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CONTENTS

Contributors to Volume 3 ix

Preface xi

Philip Rodney White xiii

Contents of Previous Volumes xix

Protoplasts and Plant Viruses

Harry Murakishi, Mark S. Lesney, and Peter Carlson

I. Virus Infection of Plant Protoplasts 1
II. Virus Replication in Plant Protoplasts 16
III. Resistance to Virus Infection 26
IV. *In Vitro* Methods for Recovering Virus Resistance 39
V. Perspectives and Problems for Genetic Engineering 47
References 48

Applications of Flow Cytometry to Tissue Culture Systems

Katharine A. Muirhead and Paul K. Horan

I. Introduction 57
II. What Is Flow Cytometry? 57
III. Cell Kinetic Analysis 66
IV. Chromosome Analysis 70
V. Cell Marker Analysis 75
VI. Cell Functional Analysis 78
VII. Detection and Cloning of Mutant and Hybrid Cells 81
VIII. Conclusions 87
References 88

Plant Tissue Culture Systems for Screening of Plant Growth Regulators: Hormones, Herbicides, and Natural Phytotoxins

J. Gressel

I. Introduction: General Considerations 93
II. Screening for Hormonal Activities 109
III. Screening for Yield Enhancement 125
IV. Screening Phytotoxic Compounds 133
V. Drift, Instability, Habituation, Phenotypic Adaptations, and Other Problems 169
VI. Concluding Remarks 171

VII. Appendix: Nomenclature of Growth Regulators 171
References 174

Microsporida in Cell Culture

STEFAN T. JARONSKI

I. Introduction 183
II. A Description of the Microsporida 184
III. Establishing Microsporida in Cell Cultures 191
IV. Observations of Microsporida in Cell Cultures 205
V. Other Techniques 213
VI. Uses of Microsporida in Cell Cultures 216
VII. Conclusion 225
References 226

Growth Factors and Hemopoietic Cell Differentiation

ANTONY W. BURGESS

I. Blood Cell Diversity 231
II. Colony Stimulating Factors: Nomenclature 233
III. Stem Cell Heterogeneity 234
IV. Production of Myeloid Cells M-CFC, G-CFC, and GM-CFC 237
V. Erythroid Cells 239
VI. Megakaryocyte and Eosinophil Production 240
VII. Production of Lymphocytes 241
VIII. Leukemic Cells 242
IX. Growth Factors 244
X. Growth Factors for Multipotential Stem Cells 246
XI. GM-CSF, M-CSF, and G-CSF 249
XII. Erythropoietic Stimuli 252
XIII. MEG-CSF and EO-CSF 253
References 255

Mosquito Cell Culture

TIMOTHY J. KURTTI AND ULRIKE G. MUNDERLOH

I. Introduction 259
II. Cell Lines 263
III. Growth Requirements 264
IV. Characteristics 276
V. Clonal Analyses 294
VI. Conclusions 296
References 298

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The Coming of Age of WI-38

LEONARD HAYFLICK

I. The Birth of WI-38 303
II. Distribution of WI-38 306
III. Who Owns WI-38? 307
IV. The Mortality of Cultured Normal Cells 310
V. The Great Virus Vaccine Substrate Debate 311
VI. Advances in Cytogerontology 313
References 315

INDEX 317

PROTOPLASTS AND PLANT VIRUSES

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I. Virus Infection of Plant Protoplasts 1
A. Introduction 1
B. Infection Requirements 5
C. Infection Mechanisms: Proposed Models 11
II. Virus Replication in Plant Protoplasts 16
A. Introduction 16
B. Infection Time Course 17
C. Synthesis of Virus-Related Proteins 18
D. Synthesis of Virus-Related RNAs 22
E. Cytopathology 23
III. Resistance to Virus Infection 26
A. Genetic Resistance 26
B. Cross-Protection 36
IV. *In Vitro* Methods for Recovering Virus Resistance 39
V. Perspectives and Problems for Genetic Engineering 47
References 48

I. Virus Infection of Plant Protoplasts

A. Introduction

The use of protoplasts in the study of plant viruses has attracted considerable attention since its inception in the late 1960s. Several reviews on this subject have been published (Zaitlin and Beachy, 1974;

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Takebe, 1975, 1977, 1983; Rottier, 1978) and have been further discussed by Wood *et al.* (1980). This article is an attempt to assess the current status of protoplasts (primarily) and of cell cultures (in some instances) in studies of virus infection, virus replication, cytopathology, cross-protection, virus resistance, and the use of *in vitro* methods and genetic engineering to recover virus-resistant plants. These areas of study proved difficult to do entirely with whole plants or plant parts. However, because protoplasts could be synchronously infected with virus, they provided a valuable alternative means of following biochemical and cytological events in relation to the virus growth cycle in a more precise manner than previously possible. Table I lists protoplasts inoculated by viruses.[1]

Before proceeding with the infection requirements, it would be desirable to consider preinfection sources of variation and how they may be minimized. Viable protoplasts—the first prerequisite for successful infection—are obtained by methods which have always been an "art." According to Takebe (1977), "Since these conditions for obtaining stable protpolasts include factors such as temperature, humidity, day length, soil and method of watering, which usually differ according to where the plants are grown, it is difficult to standardize them, and each laboratory should ascertain its best conditions for plant growth." Thus, from the outset, possibilities for wide variations are introduced between experiments in any particular laboratory as well as between experiments in different laboratories.

In the case of protoplasts derived from suspension culture, for which environment and genetic source can be controlled, an even more subtle level of experimental variation has been demonstrated. As with animal cell culture, plant virus binding has been linked to a stage in the cell replication cycle, as seen with TMV and tobacco protoplasts obtained from cell culture (Gould *et al.*, 1981). G_1- and G_2-phase protoplasts bound more virus than those from other phases. Although

[1]Abbreviations for virus and viroid names: AMV, alfalfa mosaic virus; BBWV, broad bean wilt virus: BGMV, bean golden mosaic virus; BMV, brome mosaic virus: BPMV, bean pod mottle virus; BSMV, barley stripe mosaic virus, BYDV, barley yellow dwarf virus; CaMV, cauliflower moasic virus; CCMV, cowpea chlorotic mottle virus; CEV, citrus exocortis viroid; CGMMV, cucumber green mottle mosaic virus; CMV, cucumber mosaic virus, CPFV, cucumber pale fruit viroid, CPMV, cowpea mosaic virus, CYMV, clover yellow moasic virus, PEMV, pea enation mosaic virus; PLRV, potato leaf roll virus; PSTV, potato spindle tuber viroid, PVX, potato virus X: PVY, potato virus Y; RRV, raspberry ringspot virus; SBMV southern bean mosaic virus: TEV, tobacco etch virus; TMV, tobacco mosaic virus; TNV, tobacco necrosis virus; TNcDV, tobacco necrotic dwarf virus: TRV, tobacco rattle virus; TuRoV, turnip rosette virus; TYMV, turnip yellow mosaic virus.

TABLE I

PROTOPLASTS INOCULATED WITH PLANT VIRUSES

Protoplast source	Virus	PLO requirement[a]	Reference
Tobacco mesophyll	AMV	E	Motoyoshi *et al.* (1975)
	BMV-V5	S	Motoyoshi *et al.* (1974a)
	CCMV	E	Motoyoshi *et al.* (1973a)
	CMV	E	Otsuki and Takebe (1973)
	CPMV	E	Huber *et al.* (1977)
	PEMV	S	Motoyoshi and Hull (1974)
	PVX	E	Otsuki *et al.* (1974)
	RRV	E	Barker and Harrison (1977a)
	TMV	E (NE)	Otsuki *et al.* (1972a); Zhuravlev *et al.* (1980)
	CGMMV	E	Sugimura and Ushiyama (1975)
	TRV	E	Kubo *et al.* (1975b)
	TNcDV	S	Kubo and Takanami (1979)
	PLRV	U	Takanami and Kubo (1979)
	TRV	U	Fritsch *et al.* (1978)
Tobacco suspension cells	TMV	E	Kikkawa *et al.* (1982)
Cowpea mesophyll	AMV	E	Alblas and Bol (1977); Nassuth *et al.* (1981)
	CMV	S	Hibi *et al.* (1975)
	CPMV	S	Beier *et al.* (1981)
	CYMV	S	Rao and Hiruki (1978)
	TMV	S	Koike *et al.* (1976)
Corn mesophyll	BMV	NS	Furusawa and Okuno (1978)
Wheat mesophyll	BMV	S	Furusawa and Okuno (1978)
Oat mesophyll	BMV	S	Furusawa and Okuno (1978)
	BYDV	U	Barnett *et al.* (1981)
Barley mesophyll	BMV	S	Okuno *et al.* (1977)
	BSMV	U	Chiu and Tien (1982)
Japanese radish mesophyll	BMV	S	Furusawa and Okuno (1978)

(continued)

TABLE I (*Continued*)

Protoplast source	Virus	PLO requirement[a]	Reference
Tomato mesophyll	CPFV (viroid)	U	Muhlbach and Sanger (1977)
	PSTV (viroid)	U	Muhlbach *et al.* (1977)
	CEV (viroid)	U	Muhlbach *et al.* (1977)
	TMV	E	Motoyoshi and Oshima (1975)
Potato mesophyll	PLRV	—	Barker and Harrison (1981)
Broad bean mesophyll	BBWV	E	Kagi *et al.* (1975)
Petunia mesophyll	TMV	E	Takebe (1977)
Cucumber mesophyll	CMV	U	Maule *et al.* (1980a,b)
Chinese cabbage mesophyll	TYMV	E	Renaudin *et al.* (1975)
	TMV	E	Takebe (1977)
Chenopodium mesophyll	BMV	U	Okuno and Furusawa (1979)
Bean mesophyll	BGMV	U	Bajet and Goodman (1981)
	BPMV	S	Lesney and Murakishi (1979a)
	CPMV	E	Lesney and Murakishi (1979a)
	SBMV	E	Lesney and Murakishi (1979a)
Turnip mesophyll	CaMV	U;E	Howell and Hull (1978); Yamaoka *et al.* (1982)
	TuRoV	E	Morris-Krsinich *et al.* (1979)
	BMV	U	Maekawa *et al.* (1981)
Vinca suspension cells	CMV	U	Takebe (1975)
	TMV	U	Takebe (1975)
Soybean suspension cells	CPMV	E	Jarvis and Murakishi (1980)
	SBMV	E	Jarvis and Murakishi (1980)
	BPMV	S	Lesney and Murakishi (1981a,b)
	CCMV	E	Lesney and Murakishi (1980)
Petunia suspension cells	Ti-plasmid	U	Davey *et al.* (1980)

[a] E, essential; NE, nonessential; S, stimulatory; NS, nonstimulatory; U, used but essentially not known.

binding was not studied further (to include infection), it seems reasonable to assume that this could prove critical to the kind of infection obtained, because attachment of virus to protoplast is a first step in infection.

Another source of variability is the specific infectivity of the virus. Storage of some viruses can lead to decreased infectivity with time (Matthews, 1981). Methods of purification can change infectivity, including the presence or absence of neutral salts (Matthews, 1981). In comoviruses CPMV and BPMV, the state of infection at which the source plants are harvested for virus purification can have a strong effect on infectivity, apparently because of host-mediated modification of virus coat proteins (Niblett and Semancik, 1970).

B. Infection Requirements

1. Physical

These factors include virus and protoplast concentration, osmoticum, temperature, and the duration or time of virus–protoplast interaction.

One of the characteristics of different virus–protoplast interactions is the virus concentration curve, generally a classic rectangular hyperbola of infection versus virus concentration. The similarity of the virus concentration curve to the Michaelis–Menten curve seen in enzyme kinetics is striking and has been noted previously (Lesney, 1980). Table II depicts virus–protoplast interactions for which such "simple" curves have been demonstrated.

TABLE II

VIRUS–PROTOPLAST SYSTEMS WITH "SIMPLE" INFECTION CURVES

Protoplast source	Virus	Reference
Tobacco mesophyll	BMV-V5	Motoyoshi *et al.* (1974b)
	CPMV	Huber *et al.* (1977)
	PEMV (without PLO)	Motoyoshi and Hull (1974)
	PVX	Otsuki *et al.* (1974)
	TNcDV	Kubo and Takanami (1979)
Cowpea mesophyll	CPMV	Hibi *et al.* (1975)
Wheat mesophyll	BMV	Furusawa and Okuno (1978)
Tomato mesophyll	TMV	Motoyoshi and Oshima (1975)
Soybean suspension cells	CPMV	Jarvis and Murakishi (1980)
	BPMV (minus $CaCl_2$ and PLO)	Lesney and Murakishi (1981a)

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Had such studies been done with mathematical analyses similar to those used in kinetic methodology comparisons within and between systems could have been made on a more precise level on the basis of their responses to virus concentration, pH, and amendments, as well as to temperature and other physicochemical phenomenona. Such a kinetic method has been instrumental in determining mechanism in enzyme kinetic systems (Wong, 1975).

Several problems prevent this kind of analysis of published data. Often virus concentration curves are omitted entirely or are presented without statistical analysis, or too few points are presented for mathematical analysis. In addition, systems studied have either insufficient cofactors or undefined inhibitors present, a situation preventing use of the "simple" kinetics. Some of the cofactor interactions which can lead to "aberrant" curves are discussed in the following section on biochemical parameters. Table III shows virus systems where complex interactions such as these seem to be indicated.

Another physical requirement is the proper osmoticum. Osmotica used have generally been mannitol and sorbitol. It was reported that using osmotic shock (i.e., increasing osmotic concentration sharply during inoculation) has significantly increased infection in monocotyledonous protoplasts with BMV (Okuno and Furusawa, 1978a).

Temperature effects during inoculation have also proved complex and mystifying. Cold temperatures have been reported to stimulate (Alblas and Bol, 1977), inhibit (Jarvis and Murakishi, 1980), or have no effect (Lesney and Murakishi, 1981b) on infection of protoplasts, the result depending on the host–virus combination.

The requisite time of virus–protoplast interaction for infection to take place was within 10 to 15 minutes of initial mixing (Takebe, 1977). Time has also been shown to be important in the preincubation

TABLE III

VIRUS–PROTOPLAST SYSTEMS WITH "COMPLEX" INFECTION CURVES

Protoplast source	Virus	Reference
Tobacco mesophyll	AMV	Motoyoshi *et al.* (1975)
	PEMV (with PLO)	Motoyoshi and Hull (1974)
	RRV	Barker and Harrison (1977a)
Cowpea mesophyll	AMV	Alblas and Bol (1977)
	CYMV	Rao and Hiruki (1978)
Turnip mesophyll	TuRoV	Morris-Krsinich *et al.* (1979)
Soybean suspension cells	BPMV (with $CaCl_2$ and PLO)	Lesney and Murakishi (1981b)

of virus with stimulatory amendments such as poly (L-ornithine) (PLO), and the sequence or the order in which amendments are added (Takebe, 1977; Lesney, 1980). These will be discussed later.

2. Biochemical

As mentioned earlier, there are many possible reasons for the lack of a simple kinetic relationship between virus concentration and protoplast infection. Researchers have shown that some of the aberrations were due to insufficient or excessive quantities of the various biochemical cofactors used. Foremost among these factors studied were buffers, polyions, and neutral salts. Table I lists the various virus–protoplast systems where these factors have been examined and gives some hint as to the complexity of their effects.

Buffers have been used for virus purification, for particle stabilization, and for protection against degradative enzymes associated with the host (Matthews, 1981). Specific buffers are stimulatory to virus infection (e.g., the "phosphate effect") (Yarwood, 1952). It was not surprising that phosphate buffer had similar effects on protoplasts (Kubo *et al.*, 1976). Stabilization and protection of the virus particle are well-known properties of buffers. However, of potential importance to the mechanisms of infection are those buffers which can stimulate or inhibit infection, independent of pH effects. For example, two different buffers at the same pH may show striking differences in effect, or buffers may show pronounced pH effects over ranges not likely to affect virus stability.

Such phenomena have indeed been documented in several protopolast–virus combinations. Some systems show a marked preference for citrate over phosphate buffer at low pH (Okuno and Furusawa, 1978a) and vice versa at higher pH (Kubo *et al.*, 1976). This might be expected to be the result of differences in the optimal buffering regions of the buffers. But, such a simple relationship did not hold when PLO interactions were added to the equation. For both TRV and RRV in tobacco, Tris-chloride, pH 8.0, was equally as effective as potassium phosphate, pH 6.0; but at suboptimal PLO levels, Tris was much more effective than phosphate for TRV infection.

Some systems show sharp pH dependence over regions where the virus itself is completely stable *in vitro* and throughout storage and purification, and in regions not physiologically detrimental to the protoplasts themselves (Motoyoshi *et al.*, 1973a; Lesney, 1980). In many cases, as with CPMV and cowpea, using citrate/pH 5.2 (Hibi *et al.*, 1975) versus CPMV and soybeans, using phosphate/pH 6.3 (Jarvis and Murakishi, 1980), it was the combined virus–protoplast pair rather

than either partner separately which determined the best kind of buffer and best pH. Thus, an interaction was the source of the buffer effect and not simply a stabilization phenomenon. Buffer concentration optima for the same buffers existed for different viruses but, in general, concentrations higher than the optimum were inhibitory. An example of the possible complexities is that found in the soybean protoplast system with two taxonomically related viruses, CPMV and BPMV. In this example, two different pH values were required for the same protoplast system, using the same buffer (phosphate). Each virus showed a strong, bell-shaped peak: CPMV at pH 6.3 and BPMV at pH 5.6, with moderate overlap. Yet the former required PLO for infection and the latter did not. High levels of infection were achieved with BPMV in the absence of both buffer and PLO entirely, although both were stimulatory. An interesting facet of the buffer effect in the BPMV–soybean system is that the stimulatory effect of the buffer on infection occurred only when the buffer was preincubated with the virus prior to infection and not when it was added with the protoplasts (Lesney and Murakishi, 1981b). This result would seem to indicate that the buffer was acting on the virus particle itself. Since this occurred in the absence of PLO, it differs from those cases where such preincubation was necessary to form aggregates of a PLO–buffer–virus complex (Mayo and Roberts, 1978).

In many instances, polycations were required for the infection of plant protoplasts. Basically, viruses can be divided into two groups: those that require PLO for infection and those that are merely stimulated by it. But these two groups are not determined by the viruses alone. For example, PLO was necessary for CPMV infection of tobacco (Huber *et al.*, 1977) or soybean (Jarvis and Murakishi, 1980) protoplasts, but not for CPMV infection of cowpea protoplasts (Hibi *et al.*, 1975). In contrast, BPMV did *not* require PLO for the infection of several monocotyledonous hosts and radish (Furusawa and Okuno, 1978). Similarly, BPMV had no PLO requirement in bean and cowpea mesophyll protoplast systems (Lesney, 1980). But the host cells were not the primary arbiters. In many cases, for the same host cell, there were certain viruses that required PLO for infection, whereas others did not.

An even more puzzling phenomenon has been observed: Viruses which required PLO for the infection of a particular kind of protoplast nearly always required preincubation with the polycation to be effective (Takebe, 1977). In contrast, those viruses which did not require PLO but were merely stimulated by its presence were actually inhib-

ited when preincubation occurred (Okuno and Furusawa, 1978a). Thus, whatever effect occurred in combination with PLO in this virus–protoplast interaction, it was a very ordered effect resembling enzyme kinetic studies where cofactors are involved in the reaction. The "reaction" in this case is the passage, by whatever means, of infectious virus from outside to inside the host cell.

The most frequently ascribed mechanism of PLO–virus interaction is that of charge balancing. Viruses which did not require PLO for infection of protoplasts have higher isoelectric points than those viruses which did require a polycation. In the soybean protoplast system, for example, CPMV, which is PLO dependent, has an isoelectric point between 3.7 and 4.5 (Van Kammen and de Jager, 1978), similar to that of viruses which required PLO. BPMV, which is PLO independent, has a higher isoelectric point—between 4.8 and 5.3 (Semancik, 1972), similar to that of those viruses which did not require PLO. Such data have been used to suggest that the polycation acts as a charge balancer, making the negatively charged viruses sufficiently positive so that they can approach the negatively charged membranes of the protoplasts (Takebe, 1977).

Together with charge balancing has been included the concept of virus aggregation, briefly referred to earlier. In this case, PLO acts to bind together viruses in aggregates of a few to hundreds of particles, and there is an optimum aggregate size and charge for infection (Mayo and Roberts, 1978).

In the next step, the effect of PLO is more controversial and involves the crux of the infection mechanisms. Evidence has been presented that PLO stimulates complex lesions in the plasmalemma, a process which leads to virus entry (Burgess *et al.*, 1973a); whereas it has also been demonstrated that PLO stimulates apparent endocytosis in plant protoplasts, as it is seen to do in animal cells (Suzuki *et al.*, 1977).

A more recently studied phenomenon is the effect of adding various neutral salts to the infection medium. These salts stimulate or inhibit infection, the result depending on which particular virus or protoplasts are involved. Further adding to the complexity of the entire infection process is the fact that the same interaction linkages seen with PLO or buffers do not occur in the presence of neutral salts. For example, $CaCl_2$ has been shown to be about equally stimulatory to CPMV, SBMV (Jarvis and Murakishi, 1980), BPMV (Lesney and Murakishi, 1981b), and CCMV (Lesney and Murakishi, 1980) in the soybean protoplast system, even though a broad range of differences exists in PLO requirements, temperature effects, buffer, and pH optima for these

viruses. $CaCl_2$ stimulation was also observed for CPMV and BPMV in bean mesophyll protoplasts (Lesney, 1980).

$MgCl_2$ has also been shown to be stimulatory but to a lesser degree than the calcium salt (Jarvis and Murakishi, 1980; Lesney and Murakishi, 1981b). Both divalent cations were seen to be strongly inhibitory to BMV infection of wheat, barely, maize, and Japanese radish protoplasts (Furusawa and Okuno, 1978), although this was at salt concentrations 10-fold higher than those seen as stimulatory in the soybean and bean systems.

In investigating the mode of action of these neutral salts, Jarvis and Murakishi (1980) reported that a stimulatory effect was seen only when the calcium salt was present during the inoculation period. It had no stimulatory effect as a preinoculation wash of protoplasts or as a postinoculation wash. Furthermore, use of the salt in a preinoculation wash was found to be inhibitory at the concentrations tested. Thus, the calcium effect was suggested to be part of an interaction during the infection process itself and not a stimulus solely to the protoplasts or to postinfection factors. The inhibition caused by the preinoculation wash with calcium salt might be associated with the observed phenomenon in other plant plasmalemma systems, in which $CaCl_2$ can cause membrane rigidity (Galun, 1981) which could then prove nonamenable to virus entry.

In studying the salt effect further, Lesney and Murakishi (1981b) found that both $MgCl_2$ and $CaCl_2$ had maximal stimulatory effects only when preincubated with the virus. This seemed to eliminate the possibility that the salts had a direct effect on the protoplast membrane. The probability of such salts acting as a virus stabilizer also seemed unlikely since neither CPMV nor BPMV required divalent cations for their stability or long-term storage (Bancroft, 1962; Semancik, 1972). However, such a stabilizing effect cannot be ruled out, especially in the light of the intimate association and requirement of divalent cations for stabilizing such viruses as TuRoV and SBMV (Hull, 1977; Hsu *et al.*, 1976).

Whatever the mechanisms, it appears evident that the selectivity of the divalent cation involved is readily demonstrated by the differentiation of results seen between $MgCl_2$ and $CaCl_2$. The latter was much more stimulatory in all cases tested, and $NiCl_2$ was shown to be wholly inhibitory to infection (Jarvis and Murakishi, 1980). There was no difference seen here between use of the sulfate or chloride salts, a finding indicating that the activity was most likely associated with the cation. Use of monovalent cation salts and EDTA proved somewhat inhibitory.

C. Infection Mechanisms: Proposed Models

Two main theories developed as to the mode of plant virus infection of protoplasts. These are the "pinocytosis" and the "wounding" theories of protoplast infection. Not only is this divergence of opinion directed at the phenomenon of virus infection of protoplasts per se, but it may also represent a fundamental question into the naturalness of the protoplast system as a whole for the study of such subjects as membrane composition, ion transport, cell wall regeneration, virus resistance, and other surface-mediated cell phenomena. The naturalness or artificiality of the infection process under these conditions becomes of interest, not only in plant virology, but in physiological studies as well.

The origin of these opinions may be traced to a historical background of the study of the mechanisms of plant virus infection, a study which progressed from whole plants to plant parts to tissue culture and finally to protoplast systems. The earliest studies concentrated on such aspects as the number, kind, and lifetime of infectible sites formed on intact leaves as the result of mechanical inoculation. The use of abrasives was seen to dramatically increase infection. Abrading the leaf with carborundum or Celite was thought to serve as an aid for breaking through the cell wall. As discussed by Matthews (1970), this abrasion produces wounds which penetrate through the intact leaf surface and expose the actual infectible sites. Evidence has been presented to implicate ectodesmata in permitting virus entry into the cells, a situation necessitating merely the breaking of the cuticle to allow access to the channels (Brants, 1966; Thomas and Fulton, 1968).

The "phosphate effect" discovered by Yarwood (1952) demonstrated the sensitivity to chemical additives of the infection process in whole plants. In this instance, the addition of dipotassium phosphate increased the infectivity of several viruses on bean leaves. For some viruses, 10 m*M* $MgCl_2$ greatly enhanced the phosphate effect (Kado, 1963). Phosphate increased the adsorption of TMV to cell debris *in vitro* (Taniguchi, 1966).

Spraying, washing, or dipping leaves in water within 2–4 hours after inoculation can substantially decrease lesion number (Yarwood, 1955). It was suggested that this effect was due to dilution of the ions necessary for attachment or penetration of the virus. Matthews and Proctor (1956) found that spraying $Mg(NO_3)_2$ solution and certain other metal salts onto leaves within a few hours after inoculation greatly increased infectivity. Air drying within 1 second increased more than 100-fold the number of local lesions seen on cowpea leaves

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