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BEHAVIOR AND EFFECTS OF APPLE MOSAIC VIRUS IN CULTURED PLANT TISSUE

A Thesis Presented

By

CHARLES D. HURWITZ

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of
MASTER OF SCIENCE

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Plant Pathology

BEHAVIOR AND EFFECTS OF APPLE MOSAIC VIRUS

IN CULTURED PLANT TISSUE

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CHAPTER I

INTRODUCTION

The study of viruses of fruit trees has lagged behind that of viruses of herbaceous plants primarily because of the presence in fruit trees of large amounts of secondary compounds which interfere with virus study. Tissue culture lends itself well to the study of several aspects of plant virology because it makes possible the exposure of host cells to completely defined environmental conditions, nutrients, and hormones. It has been shown that by manipulating these factors it is possible to affect both virus titer and host susceptibility, and also the production of secondary compounds which may affect virus multiplication and transmission.

Very little work has been done on the study of tree viruses in cultured plant tissue, and even less in tissue derived from fruit trees. Moreover, most general information on plant tissue culture, especially protoplast isolation, has been derived from work with herbaceous plants.

Most previous work involving viruses in cultured plant tissue used a bioassay of host plants to test for the

presence or absensce of virus. Most viruses of fruit trees however, are difficult or impossible to transmit mechanically. The serological technique known as the enzyme-linked immunosorbent assay (ELISA) has become widely used in the study of plant viruses over the past several years, but examples of its use to detect virus in cultured plant tissue have been rare. In addition, little work has been done on testing of callus for the presence of virus by grafting or tissue implantation.

The present study was undertaken with the following objectives in mind. First, to further define conditions for apple callus and suspension culture, and to work out a protocol leading to the isolation of protoplasts from cultured apple tissue. Second, to study the behavior of a known, fairly well defined fruit tree virus (apple mosaic virus) in cultured tissue of its tree host. Third, to compare callus cultures from different sources for their ability to support virus replication. Fourth, to monitor virus presence and determine its effects over time in both apple and cucumber callus. And fifth, to determine the potential use of ELISA for detecting virus in callus, cell suspensions and protoplasts, and to compare it to the bioassay method for detecting AMV in cultured apple and cucumber tissue.

It is expected that information obtained on the above

objectives will lead to the development of a model approach for studying other known fruit tree viruses, and for determining the cause of several virus-like fruit tree diseases of as yet unknown etiology.

CHAPTER II

LITERATURE REVIEW

Viruses of fruit trees are capable of causing large economic losses. Apple tree viruses such as apple mosaic and star crack have been reported to cause fruit yield losses up to 90% (Meijneke *et al.*, 1963). Besides affecting fruit yield, some viruses of fruit trees also kill buds and young twigs, cause dwarfing, and reduce fruit quality (Smith, 1972). Often, fruit tree viruses are latent and the losses they cause are not always evident.

The study of fruit tree viruses has lagged behind that of viruses of herbaceous crops primarily because woody plants contain large amounts of secondary compounds which make mechanical transmission and study of fruit tree viruses difficult, and in many cases impossible (Fulton, 1966).

Apple mosaic virus (AMV) is a member of the ILAR (isometric labile RNA) virus group. It is distributed worldwide (Fulton, 1972), and can infect several kinds of plants including birch (Gotlieb and Berbee, 1973), peach (Kirkpatrick, 1955), horsechestnut (Sweet and Barbara, 1979), plum (Posnette and Elenberger, 1957), cucumber and

rose (Fulton, 1972). AMV can be transmitted by grafting (Posnette and Cropley, 1956), and with difficulty, through sap (Fulton, 1972).

AMV occurs in several strains some of which can cause significant economic losses. In New Zealand, one strain of AMV reduced tree size of "Freyberg" apple by 42%, and yield by 26% (Chamberlin _et al._, 1971). In addition, AMV is thought to be at least partly responsible for causing birch decline in the northeastern United States and Wisconsin (Gotlieb and Berbee, 1973).

AMV has been purified from cucumber cotyledons (Fulton, 1967), and antiserum has been prepared against it (Fulton, 1968; Casper, 1973). The enzyme-linked immunosorbent assay (ELISA) has been used to assay for AMV in apple (Barbara, 1980), horsechestnut (Sweet and Barbara, 1979), and birch (Hardcastle and Gotlieb, 1980). Through ELISA, AMV could be detected in all parts of infected apple trees in May but not after July (Barbara, 1980.)

Plant tissue culture has become increasingly important in many areas of plant science, including plant pathology (Ingram and Hegelson, 1980). Most work in plant tissue culture has been done with herbaceous plants, but recently an increasing amount of work has been done on fruit trees

(Kochba and Spiegel-Roy, 1977; Anonymous, 1980.), and forest trees (Bonga and Durzan, 1982.)

Apple tissue culture has been used to study sorbitol metabolism (Chong and Taper, 1972, 1974), dwarfism (Messer and Lavee, 1969; Lane et al., 1982), fruit growth in tissue culture (Letham, 1958; Saad and Boone, 1964; Wallner, 1974), adventive embryony (Eicholtz et al., 1979), cytology (Mu and Liu, 1979), growth controlling properties (Schneider, 1978), morphogenesis (Mehra and Sachdeva, 1979), and several aspects of apple propagation through meristem tips and buds (Lane, 1978; Jones et al., 1979; Glimeroth and Richter, 1980; Snir and Erez, 1980; Werner and Boe, 1980). Although regeneration of whole trees from callus has been successful with some dicotyledenous woody species, e.g., almond (Mehra and Mehra, 1980), poplar (Berbee et al., 1972), aspen (Wolter, 1968), elm (Durzan, 1975), and citrus (Kochba and Spiegel-Roy, 1977), no consistent organogenesis has been reported from apple callus cultures (Mehra and Sachdeva, 1979). It has been suggested (Mu and Liu, 1979) that the low capacity of apple callus for regeneration may be due to the great variations in ploidy found in callus derived from the endosperm of apple seeds.

With the exception of citrus (Vardi et al., 1975, 1982) little work has been done on the isolation and

culture of protoplasts from callus derived from fruit tree tissue. Apple protoplasts have been isolated from apple fruit for use in studies on ethylene synthesis (Matto and Lieberman, 1977, Anderson et al., 1979) and from apple cotyledons to examine the subcellular localization of sorbitol-6-phosphate dehydrogenase (Yamaki, 1981).

The multiplication of viruses and viroids in callus derived from systemically infected plants, and the inoculation of callus and suspension cells with purified virus have been studied with several viruses including, potato viruses X and Y (PVX and PVY) (Svobodova, 1966; Wang, 1975, 1977), sugarcane mosaic virus (Dean, 1982) and southern bean mosaic virus (SBMV) (White et al., 1977; Wu and Murakishi, 1978, 1979; Wu et al., 1982), and the viroids causing potato spindle tuber (PSTV) (Zelcer et al , 1981), and citrus excocrtis (CEV) (Semancik, 1978; Marton et al , 1982). The majority of work, however, has been done with tobacco mosaic virus (TMV) (White, 1934; Kassanis et al., 1958; Wu et al., 1960; Beachy and Murakishi, 1971; Pelcher et al., 1972). With the exception of CEV (Semancik et al., 1978; Marton et al., 1982) no other work has been published on the behavior of viruses or viroids of fruit trees in cultured plant tissues, particularly in callus derived from woody plants.

In callus derived from systemically infected plants,

virus often appears to be lost after a few subcultures. Thus, in callus derived from leaf mid-ribs taken from systemically infected plants, tomato ringspot virus (TomRSV) was lost after twenty weeks (Reinert, 1966). Also, tobacco callus, that had been inoculated with TMV by rubbing its surface with virus, became virus-free after several subcultures (Kassanis et al., 1958).

Other studies, however, have shown that virus persists in callus cultures through many subcultures though virus titer may decrease. Thus, a callus culture initiated from a crown gall growing on the stem of a tobacco plant systemically infected with TMV (Morel, 1948) remained infective for at least nine years, with virus titer aproximately 1/30 that of sap from systemically infected plants (Kassanis, 1957). Reinert (1966) found that tobacco etch virus (TEV) concentration did not decrease after twenty weeks growth in callus, while the concentration of cucumber mosaic virus (CMV) and tobacco ringspot virus (TRSV) decreased.

At least two viroids have been shown to replicate successfully in both callus and suspension cultures derived from systemically infected plants. CEV reached high titers in callus cultures initiated from crown gall tumors (Semancik 1978), and in cell suspension and callus cultures derived from non-tumorous tissue from systemically infected

tomato plants (Marton *et al.*, 1982). Similarly, PSTV was found to replicate for at least 1.5 years in suspension cultures initiated from systemically infected plants (Zelcer *et al.*, 1981).

Regeneration of plants or organs has been achieved from several callus cultures derived from systemically infected plants. Callus from virus-infected geranium (Pillai and Hildebrandt, 1968), gladiolus (Simonsen and Hildebradt, 1971), potatoes (Wang, 1975, Wang and Huang, 1977) sugarcane (Dean, 1982), and poplar (Berbee and Hildebrandt, 1972; Berbee and Castello, 1976) have successfully regenerated symptomless leaves. But in several of these studies regenerated plants were judged virus-free only on the basis of lack of symptoms and were not assayed for virus either serologically or by bio-assay.

Svobodova (1966) regenerated stems from callus derived from tobacco plants systemically infected with PVY. When kinetin was omitted from the media, the regenerated stems were virus-infected. When the medium included kinetin, regeneration was faster and the regenerated stems were virus-free. When tobacco plants were regenerated from single cells with TMV inclusion bodies, the regenerated plants developed mosaic symptoms (Chandra and Hildebrandt, 1967). Similarly, when protoplasts from the mesophyll of potato plants systemically infected with PVX were

regenerated into intact plants, 92.5% of the regenerated plants were infected (Shepard, 1975). When the protoplasts were treated with Virazole (an antiviral compound) they regenerated virus-free plants (Shepard, 1977).

Virus infections have varying effects on cultured plant tissues. TEV reduced the rate of growth of infected callus, while TRSV, TomRSV, and CMV did not slow down callus growth (Reinert, 1966). Tobacco callus cultures developed reddish-brown local lesions when inoculated with TMV in a vortexer (Beachy and Murakishi, 1971). In addition, inclusion bodies have been reported in TMV infected callus cultures (Singh and Hildebrandt, 1967). Wu et al., (1982) reported that the cytopathology of soybean callus cells infected with SBMV differs from that of virus-infected intact soybean plants.

Virus infection also seems to have varying effects on the regenerative capacities of cultured cells. Thus, TMV had no effect on the regeneration of tobacco plants from infected callus cultures (Sacristan and Melchers, 1969). In contrast, virus-like particles were found in cultured Strepanthus cells that had lost the ability to regenerate (Sjolund and Shih, 1970), however, no cause-effect relationship was proven. On the other hand, PVY and PVX have been shown to reduce the organogenic ability of infected callus cultures (Wilson and Eisa, 1975). Single

infection with either PVY or PVX had no effect on regeneration of tobacco protoplasts into intact plants, however, double infection with both PVY and PVX markedly reduced protoplast regenerative capacities (Shepard, 1975).

Viroid infections also have varying effects on infected callus and suspension cells. CEV has no effect on the growth rate of viroid-infected suspension cultures at moderate temperatures. However, viroid-infected cells have a better capacity for growth at high temperatures than healthy cells (Marton, 1982). In contrast, PSTV markedly reduces the growth rate of viroid-infected tomato suspension cells (Zelcer et al., 1981).

Initial attempts at inoculating cultured plant tissue with purified virus proved generally unsuccessfull. Kassanis et al., (1958) trying to inoculate tobacco callus with TMV by superficial abrasion and by needlepricking, found that, while needlepricking was more effective, neither method resulted in high efficiency of infection.

Murakishi et al., (1970, 1971) improved the callus inoculation technique by vortexing 300 mg of friable tobacco callus in the presence of TMV and incubating the inoculated callus on agar. This procedure resulted in high efficiency of infection and, after seven days, the virus yields from inoculated callus cells were aproximately equal to yields obtained from leaves of systemically infected

plants. Similar results were obtained with SBMV and soybean callus (Wu and Murakishi, 1978), except that liquid- grown cells proved to be more susceptible to inoculation than callus grown on solid media, and virus multiplication was most effective in rapidly proliferating cells. When optimum inoculation and incubation conditions were used, soybean tissue culture proved to be a better host for SBMV than intact soybean plants. Suspension cultures of Trifolium have been inoculated successfully with purified clover yellow mosaic and clover yellow vein viruses (Jones et al., 1981). The reaction of the infected callus generally corresponded with the degree of susceptibility or resistance of the host plants.

Various physical and chemical factors such as pH, temperature, and various additives seem to affect viral replication in callus and suspension cultures. Hildebrandt et al., (1954) showed that tobacco callus infected with TMV produced the most local lesions when grown at 24 to 37 C at a pH of 8.3 to 8.4. Callus grown on media with pH values greater than 9.3 produced no local lesions. Virus multiplication in cultured plant tissue seems to be repressed by compounds such as cytokinins (Milo and Scrivastava, 1969), phosphates (Kassanis, 1957), 6-methylpurine (Kurtzman et al., 1960), Virazole (Shepard 1977), napthalene acetic acid (NAA) (Kutsky and Rawlins,

1950), and indole butyric acid (IBA) (Kutsky, 1952). On the other hand, 8-azaguanine and thiouracil (Wu et al., 1960) increased virus infectivity per weight of tissue by favoring virus (TMV) synthesis while, at the same time, inhibiting callus growth. N6-isopenthyladenosine favored both callus growth and virus replication (Milo and Scrivastava, 1969).

Virus in cultured plant cells has been assayed primarily by the local lesion method, but in some cases assaying involved virus purification (Murakishi et al., 1971), or tissue implantation (Dimock et al., 1971, Ben Jaakov et al., 1973). Monitoring of virus levels in cultured plant tissues has never been done through the use of ELISA.

Many fruit tree viruses are extremely unstable and are inactivated by phenolics and, perhaps, other secondary compounds (Mink, 1965; Fulton 1966; Mink and Saksena, 1971). It has been shown, however, that by manipulating conditions such as light (Forrest, 1969; Hahlbrock et al., 1976), temperature (Rutland, 1969), hormones (Ibrahim et al., 1971), and vitamins (Hagimoril, 1982), it is possible to affect the production of such secondary compounds in callus and suspension cultures.

Callus culture and *in vitro* regeneration of cucurbits has been achieved (Jelaska, 1972, 1974; Wehner and Locy, 1981). Wehner and Locy (1981), working with hypocotyl and cotyledon explants from 85 cultivars of cucumber (*Cucumis sativus* L.), found that none of the hypocotyl pieces from any of the cultivars formed shoots, while 11 of the 85 formed roots. On the other hand, when cotyledon explants were taken, 28 of the cultivars formed shoots and 27 formed roots.

Crown gall is a plant disease characterized by the formation of tumors. The disease is caused by the bacterium *Agrobacterium tumefaciens.* Upon infection, the bacterium injects a plasmid (the Ti plasmid) into plant cells which then multiply uncontrolled and produce a tumor (Van Larbeke *et al.*, 1974; Watson *et al.*, 1975). Tumor formation on different plants seems to be strain specific (Anderson and Moore, 1979).

Tumor induction and morphology are due, at least in part, to elevated levels of auxins and cytokinins (Amasino and Miller, 1982), but studies in this area have been conflicting (Weiler and Spanier, 1981; Pengelly and Meins, 1982). Crown gall cells are able to grow autonomously in vitro without the addition of phytohormones; they

apparently contain high levels of the cytokinin ribosyl-trans-zeatin and the auxin indole acetic acid (IAA) (Naikjam et al., 1979), and high levels of auxin protectors, which is also charateristic of meristematic tissue (Stonier, 1969).

Crown gall tissue can be grafted both in vitro (deRopp, 1948) and in vivo (Braun, 1953; Braun and Wood, 1976), and intact plants can be regenerated from crown gall tumors growing in vitro (Einset and Cheng 1979).

CHAPTER III

MATERIALS AND METHODS

The Virus

Apple mosaic virus (AMV) was obtained from apple trees growing in the University Fruit Research Orchard in Belchertown, Massachusetts. Scionwood of Golden Delicious was grafted onto seedling rootstocks growing in 15 cm pots. The grafted trees were inoculated with two AMV-infected buds obtained from McIntosh apple trees.

AMV-infected *Vinca rosea* leaves were kindly supplied by Dr. Robert W. Fulton of the University of Wisconsin. The virus was maintained by inoculating the cotyledons of 7-to-10-day-old Marketer (Burpee Seed Co.) cucumber seedlings with sap of AMV-infected *Vinca* leaves or cucumber cotyledons. The sap was obtained by crushing the tissue with a mortar and pestle in a 1:10 weight/volume ratio with inoculation buffer consisting of 0.03M phosphate buffer containing 0.02M 2-mercaptoethanol at pH 8.0 (Fulton, 1967). Cotyledons to be inoculated were dusted with 600-mesh carborundum, and the inoculum was applied by

gently rubbing the cotyledons with a gloved finger. The cotyledons were then rinsed with tap water.

Crown Gall Initiation

A strain of *Agrobacterium tumefaciens* (strain A49) known to induce tumors in apple trees was kindly supplied by Dr. Larry W. Moore of Oregon State University. The bacteria were maintained on nutrient agar (Difco) slants supplemented with 2% yeast extract. To prepare inoculum, 125 ml flasks containing 0.8% nutrient broth and 0.2% yeast extract were seeded with bacteria and placed in a water bath shaker at 28 C for 36 hours. Just prior to inoculation, cells were spun, washed, and resuspended in distilled water. Apple seedlings were inoculated by wounding stems in three places and infusing the wounds with bacterial suspension using a Pasteur pipette. The wounds were then wrapped with 2-3 layers of parafilm.

The Nutrient Media

For apple callus. After preliminary experiments, a modified Linsamier and Skoog's (1965) medium was used for apple callus initiation and growth. The medium consisted of Murashige and Skoog's salt mixture (Gibco) combined with

30 g/l sucrose, 8 g/l agar (Difco), 200 mg/l myo-inositol, 0.2 mg/l thiamine HCl, 2 mg/l NAA, 1 mg/l 2,4-D, and 0.2 mg/l kinetin. For liquid culture the same medium was used minus agar.

For in vitro culture of crown gall tissue, the same medium as above was used except that all phytohormones were omitted, and 80 mg/l penicillin G, 100 mg/l cephaloridin, 6.5 mg/l polymyxin B and 50 mg/l neomycin sulfate were added through a Millex (Millipore Corp.) filter after autoclaving to prevent the growth of *Agrobacterium tumefaciens* in culture. After two subcultures, all antibiotics were omitted from the medium.

Cucumber callus. For growth of cucumber callus in culture the medium of Wehner and Locy (1981) was used. This consisted of Murashige and Skoog salts (Gibco) with 30 g/l sucrose, 200 mg/l myo-inositol, 0.2 mg/l thiamine HCl, 8g/l agar, 1 mg/l NAA, and 1 mg/l 6-benzylamino- purine.

All solid media were adjusted to pH 5.7 with KOH and autoclaved for 15 minutes at 15 psi prior to use.

Initiation of Plant Tissue Cultures

Apple callus cultures were initiated from virus-free and AMV-infected leaves, buds, fruit, petals, and crown galls, and from virus-free stems and seeds.

For initiation from leaves 9 mm diameter leaf discs were obtained with a number four cork borer from healthy leaves and from mosaic areas of symptomatic expanding leaves collected in April and May. To initiate cultures from fruit, immature fruits were picked from healthy and AMV-infected apple trees in July. The fruit was washed, and a cylinder 2 cm long by 9mm in diameter was removed from the fruit with a number four cork borer. A section 2 mm long was aseptically removed from the middle of the cylinder and placed on the medium without surface sterilization. For culture initiation from petals, petals were picked from symptomatic and healthy trees in April and May. The petals were cut in half, surface sterilized, and placed on the medium. Culture initiation from twigs was accomplished by removing twigs from young branches, surface sterilizing for 5 minutes, peeling the bark, and then aseptically splitting the twig longitudinally and placing it cut-side down on the medium. Seed cultures were initiated from seeds taken from apples that had been in cold storage for at least six months. The seeds were removed from the apples, soaked in distilled water for two days, and then placed in a 10% solution of commercial bleach for ten minutes. The seed coats were then removed and the seeds placed on the medium.

Crown gall tissue cultures were initiated from

expanding galls removed 4-5 weeks after they first appeared. The galls were rinsed several times in distilled water, and the outside layers of the gall were trimmed off with a scalpel. The galls were then cut into pieces weighing 100-200 mg, surface sterilized in a 10% bleach solution for seven minutes, rinsed three times in distilled water, and placed on crown gall tissue nutrient medium.

Cucumber callus was initiated by removing 9 mm diameter leaf discs from healthy and AMV-inoculated cotyledons, surface sterilizing the leaf discs and placing them on cucumber tissue nutrient medium.

All tissue cultures were grown in the dark at 25 C and transfered to fresh medium monthly. All operations were carried out under sterile conditions in a laminar air flow hood.

Initiation of suspension cultures in liquid media. After 3-4 months in culture on solid media, most apple calluses began to exhibit light friable growth. This growth was removed with sterile scalpel and forceps and placed in the liquid medium. Suspension cultures were also initiated by placing two-week-old apple seedlings, which had been produced from apple seeds on water agar, directly into suspension medium. Cultures were maintained in 125 and 250 ml flasks on rotary shakers at 125 rpm at room temperature and under natural day-night light conditions. At

approximately two week-intervals, 1/4 of the volume of each flask was removed and placed in fresh medium.

Isolation of Protoplasts

For protoplast isolation from suspension cells, 7-day-old supension cultures derived from leaf discs were poured into conical centrifuge tubes and spun at 50g for 10 minutes. The supernatant was poured off, and the pellet was resuspended in 7 ml of a filter-sterilized solution containing 2% Cellulysin (Calbiochem) and 0.5% Macerase (Calbiochem) dissolved in 0.7 M mannitol. The mixture was placed in a Petri dish which was then sealed with Parafilm, and placed on a rotary shaker at 80 rpm at 25 C. For isolation of protoplasts from callus, 200-300 mg of friable callus was removed from actively growing callus cultures, cut up into small pieces with a scalpel and suspended in the same enzyme mixture as used for protoplast isolation from suspensions.

Following incubation of the suspension cells or callus pieces in the enzyme mixture for 4-5 hours, the cell walls of most cells were digested and they released protoplasts. The protoplasts were first filtered through Miracloth to remove debris and undigested cells, and were then washed three times in a 0.7 M mannitol solution containing 0.01M

CaCl. Protoplast samples were then stained with 0.025% Evans Blue (J.T. Baker Chemical Co.) to assess viability, and with 0.1% Calcofluor White (Polysciences) to confirm the absence of a cell wall. After counting of protoplasts in a few samples with a haemocytometer, the protoplasts were resuspended at a concentration of 10,000/ml in the apple suspension medium supplemented with 0.5 M mannitol, and 0.01% CaCl in a Petri dish, and cultured in the dark at 25 C.

Regeneration studies. Four-month-old callus, derived from healthy opening buds and from seeds, was placed on media containing MS salts (Gibco), 30 g/l sucrose, 7 g/l agar, 0.2 mg/l thiamine HCl, 200 mg/l myo-inositol, and the following phytohormone combinations:

Benzyladenine (mg/l)	Giberillic acid (mg/l)
0	0
1.0	0
1.5	0
2.0	0
1.0	.5
2.0	1

Each treatment was replicated three times and all treatments were kept under 17 hours light and 7 hours darkness at 25 C.

The Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed by the indirect method in accordance with the procedures of Clark and Adams (1977). AMV antiserum was obtained from the American Type Culture Collection.

Purification of AMV gamma globulin. The AMV gamma globulin was purified from the antiserum by precipitation with saturated ammonium sulfate, centrifugation, and overnight dialysis in 1/2 strength PBS. The gamma globulin (IgG) was further purified by passing the dialyzed material through a 50 cm long column of DE 22 cellulose (Whatman). The sample was loaded onto the column, and the column was washed with 1/2 strength PBS until all purified IgG protein was eluted. The peak fraction had an optical density of 1.02 at 280 nm. The four fractions with the highest optical density were combined and diluted to give an optical density of 0.5, which corresponds to a protein concentration of 0.35 mg/ml. After addition of sodium azide to prevent growth of microorganisms, the purfied IgG was stored at 4 C in a siliconized glass tube.

Preparation of samples for ELISA. Apple leaves were macerated in a mechanical leaf crusher and diluted in approximately 9 volumes of coating buffer. Callus and suspension cultures were macerated with a tissue homogenizer in 9 volumes of coating buffer. Protoplasts were placed in coating buffer in which they burst within

2-3 minutes.

Preliminary experiments showed that a 1:1,000 dilution of both AMV gamma globulin and goat antirabbit-IgG alkaline phosphate conjugate (Sigma) gave optimum results. Samples were added in 0.2 ml aliquots to polystyrene Gilford ELISA plates. At the end of the procedure, the optical density of the samples was determined at 405 nm using a Gilford EIA manual reader.

Bioassays

Mechanical inoculation. Twenty attempts were made to inoculate 7-to-10-day-old "Marketer" and "Wisconsin SMR-18" cucumber seedlings (*Cucumis sativus* L.) (Burpee Seed Co.), with AMV-infected apple and cucumber callus. Callus was ground with a mortar and pestle in a 1:10 weight:volume ratio with inoculation buffer (Fulton, 1967). Several attempts were also made to inoculate cucumber cotyledons using AMV-infected protoplasts as inoculum. In addition, mechanical inoculations were made by utilizing liquid nitrogen in place of inoculation buffer according to the methods of Ragetli *et al.* (1973).

Tissue implantation. The tissue implantation method of Ben Jaakov *et al.* (1973) was used in an attempt to screen apple callus cultures for AMV presence and infectivity.

AMV-free Golden Delicous grafts growing on apple seedlings were used as AMV indicators. Three holes, two in the scion and one in the rootstock, were made with a 15 gauge sternum needle. Plugs of callus derived from AMV-infected plants were then taken up with the cannula of the needle, and were subsequently inserted into the holes in the tree, and the holes were covered with Parafilm.

Grafting. Callus cultures were also screened by conventional grafting.Three T-shaped slits were made in the stems of AMV-free Golden Delicous grafts as above. The flaps of bark were pulled to open the slit and a small amount of callus was placed underneath. The bark was then allowed to take its normal position and the wound was covered with Parafilm. Five tumorous and five non-tumorous apple calluses were assayed by grafting in August 1982, and 27 calluses in March 1983.

In March 1983, calluses were assayed for AMV by tissue implantation and by grafting on two separate Golden Delicous grafts. After 6 weeks, all grafts were screened with ELISA.

CHAPTER IV

RESULTS

Initiation and Growth of Cultured Apple Tissue

All apple tissue sources (leaf, twig, bud, petal, fruit, seed, crown gall) produced callus on the modified MS medium (Fig. 1). Callus from all sources grew well on the same medium. For example, after eight months growth, both tumorous and non-tumorous calluses weighed an average of 12 grams, which was approximately 1,000 times the initial explant weight (Fig. 2).

Callus suspensions were readily produced from callus of friable consistency which had been sub-cultured 3-4 times on solid media. Suspensions consisted mainly of clumps of 20-30 cells with occasional single-cells. Cell shape varied from oval to elongate. No chloroplasts were observed in the cells (Fig. 3). Ten-day-old apple seedlings placed directly into liquid suspension medium yielded a true single cell suspension within four weeks (Fig. 4). The suspension cells were all elongated, measuring approximately 50 X 500-600 micrometers, and lacked chloroplasts.

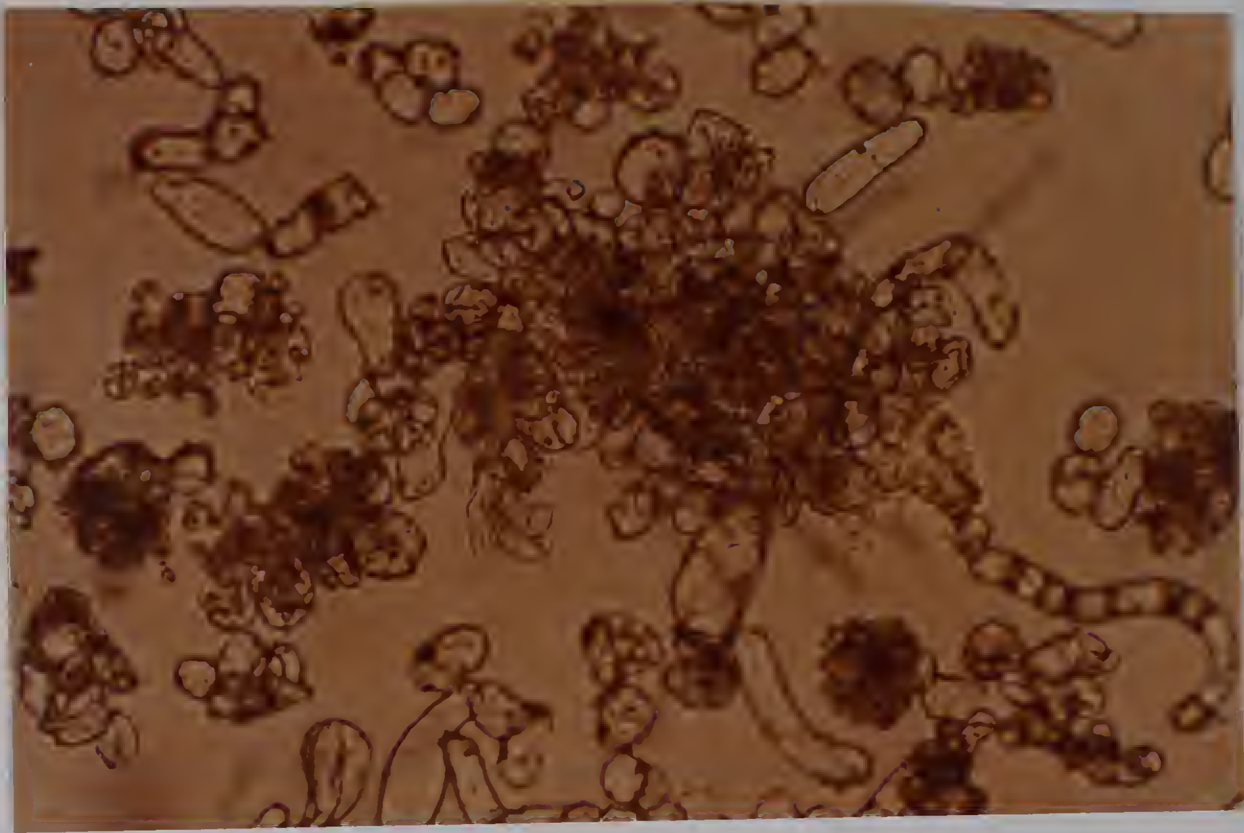
Figure 1. Callus growing from apple twig section kept on nutient medium for 4 weeks.

Figure 2. Eight-month-old crown gall callus derived from apple mosaic virus-infected tree.

1 2 3 4 5 6 7 8

Figure 3. Suspension cells derived from apple leaf callus.

Figure 4. Suspension cells derived from apple seedling kept in liquid nutrient medium.



When these cells were poured onto solid apple callus growth medium they produced callus colonies of 200-300 cells of which became visible to the naked eye after about four weeks. These colonies ceased growth 2-3 months after they had been poured onto the solid medium.

All attempts at inducing organogenesis in apple callus proved unsuccessful.

Isolation of Protoplasts From Cultured Apple Cells

Protoplasts were successfully, and repeatedly, isolated from apple callus tissue cultured on either solid media or in liquid suspension. Protoplasts could be isolated only from actively growing callus and suspension cultures that were less than six months old. Callus cultures that yielded protoplasts when they were four months old, failed to release protoplasts when they were 8 months old. When four-month-old leaf callus was used for protoplast isolation, about 75% of the callus cells yielded protoplasts. Approximately 100,000 protoplasts were produced per gram of callus tissue placed in the enzyme mixture. Callus suspension cultures were even more efficient producers of protoplasts; approximately 90% of the callus suspension cells placed in the enzyme mixture yielded protoplasts. Staining with Calcofluor White

confirmed that all cell wall material was removed from the protoplasts. Protoplast viability at isolation was 95-98%, as determined by Evans Blue staining. Protoplasts ranged in size from 30-60 micrometers, lacked chloroplasts, and had large vacuoles.

When protoplast isolation was attempted from the true single-cell suspension obtained from the cultured apple seedlings, no protoplasts were produced even when Cellulysin concentration was increased from 2% to 8%, and incubation periods of the cells in the enzyme solution were extended to 24 hours.

Protoplasts were successfully maintained contaminant-free in culture for up to two weeks. At two weeks, 75-80% of the protoplasts were living. Approximately 60% of the protoplasts began to show cell wall regeneration within 48 hours of isolation. By the sixth day, 5-10% of the regenerated cells began to divide, but they usually stopped dividing after they had produced 4-8 cells.

Monitoring of AMV in Apple and Cucumber Tissue via ELISA

AMV was detected via ELISA in apple leaf, leaf and crown gall callus, callus-derived suspension cultures, protoplasts, and cucumber callus (Table 1, Fig. 5).

Table 1

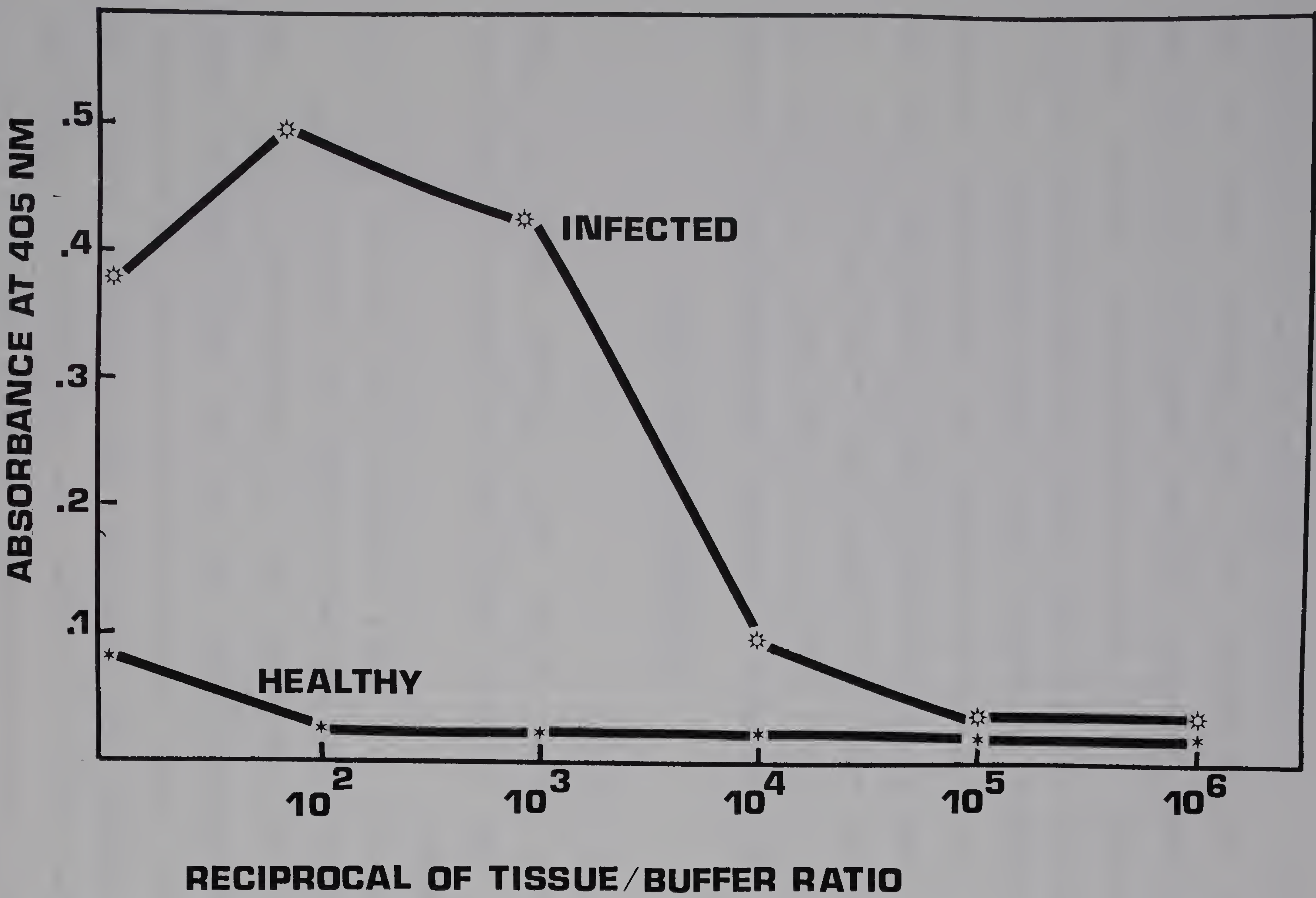
Typical ELISA readings (Absorbance at 405 nm)

of AMV-infected and non-infected

apple and cucumber tissue

	Non-infected	Infected
Apple leaf	0.09	0.380
Apple leaf callus	0.07	0.475
Apple cell suspension	0.05	0.410
Apple protoplasts	0.02	0.170
Cucumber cotyledons	0.02	0.646
Cucumber callus	0.08	0.375

Figure 5. ELISA readings of 6 dilutions of AMV-infected and non-infected apple callus.


ABSORBANCE AT 405 NM
.1
.2
.3
.4
.5
INFECTED
HEALTHY
10^2
10^3
10^4
10^5
10^6
RECIPROCAL OF TISSUE/BUFFER RATIO

ELISA readings being present in apple leaves but not in apple callus, To check the possibility of substances interfering with an infected apple callus was cut in two equal halves; in preparing them for ELISA, to one half was added coating buffer, and to the other half was added coating buffer and sap from healthy apple leaves. When these two samples were assayed for AMV by ELISA, no difference in ELISA readings was detected in the two samples.

AMV in apple leaves could be detected via ELISA until August, but not later, when emerging leaves no longer showed symptoms.

Bioassays of Apple Callus

All attempts to transmit AMV mechanically from apple callus, apple suspension cells, apple protoplasts and cucumber callus to cucumber plants were unsuccessful.

When 5 AMV-infected calluses of crown gall origin and 5 of leaf origin were grafted onto healthy apple seedlings in August, 3 months after callus initiation, all 5 seedlings that had been grafted with AMV-infected callus of crown gall origin began to display mosaic symptoms within 4-5 weeks. No mosaic symptoms appeared on any of the trees that had been grafted with AMV-infected callus of leaf origin. In addition, seedlings grafted with AMV-infected

crown gall callus began to produce visible crown gall tissue at the grafted areas 6-8 weeks after grafting. Trees grafted or implanted with AMV-infected callus in March were still symptomless six weeks later. When assayed for AMV by ELISA, all trees tested virus-free. No tumor or other growth was observed in the grafted or implanted areas, suggesting that the grafts were unsuccessful.

Effects of AMV Infection on Apple and Cucumber Tissue

No differences were seen in the color of AMV-infected and non-infected callus of either apple or cucumber. Similarly, there was no difference in the growth rates of AMV-infected and non-infected apple callus over time (Fig. 6). There was however, a significantly lower growth (at the P=0.05 level) of AMV-infected than of non-infected cucumber callus. The differential growth began when the callus was eight weeks old and continued thereafter (Fig. 7). Both AMV-infected and uninfected calluses developed roots with similar frequencies (Fig. 8).

Figure 6. Rates of growth of healthy and AMV-infected apple callus over time.

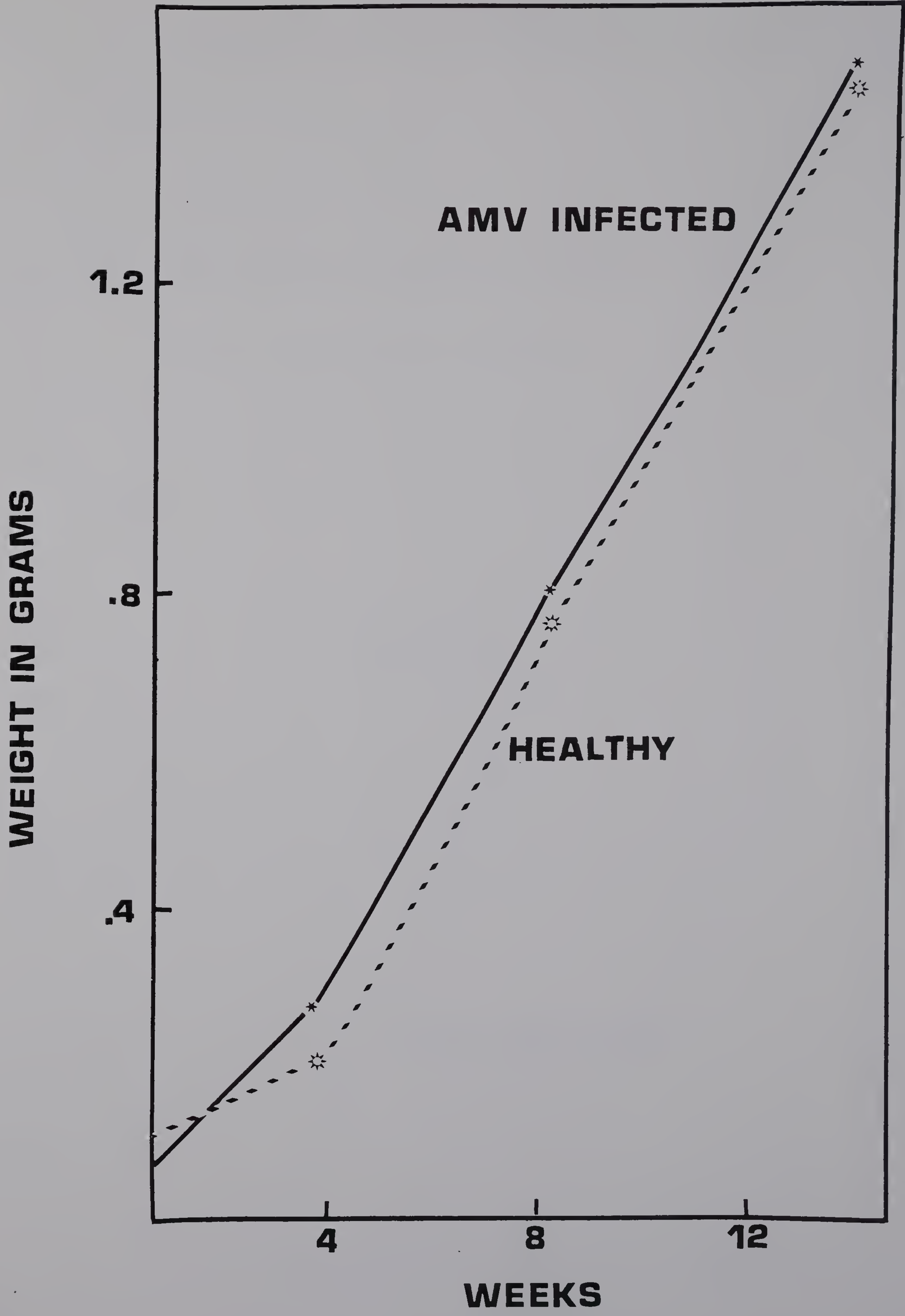
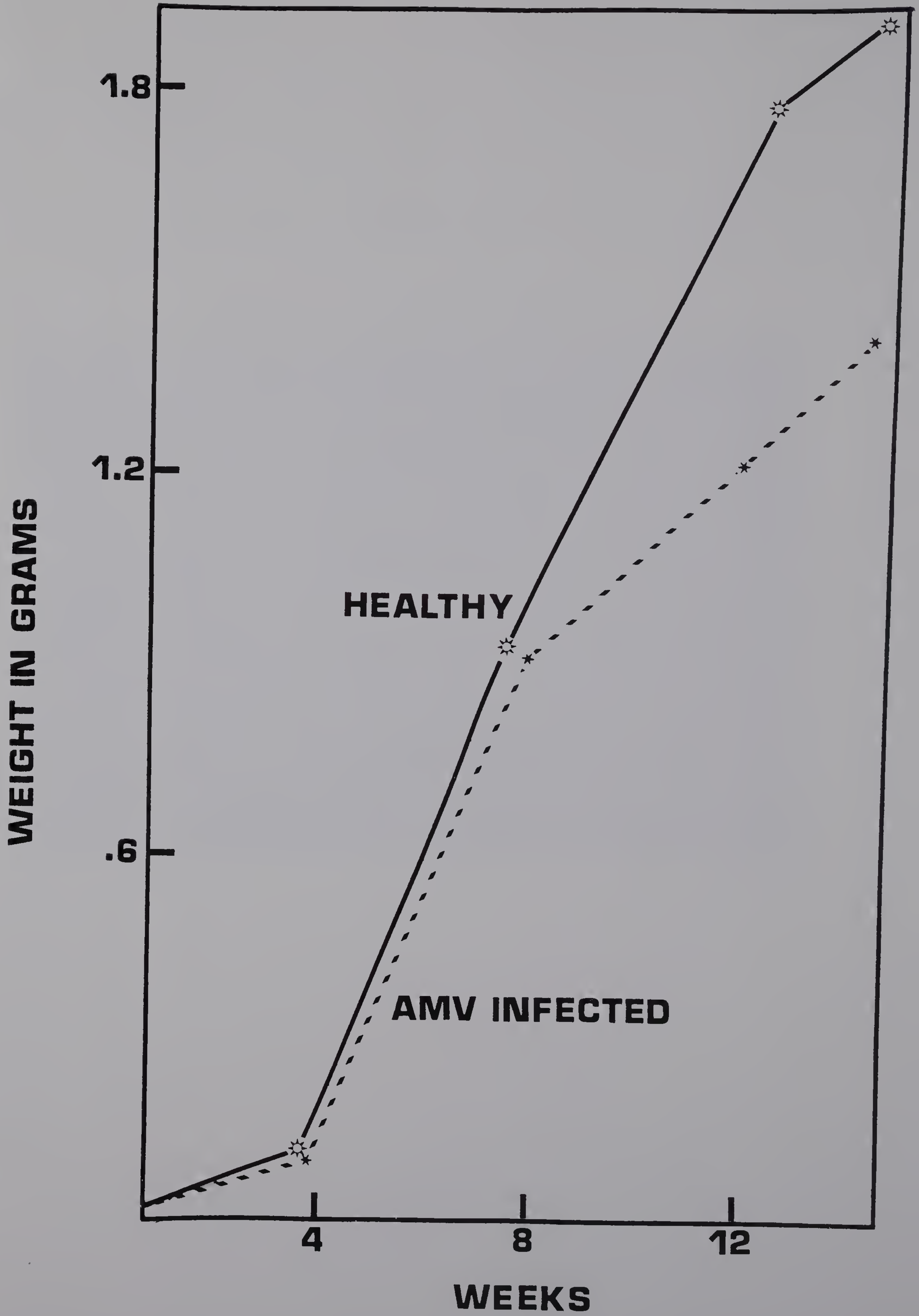
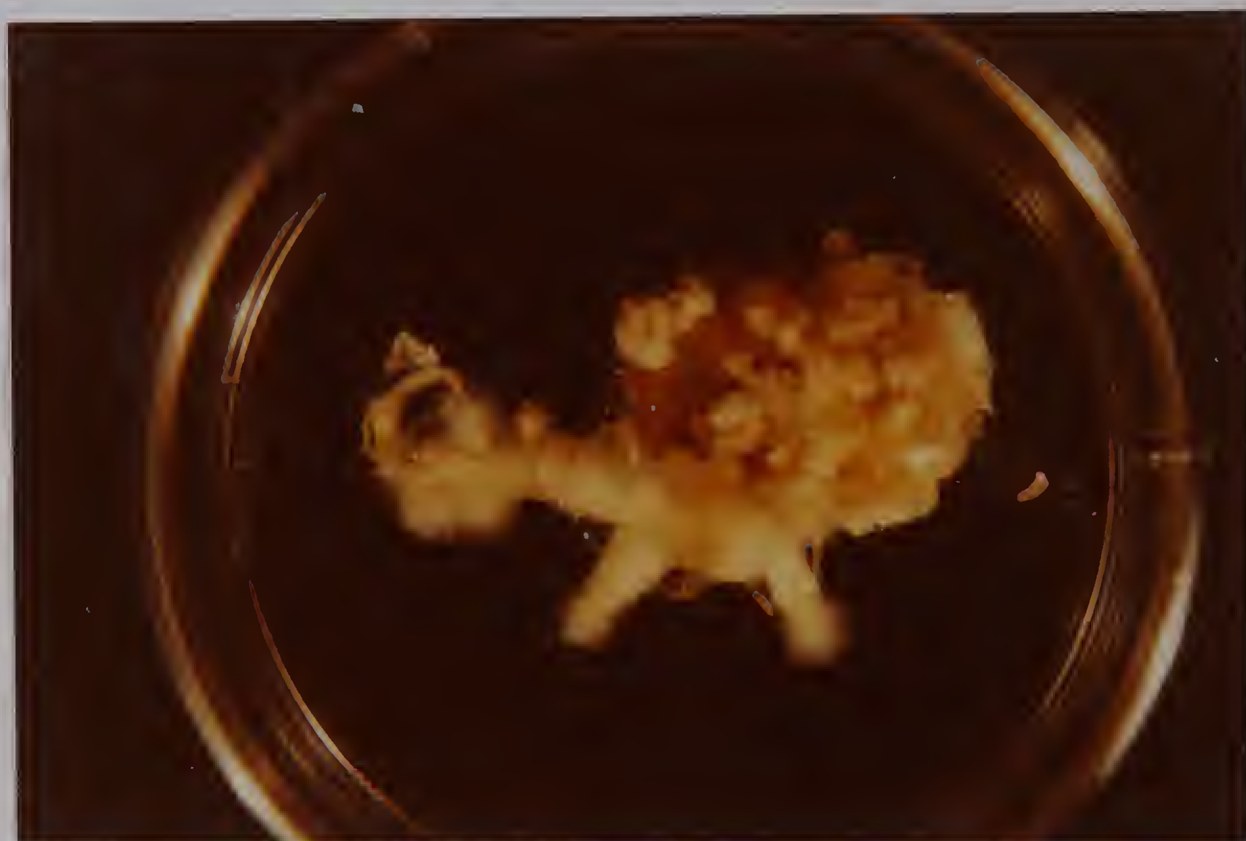

AMV INFECTED
HEALTHY
1.2
.8
.4
WEIGHT IN GRAMS
4
8
12
WEEKS

Figure 7. Rates of growth of healthy and AMV-infected cucumber callus over time.



1.8
1.2
.6
WEIGHT IN GRAMS
HEALTHY
AMV INFECTED
4
8
12
WEEKS

Figure 8. Roots forming from cucumber callus after 6 weeks in culture.



Persistence of AMV in Callus Tissue

Fifteen of 22, or 68% of the 3-month-old calluses tested by ELISA were shown to be AMV-infected (Table 2). When the calluses were 9 months old, only 10 of 27, or 37% of them were shown by ELISA to be AMV-infected (Table 2). The AMV-infected 9-month-old calluses included 3 of 8 crown gall calluses (37.5%), and 7 of 19 (36.8%) leaf disc-derived calluses (Table 3). Two of the leaf calluses and one of the crown gall calluses consistently gave high ELISA readings throughout the course of the study. When two calluses derived from fruit and two derived from petals of AMV-infected trees were assayed for AMV by ELISA when they were three months old and again when they were 9 months old, all of them were shown to be free of virus. When seven cucumber calluses were assayed after two months in culture, 5 of the 7, or 71.4% were shown to be AMV-infected.

Table 2

Detection of AMV by ELISA on 3 and 9-month-old apple callus tissue

Callus age	Number of Calluses Tested	Number of Calluses Infected	% Infected
3 Months	22	15	68
9 Months	27	10	37

Table 3

Detection of AMV by ELISA on 9-month-old callus tissue originating from apple leaves or crown galls

Callus Origin	Number of Calluses Tested	Number of Calluses Infected	% Infected
Crown Gall	8	3	37.5
Leaf	19	7	36.8
Total	27	10	37

CHAPTER V

DISCUSSION

Initiation of apple tissue cultures has already been reported from a variety of explant sources. However, each study has used different growth media and different growth conditions. The main study dealing with apple callus culture (Mehra and Sachdeva, 1979) concluded that consistent callus growth could only be achieved by initiating cultures on White's medium followed by transfer of the cultures to MS medium. The present study shows that consistent apple callus growth can be achieved by culturing a wide variety of apple tissues directly on the modified MS growth medium (Fig. 1,2).

Organogenesis from callus obtained from various explant sources was unsuccessful regardless of the hormone regimes used. This was, perhaps, caused by the extensive changes in ploidy levels found in cultured apple cells, which have been speculated to adversely affect organogenesis (Mu and Liu, 1979), or by lack of exact balance of hormones and of the proper carbohydrate and vitamin sources (Berbee _et al._, 1972; Durzan and Lopushanski, 1975). No doubt, when the proper combination

is found, consistent organogenesis from apple callus cultures will be possible.

Suspension cultures of apple callus consisted of aggregates of 10-30 cells with occasional single cells (Fig. 3). Various techniques that have been utilized to achieve true single-cell suspensions have met with varying success (Kubek and Shuler, 1978). By placing two-week-old apple seedlings directly into liquid suspension medium, true single-cell suspension cultures were achieved (Fig. 4). These cells were living and functioning as shown by their ability to produce discrete colonies when placed on solid medium. The cell walls of these cells, however, were not digested by enzyme concentrations that were successfull in releasing protoplasts from callus-derived suspension cultures, suggesting that their cell wall constituents differ from those of callus-derived suspension cells.

Protoplast isolation from cultured cells offers several advantages over isolation from intact plants. For example, conditions for protoplast isolation can be more easily standardized from cultured cells; cultured cells are harmed less by the isolation process (Fowke and Gamborg, 1980), and cultured cell-derived protoplasts regenerate cell walls with greater frequency (Vasil and Vasil, 1980). An additional advantage to the use of callus derived from

leaf cells rather than from cotyledons of self-sterile trees such as apple is the fact that cotyledons yield protoplasts whose genome is different from that of the cultivar.

Although the procedure described here yields large numbers of fruit tree protoplasts that can regenerate cell walls, divide to produce small clumps of cells, and remain viable for up to two weeks, it should be noted that protoplasts could be obtained only from relatively young, actively growing callus cultures. Callus cultures that were no longer actively growing, and single-cell suspension cells derived from the liquid cultures of seedlings did not produce protoplasts regardless of the enzyme concentrations and incubation times used. Nevertheless, the ability to produce almost unlimited numbers of protoplasts at any time of the year from apple callus may prove extremely useful not only for physiological studies (Galun, 1981), but also for studying the replication and transmission of viruses of fruit trees and other woody plants.

This study shows that ELISA can be used effectively for detecting AMV in cultured plant tissue. Application of ELISA to apple and cucumber callus, and to apple suspension and protoplast cultures could distinguish AMV infected from non-infected tissues (Table 1). ELISA readings remained unchanged or increased while tissue was diluted up to a

certain point (Fig. 5), then decreased with tissue dilution, probably because of the presence in the apple tissue and subsequent dilution of substances which interfere with the serological reaction. Similar interference with ELISA reactions by plant sap has been observed with potato leaves (Cadeno-Hinojosa and Campbell, 1981). In addition, similar effects were observed when purified AMV, to which various dilutions of birch tree extracts had been added, were tested by ELISA (Hardcastle and Gotlieb, 1980). As the concentration of the interfering substances decreased, ELISA readings increased. On the other hand, since virus concentration also decreases with dilution, ELISA readings eventually decrease at higher dilutions.

Detection of AMV in apple trees, up to August but not later confirms earlier findings (Barbara, 1980). In contrast to the situation in intact trees, however, in the present study, AMV could be detected in apple callus for at least ten months. Thus, for some aspects of AMV study, which require long-term monitoring of virus presence, a tissue culture system might be preferable.

When AMV-infected tumorous and non-tumorous callus was grafted onto young trees in August, only trees grafted with AMV-infected tumor tissue developed AMV symptoms. This may have resulted from the fact that the crown gall callus

grafts "took", as shown by the development of crown galls at the infected areas, while the leaf callus apparently did not "take". That the tissue growing from the grafted areas was of crown gall-derived callus origin, and was not caused by any A. tumefaciens bacteria that may have been present in the grafted callus, is indicated by two facts. First, all grafted crown gall calluses had been grown on antibiotic-containing medium, and then, when parts of them were transfered to antibiotic-free medium, no A. tumefaciens colonies were seen. Second, the galls growing from the grafted area were apparent after only 1.5-2.0 months, while when trees were inoculated with A. tumefaciens it took at least four months for galls to appear.

When 9-month-old AMV-infected callus was grafted or implanted on apple trees in March, none of the trees had developed AMV symptoms on the leaves, or crown galls at the area of grafting, 8 weeks later. When tested for AMV by ELISA, all trees appeared to be free of AMV, although ELISA tests showed that several of the calluses from which graft material was taken still contained virus. It is not known why the crown gall calluses "took" in August but not in March, however, it must be noted that the crown gall callus used for grafting in March had been in culture for 9 months and may have become dependent on a component of the apple

growth medium and was thus unable to grow in the grafts without that component.

Mechanical transmission of AMV from AMV-infected apple callus or protoplasts to cucumber plants was unsuccessful. This may be due to the fact that some strains of AMV, and most woody plant viruses, are difficult or impossible to transmit mechanically (Fulton, 1972), possibly as a result of secondary compounds produced by the plants which hinder transmission (Fulton, 1966) and/or inactivate the viruses (Mink, 1965; Mink and Saskena, 1971). Production of secondary compounds in cultured plant tissue has been shown to be influenced by environmental and nutritional conditions (Forrest, 1969; Hagimoril, 1982). It is hoped that, by manipulating these conditions, production of inhibitory compounds can be reduced, thus facilitating virus transmission.

The results from AMV transmission and ELISA tests indicate that ELISA is more reliable for monitoring AMV in apple tissue than are the bioassays. This finding differs from the results of implantation experiments done with chrysanthemum callus, in which two chrysanthemum viruses could be easily transmitted by implanting virus infected callus into plants (Ben Jaakov _et al._, 1971). It should be pointed out, however, that AMV requires actual graft union in order to be transmitted, whereas the chrysanthemum

viruses are sap transmitted (Ben Jaakov et al., 1971) and may have been transmitted that way when the callus came in contact with the host tissue.

According to ELISA results, 68% of the apple calluses derived from AMV-infected plants were still infected with AMV 3 months after initiation. Six months later however, only 37% of the calluses had virus levels detectable by ELISA (Table 2). These findings are consistent with those of studies on several other viruses such as PVX and PVY (Wilso and Eisa, 1975), TMV (Kassanis, 1957; Hansen et al., 1966; Hirth and Durr, 1971), TRSV, CMV, and TomRSV findings are consistent with those of studies on several other viruses such as PVY and PVX (Wilson and Eisa, 1975), TMV (Kassanis, 1957; Hansen (Reinert, 1966), in which virus levels often dropped sharply after several subcultures, frequently dropping below detectable levels. On the other hand, several of the apple callus cultures continued to have high AMV titers, as shown by consistently high ELISA readings. This finding agrees with that of Hirth and Durr (1971) who found that, while TMV levels dropped in most callus cultures, in others virus levels stayed the same.

Decrease of virus titer in callus tissue has been attributed to several factors including phytohormones (Kutsky 1950, 1966), low protein levels (Kassanis, 1957), and the failure of the virus to spread to all of the cells

(Hansen and Hildebrandt, 1966). AMV spreads irregularly and erratically in infected apple trees (Fridlund, 1979), and it is likely that its spread in apple callus is similarly erratic. Some of the differences in virus multiplication in different calluses may be due to genetic changes in the callus tissue since plant cell culture has been shown to generate variation in cultured plant tissue (Larkin and Scowcroft, 1981). For example, 65 clones of potato regenerated from mesophyll protoplasts exhibited statistically significant variation in 22 characters (Secor and Shepard, 1981). Similarly, it may be possible that, with increasing time in culture, apple callus may undergo genetic changes which could increase or decrease its ability to support virus replication.

The disappearance of AMV from many callus cultures may have implications for producing virus-free stock. Virus-free plants have been regenerated from virus-infected callus cultures in several studies (Wang, 1975;, Berbee and Castello, 1976; Dean, 1982), and increases in vigor and disease resistance in plants regenerated from some callus cultures may be due in part to elimination of latent viruses (Larkin and Scowcroft, 1981). It may be possible in the future, by regenerating apple trees from callus tissue, to produce trees free of symptom-inducing and of latent viruses.

On the other hand, callus cultures which have been shown to maintain high virus titer may prove useful for virus studies including purification, transmission, and testing of antiviral compounds.

CHAPTER VI

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