presumptive evidence that the materials that leak out of the affected cells have bactericidal properties (*36*).

Physiological Changes Associated with HR

Because the HR involves changes in membrane integrity, one of the first, measurable, physiological changes in inoculated tobacco leaf tissue is an increase in electrolytes that leak out of the cells and into the suspending solution (*6*). A sharp increase in conductivity coincides with the appearance of the HR. Other physiological changes also occur during the latent period. Although the host is symptomless, respiratory metabolism increases, the maximum rate occurring just before tissue collapse occurs (*32*). Such temporary respiratory increases may reflect the initial effect of the loss of integrity of mitochondrial membranes. The inactivity of polyphenoloxidase, peroxidase, or other oxidases does not increase significantly enough to contribute to the increase in oxygen consumption. In addition, phosphatidases and proteases, which could be involved in membrane breakdown, do not increase during the latent period (*12, 33*).

Only minor increases are shown in RNase in the supernatant fractions of tissue extracts undergoing the HR. Kiement (*17*) argues that marked changes in these various enzymes, which do occur when the HR is induced by tomato mosaic virus (TMV), are absent in the bacterially-induced HR because the latter reaction occurs too rapidly. Alternatively these changes might not occur with bacterial

inocula because the mechanisms that induce the HR are different from those involved in local lesions initiated by viruses.

The properties of the intercellular fluid before, during, and after the HR development are of considerable interest. The method to obtain this fluid, described by Klement (*16*), is simple, but, for reasons that are not readily apparent, has had limited applications. In this method, the fluid is spun out of the leaves by centrifugation after infiltrating the leaves with water or buffer. Because considerable dilution results from this procedure, the fluid must be concentrated (to about one-fourth original volume) to simulate the osmotic potential of the fluid *in vivo*. Even in the dilute form, however, the fluid provides an adequate medium for such bacteria growth as *P. tabaci* (*16*).

Fluids from tobacco leaves infiltrated with an incompatible strain of *P. solanacearum* became inhibitory to bacterial growth only when extracted 12 to 18 h after inoculation (*29*). The compounds responsible for this inhibition have not been identified—but are heat-stable and of low molecular weight.

Such inhibitory compounds may help explain why bacterial populations begin to decrease at about the same time that electrolyte leakage first becomes evident and why these populations drop so rapidly before complete collapse and desiccation of the leaves occur.

Nature of the HR Inducer

The bacterially induced HR is particularly suitable for investigations on the nature of the substance(s) that induces the reaction. The reaction is predictable, and numerous fractions can be assayed conveniently on a single leaf. The bacteria can be grown separately from the host and vast amounts of cell constituents can be obtained by relatively simple fractionation procedures. Unfortunately, these procedures have not resulted in the elucidation of the inducer. By osmotic shock, Gardner & Kado (*5*) isolated a factor from *Erwinia rubrifaciens* cells that reproduced the symptoms of the HR. Sequeira & Ainslie (*38*) reported also that a high-molecular weight fraction from *P. solanacearum* induced a reaction indistinguishable from the typical HR. Unfortunately, these reports have not been published in detail and have not been confirmed.

Because many toxic compounds present in bacterial extracts can cause collapse of tobacco leaf tissues, distinguishing this reaction from that typical of the HR is difficult. However extracting the inducer only from those strains that are incompatible in tobacco should be possible. When this criterion is applied, none of the published reports appears to refer to a specific inducer. Possibly, cell walls of all gram-negative, plant pathogenic bacteria contain the inducer but are prevented from exerting action on the host because compatible strains produce a capsule or extracellular slime that covers potential binding sites. Such strains do not induce the HR, but extracts that do not contain the soluble capsule, but retain cell wall components, would be effective inducers of the HR.

Incompatible bacteria, once they attach to an infectible site on the host cell wall, could become metabolically activated and release a highly unstable toxin that would interact with a "sensitivity locus" on the host cell (*3, 37*). At one time, this toxin was thought to be simply ammonia released by the metabolic activities of the bacterium (*25, 26*), but careful measurements of the amounts of ammonia produced in the tissues undergoing the HR failed to support this hypothesis (*7*).

Because a broad spectrum of compounds that contain a reactive SH group (thioglycolic acid, mercaptoethanol, dithiothreitol, and others) cause a toxic reaction on tobacco leaves that mimicks the HR, it has been suggested that the HR results from cleavage of the S-S bonds in membrane proteins (*6, 19*). This is a plausible hypothesis, but components that have this capacity to break S-S bonds have not been isolated from plant pathogenic bacteria.

HR Prevention

Numerous reports exist that the HR can be prevented or interfered with by various treatments. These include high temperature (*44*), exposure to long periods of darkness (*29*), cytokinins (*34*), and preinoculation with either live (*35*) or heat-killed bacteria (*30*). The effects of these treatments are of interest because they may help to elucidate the nature of the HR. The effect of heat-killed bacteria is of particular interest because competition for nutrients between the bacteria in the initial and in the challenge inoculations is prevented. Tobacco leaves pretreated with heat-killed bacterial cells become refractory to infection by either compatible (*23*) or incompatible (*30*) bacteria. Although incompatible bacteria do not cause the HR in protected leaves, the reaction does not shift to compatibility. Rather, a systemic response results that protects the plant from invasion by a variety of plant pathogens, including bacteria and viruses (*22, 27, 30*).

The systemic, protective response induced by heat-killed bacteria is (a) dependent on the concentration of bacteria in both initial and challenge inoculations; (b) time-dependent—the reaction requiring between 7 and 18 h for complete protection; (c) light-dependent—the action spectrum consisting of a bimodal curve with peaks at 450 to 500 nm and 600 to 660 nm (Kraus, unpublished), reminiscent of the spectrum for chlorophyll b; (d) temperature-dependent—protection being inhibited in plants grown at 32 C or higher; (e) persistent—protection reaching a maximum within 60 h and then decreasing gradually for several days; and (f) nonspecific—both in terms of the bacteria that induce the protective response and in terms of the bacteria that are prevented from multiplying in the protected tissues (*30, 41*).

The acquisition of resistance appears to depend on the same type of recognition phenomenon between outer constituents of the cell walls of host and parasite that we described for HR induction. Heat-killed bacteria are rapidly attached to and enveloped by the host cell wall, and large amounts of granular material accumulate around the dead bacterial cell and on the inside of the host cell wall. Recent work has established that the lipopolysaccharide layer (LPS) of the outer wall of gram-negative bacteria is the inducer of resistance (*10, 31*). Because gram-positive bacteria are ineffective as inducers of disease resistance, and all gram-negative bacteria tested so far (including several saprophytes) are active inducers, these results are consistent with the hypothesis that LPS is the active molecule.

The LPS isolated in pure form from *P. solanacearum* is an effective inducer of disease resistance in tobacco, but its activity is completely destroyed by treatment with almond emulsin, which contains a mixture of β-glycosidases. Chemical modifications of the LPS molecule indicate that the active moiety is the lipid A portion, but it must be present in a form sufficiently soluble to attach to a putative receptor in the host cell wall.

Purified LPS can be visualized readily by electron microscopy. When introduced into tobacco leaves, it attaches to mesophyll cell walls and induces ultrastructural changes in the host cell similar to those resulting from attachment of the entire bacterium (*10*). A lectin, isolated from tobacco cell walls, exhibits high binding affinity for LPS and causes rapid precipitation of this compound *in vitro* (*39*). The lectin binds specifically to internal *N*-acetyl glucosamine groups; such groups are major constituents of the lipid A portion of LPS. Thus, a plausible hypothesis is that this cell wall lectin constitutes the binding site for bacterial LPS in the plant and is responsible for the initial recognition event that results in a resistant response.

Although the molecular interactions that result in attachment of bacteria to tobacco cell walls are reasonably well understood, information is limited concerning the nature of the mechanism that results in a resistant response (*40, 42*). When live, incompatible bacteria are used in the challenge inoculation, the number of cells is reduced rapidly, they grow in chains, and do not attach to the host cell wall. Antibacterial substances appear in the intercellular fluid at the time when protection first becomes evident (*36*). These compounds are produced only in plants kept in the light, are heat-stable, and of low molecular weight. Intercellular fluids extracted from protected tissues contain, in addition to these inhibitors (possibly terpenoids), substantial amounts of salts, amino acids, sugars, and other compounds that affect bacterial growth. The increase in osmotic pressure is sufficient to explain some of the inhibitory effects of intercellular fluids. These complex effects have not been resolved as yet, but it is clear that terpenoids contribute most of the inhibitory effect.

Tobacco leaves that have been treated with heat-killed bacteria also show marked increases in peroxidase (*27, 36*). This increase appears to be caused by a light-dependent transfer of peroxidase from the leaf cell to the intercellular fluid. Increases in peroxidase can be detected in tissues distant from the site of initial inoculation and are correlated with the appearance of resistance. In addition, a lectin that binds to bacterial LPS can be extracted from protected leaves by infiltration with saline and is present at concentrations considerably greater than normal (Graham & Sequeira, unpublished). How all of these factors interact to create an environment that is unfavorable for multiplication of plant pathogenic bacteria is unknown.

Discussion

This brief review of some of the literature pertaining to the HR induced in tobacco by plant pathogenic bacteria gives ample evidence of substantial progress in the understanding of a reaction that was essentially unknown until 1963. This rapid progress is the result of applying simple, but effective, techniques developed by Klement to introduce bacteria into the intercellular spaces of tobacco leaves and to extract the fluid in which these bacteria grow in the plant. These techniques will continue to be useful adjuncts to other, more sophisticated, techniques to study the interaction of bacteria and host cells.

That tobacco was used initially in the bacterially induced, HR study was a fortuitous but fortunate occurrence. The tobacco plant has remarkable sensitivity to the presence of plant pathogens, including bacteria, and reacts in a highly predictable fashion. It is highly adaptable for work in the laboratory and growth room, and the large intercostal spaces in the laminae facilitate the introduction not only of bacteria but of a variety of substances that affect the HR and thus help in the elucidation of the reaction itself. Extensive literature on the physiology, biochemistry, and genetics of tobacco provides information to resolve problems of induction or prevention of the HR.

The wide popularity that the tobacco plant has had for the HR study is justified but carries the inherent danger that broad generalizations will be made that are not applicable to all plants. The HR induced in pepper and bean by phytopathogenic bacteria differs in many important aspects from that induced in tobacco. Controversies regarding factors that prevent the HR in tobacco, but not in other hosts, are now glaringly evident in the literature. The systemic resistance induced by prior inoculation in tobacco is not characteristic of most of the host-parasite systems that have been described.

Techniques that will allow unraveling of many of the complex problems associated with the bacterially induced

HR in tobacco are now available. No doubt the next few years will see advances that will allow an interpretation of many unresolved problems pertaining to the interaction of tobacco and plant pathogenic bacteria. However, such solutions may be applicable only to the tobacco plant and reflect only how it has evolved and developed mechanisms to prevent the establishment of potential parasites. Such mechanisms may be vastly different from those in other plants.

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Appendix—Suppliers

Nicotiana species.
L. G. Burk, Science and Education Administration, U.S. Department of Agriculture, Tobacco Research Laboratory, RR 2, Box 16 G, Oxford, N.C. 27565.

Nicotiana tabacum—cultivar and introduction collection and breeding lines.
V. A. Sisson, Science and Education Administration, U.S. Department of Agriculture, Tobacco Investigations, Beltsville, Md. 20705.

Nicotiana tabacum cv. Havana 38 (Wis 38).
R. C. Newman, Horticulture Department, University of Wisconsin, Madison, Wis. 53706.

Nicotiana—selected species and cultivars of *N. tabacum*.
L. N. Bass, Director, National Seed Storage Laboratory, Colorado State University, Ft. Collins, Colo. 80521.

The Laboratory was established in 1958 to preserve germplasm through acquisition and storage of seed of known or potential value. Tobacco seed with adequate documentation are gladly accepted from all public agencies, seed companies, and individuals involved in plant breeding, seed research, or genetic studies. When seed are accepted for storage they become the property of the Federal Government, and the Laboratory assumes the responsibility of future maintenance unless other arrangements are made prior to acceptance.

Tobacco pathogens.
American Type Culture Collection catalog, 13th edit., 12301 Parklawn Drive, Rockville, Md. 20852.

Macerozyme R-10 and Cellulase R-10.
Yakult Biochemicals Co., Ltd., 8-21 Shingikaucho, Nishimomiya, Japan.

Phytohormones and miscellaneous fine chemicals.
ICN Life Sciences Group, 26201 Miles Rd., Cleveland, Ohio 44128.
Sigma Chemical Co., P. O. Box 14508, St. Louis, Mo. 63178.

Premixed inorganic salts, phytohormones and coconut water.
Grand Island Biological Company, 3175 Staley Rd., Grand Island, N.Y. 14072.

Mannitol.
Curtis Matheson Scientific Co., 470 Valley Dr., Brisbane, Calif. 94005.

Dextran T_{40}.
Pharmacia Fine Chemicals, Piscataway, N.J. 08854.

Rimocidin.
Pfizer Laboratories, 16700 Red Hill Ave., Irvine, Calif. 92714.

Evans blue.
ICN Life Sciences Group, 26201 Miles Rd., Cleveland, Ohio 44128.

N-Z amine type A.
Sheffield Chemical Co., Norwich, N.Y. 13815.

Potassium dextran sulfate.
Meito Sangyo Co., Ltd, The Sankeido Bldg., 1, Muromachi, 4-chome, Nihonbashi, Chau-Ku, Tokyo 103, Japan.

Polyethylene glycol (Carbowax).
Fisher Scientific Co., 585 Alpha Dr., Pittsburgh, Pa. 15238.
Polysciences, Inc., Paul Valley Industrial Pk., Warrington, Pa. 18976.

Polyvinylpyrrolidone.
Sigma Chemical Co., P. O. Box 14508, St. Louis, Mo. 63178.

Silicone oil DC200.
J. T. Baker Chemical Co, 222 Red School Lane, Phillipsburg, N.J. 08865.

Peters fertilizer.
Harry Sharp and Son, 420 Eighth Avenue North, Seattle, Wash. 98109.

Filter aids.
VWR Scientific Co., P. O. 8603, Baltimore, Md. 21240.

Miracloth.
Calbiochem, P. O. Box 12087, San Diego, Calif. 92112.

Nylon (Nitex) screening.
Toblar, Ernst and Trabber, 240 Saw Mill River Rd., Elmsford, N.Y. 10523.

BEEM capsules Spurr's epoxy resin, and EM grade acetone.
Ernest F. Fullam, Inc., P. O. Box 444, Schenectady, N.Y. 12301.

Plastic ware for cell culture.
Falcon Plastics, 1950 Williams Dr., Oxnard, Calif. 93030.

DeLong flasks, Cuprak dishes and general plastic ware.
Bellco Glass Inc., 1401 Edrudo Rd, Vineland, N.J. 08360.

Babcock centrifuge and Babcock milk test bottles.
Scientific Products, 150 Jefferson Drive, Menlo Park, Calif. 94025.

Gro-Lux lamps.
North American Phillips Lighting Corp., Hightstown, N.J. 08520.

Laminar flow hoods.
Baker Co. Inc., Sanford, Maine 04073.
Laminar Flow, Inc., 739 E. Elm St., Gonshohocken, Pa. 19428.
NuAire Inc., 4937 Boone Avenue North, Minneapolis, Minn. 55428.

Gyrotory shaker.
New Brunswick Scientific Co., Inc., P. O. Box 986, Edison, N.J. 08817.

Laboratory safety information.
Laboratory Safety at the Center for Disease Control (Sept. 1974). U. S. Department of Health Education and Welfare, Publication No. CDC 75-8118. Center for Disease Control. 1600 Clifton St. NE. Atlanta, Ga. 30333.

Guidelines for recombinant DNA research containing environmental impact and laboratory safety information.
National Institutes for Health (Director Donald S. Frederickson), 9000 Rockville Pike, Bethesda, Md. 20014.

Species Index

END