AN ABSTRACT OF THE THESIS OF

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Title: Effects of Growth-regulating Treatments on Eradication of PVX and PVS in Potato Shoot-tip Culture.

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Four experiments were conducted to evaluate the effects of growth-promoting treatments on potato sprouts and plantlets grown **in vitro**, and to determine the relationships between growth rate and eradication of PVX and PVS through shoot-tip culture.

The approach consisted of exposing plant material to potential growth-regulating treatments, selecting the more promising ones and excising the shoot-tips from associated plantlets to evaluate treatment effects on virus eradication. Treatments consisted of dark-induced etiolation and growth regulators believed to have antiviral activity.

Sprouts attached to seed pieces responded to the growth-promoting effect of GA_3. However, the presence of decay microorganisms made this approach unsuitable. Nodal cuttings from isolated disinfected sprouts or from plantlets grown under aseptic conditions **in vitro** produced more suitable plant material since growth of decay microorganisms was minimal. Nodal cuttings from plantlets developed **in vitro** responded more obviously to growthregulating treatments than those from sprouts.

Plantlet growth was favored by adding either 2,4-D at 0.1 and 1.0 µM, or GA_3 at 0.5 and 1.0 µM to the culture media, or by inducing etiolation with darkness. Plantlet growth was reduced when 2,4-D and BAP were added to the culture medium at concentrations of 10.0 µM and 100.0 µM, respectively, at these concentrations, callus growth, excessive branching, or both were observed.

Except for dark-induced etiolation, 0.5-mm shoot-tips regenerated more plantlets in less time than 0.2-mm shoot-tips. Shoot-tips excised from plantlets treated with GA_3 at 0.5 and 1.0 µM and benomyl at 25 mg ai/l grew rapidly and survived well.

Small shoot-tip size (0.2 mm) favored virus elimination, especially for PVS. Out of 25 plantlets regenerated from 0.2-mm shoot-tips, 60% were PVX-free and 48% were PVS-free while only 41.5% of 41 plantlets regenerated from 0.5-mm shoot-tips were PVX-free and 9.8% PVS-free.

Compared to other alternatives for virus eradication such as thermotherapy, GA_3 at 0.5 µM and benomyl at 25 mg ai/l in the culture medium seemed to offer a good alternative because of high survival rate, short regeneration time and moderate virus eradication.

The virus eradicative effect of some treatments was not clearly related to their stimulative effect on growth.

EFFECTS OF GROWTH-REGULATING TREATMENTS ON ERADICATION OF PVX AND PVS IN POTATO SHOOT-TIP CULTURE

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INTRODUCTION

The potato is one of the most efficient crops in converting natural resources, labor and capital into highly nutritious food and represents an important tool in the world struggle against famine. Potato production has decreased in the temperate zone during the last two decades, but total area and production have increased in the tropics and subtropics. Increased production in these latitudes reflects improved acceptance of the potato as a basic food in developing countries. The potato leads other important food crops such as wheat, sorghum, rice and corn with the highest percent increase in production in third world countries.

Despite the potato's high productivity and potential as a quality food source, it is still considered a luxury vegetable in developing countries where major production barriers still exist. Seed production and technology are two of the most important of these barriers.

Potatoes are conventionally grown from tubers, a type of vegetative propagation that ensures genetic purity through cloning but also increases the spread of tuber-borne diseases. Rigorous controls are used in seed certification programs to prevent infection and spread of diseases through seed tubers.

Tissue culture techniques have recently been incorporated into potato seed production systems to eradicate pathogens and to

increase multiplication rates, producing more healthy seed stocks in less time. In some developing countries such as Taiwan and Vietnam, tissue culture has been adopted in an effort to reduce dependence on foreign seed. Tissue culture could potentially be used in many countries where conventional seed production methods have failed due to predominant mild weather conditions which favor virus vectors.

Tissue culture has made virus elimination possible through shoot-tip culture. The method consists basically of dissecting the apical growing tissue, culturing it in an artificial nutrient medium under aseptic conditions, and providing the required environment for plantlet development.

Virus eradication through shoot-tip culture is probably favored by either low concentrations or total absence of viruses in the meristematic region. Low virus levels may be due to the lack of a differentiated vascular system which would restrict virus invasion of the meristematic region. High metabolic activity in meristems may also prevent virus replication. An alternate theory suggests that components in the tissue culture media may interfere with virus replication and thereby favor virus removal in shoot-tip culture.

Viruses are not always eradicated by isolating the shoot-tip from the infected mother plant. Success has been improved through a combination of tissue culture and thermotherapy. This procedure, however, normally relies on the use of expensive growth chambers which are not readily available in developing countries, although various inexpensive but functional alternatives to commercial growth chambers have been devised. Another disadvantage of heat therapy is that some viruses or virus strains are difficult to eradicate

because they withstand higher temperatures than the plant can tolerate.

The main goal of this research was to develop an alternative to thermotherapy for eradicating PVX and PVS, two of the most difficult viruses to eliminate from infected potato plants. This alternative must be simple and inexpensive in order to be feasible in developing countries. The specific objectives of this research were to:

1.- Evaluate effects of diverse growth-regulating treatments on growth, regeneration and virus content of plantlets **in vitro**. Treatments included dark-induced etiolation and growth regulators believed to have antiviral activity.

2.- Determine relationships among plantlet growth rate, shoot-tip size and persistence of PVX and PVS.

LITERATURE REVIEW

Significance of viruses in potato production

Virus diseases are of particular importance in potato production. Viruses are easily spread in vegetatively propagated crops like potatoes, cause severe yield losses, and are difficult to control.

Because of their economic impact, potato viruses were among the first discovered and they have been intensively studied (Want, 1972). Approximately 23 viruses have been reported to cause economic damage in potatoes (Hooker, 1981); Potato virus X (PVX), Potato virus S (PVS), Potato virus Y (PVY), Potato Leafroll virus (PLRV), Potato virus A (PVA), Potato virus M (PVM), Andean potato mottle virus (APMV), and Alfalfa mosaic virus (AMV) are among the most important. Some viruses are universally present wherever potatoes are grown while others are locally distributed.

The clonal nature of potato propagation combined with the difficulty of virus detection have contributed to the spread of virus diseases (Mellor and Stace-Smith, 1977). Viruses can be transmitted to the next crop when infected seed-tubers are used. When commercial potato production first began, farmers quickly noticed yield decreases when seed-tubers were used from successive generations, and recognized the advantages of certain production areas in producing "clean" seed. But the roles of viruses and virus vectors in so-called "potato degeneration" were not determined until the twentieth century (Want, 1972). Potato farmers are now very aware of virus diseases and their symptoms and effects.

Virus symptoms vary depending on cultivar susceptibility, virus strain, environmental conditions and other factors. Common symptoms include chlorosis, stunting, curling, corky ring spot and net necrosis, which cause poor plant growth or death in extreme cases. Some viruses, however, cause no visible symptoms, and are overlooked or misidentified due to the lack of apparent damage. These latent infections may easily be attributed to abiotic factors or physiological disorders such as mineral deficiencies, moisture stress, frost damage or poor soil drainage (Bos, 1983). Regardless of the symptoms, the most important aspect of virus infection is yield reduction.

Reports of potato yield reduction due to virus infection are not consistent, with estimates varying from 15% to 99%. Assessment of yield reduction under field conditions is difficult because healthy plants normally compensate for adjacent diseased ones by increasing their production up to 54% (Reestman, 1972; Bos, 1983). Due to the availability of virus-free plants, yield loss can now be more accurately analyzed by comparing infected plants with virus-free plants of the same clone (Mellor and Stace-Smith, 1977). Using this technique, it has been determined that freeing the cultivar King Edward of PVM produced more vigorous haulms and increased yield about 10% (Bawden and Kassanis, 1965); likewise, removing PVX and PVS from the cultivars Netted Gem and White Rose raised yields 11-38% over infected plants (Wright, 1970). Yield increases of up to 60% have been reported in virus-freeing studies of the combined effects of PVX and PVA (Gregorini and Lorenzi, 1974).

Damage from virus infection is not restricted to yield reduction. Plants infected with viruses are predisposed to other pathogens and pests and show increased sensitivity to abiotic factors due to reduction in vigor. In addition, tuber quality is also sometimes reduced affecting the market value of the commercial product. Indirect losses resulting from the cost of implementing hygienic and pest control measures to prevent the spread of viruses also adds to their economic impact (Bos, 1983).

Most virus control measures are focused on preventing infection. Use of virus-free stocks, avoidance of virus vectors and eradication of diseased plants or other sources of inoculum are commonly practiced to prevent the spread of viruses. However, after infection has occurred, the virus begins to replicate by inducing the host cells to form more virus particles (Agrios, 1978), becomes intimately associated with the plant system and, therefore, becomes more difficult to control.

Virus-host plant association is so intimate that attempts to eliminate the virus normally interfere with plant metabolism and development (Quak, 1977; Kassanis, 1980). Some techniques such as heat therapy have been used, however, to eliminate the virus after infection has occurred.

In heat or thermotherapy, propagative organs like cuttings, rhizomes, or tubers, are dipped in hot water (35°- 45°C) for a few minutes or hours, or complete plants can be kept in greenhouses or growth chambers at 35°to 40°C for several days, weeks, or months. Sometimes, the whole plant is freed from the virus, or the new tissues or organs formed by the plant during the heat treatment are

virus-free and then used as material for increase. The use of heat therapy is based on the fact that the virus is inactivated at high temperatures (Agrios, 1978).

The mechanism of virus elimination through thermotherapy is not totally clear. It appears that the rate of virus replication is reduced at high temperature while degradation continues (Mellor and Stace-Smith, 1967). Many viruses are completely unable to multiply when the temperature is raised to 36°C. There are exceptions, however, and some viruses and virus strains are able to multiply at temperatures over 36°C.

The thermal inactivation point is defined as the temperature at which the virus is inactivated **in vitro**; the temperature at which viruses are inactivated in living plants is usually lower. Thermotherapy is often more successful with young and vigorously growing plants than with old ones. These two facts suggest that high temperatures do not affect the virus directly, but instead alter metabolic processes in the plant that inactivate the virus (Kassanis, 1980).

Thermotherapy is not effective in eliminating all viruses. Experience suggests that isometric and some thread-like viruses are more easily eliminated (Quak, 1977). Another disadvantage of heat treatment is that exhaustion or death of the plant can occur because some viruses or virus strains can withstand longer periods of heat treatment than the host plant can tolerate (Cassels and Long, 1980).

Chemotherapy has also been proposed for eliminating viruses from infected plants. Antiviral chemicals have been developed to control viruses in animals and some of them have also been tested in

plants with relative success (Hansen and Lane, 1985). One of the antiviral chemicals is ribavirin (syn. Virazole) which is a synthetic guanosine analogue. Its mode of action is not completely understood either in animal cells or in plant tissues; but in animal cell culture, ribavirin is believed to reduce availability of guanosine triphosphate (GTP) and GTP-derivates for synthesis of virus DNA, RNA, and proteins (Cassels and Long, 1982). The use of ribavirin under field conditions is restricted because it is toxic to plant tissues and it is expensive.

A group of researchers at Rothamsted Experimental Station, UK, investigated effects of injected polymers on resistance to virus injury in an attempt to mimic effects of the natural protective mechanism of hypersensitivity. Results indicate that chemicals like polyacrylic acid induce resistance by the production of "resistance-associated proteins" (Kassanis 1980). Although this chemical offers very little protection against systemic viruses, which are important causes of crop losses, this discovery widens the possibility of finding an effective chemical control of virus diseases. Other chemicals have been tested, including benzoic and salicylic acid, from the aspirin group, and some plant auxins and cytokinins. The aspirin group of chemicals appears to be effective against viruses that invade the plant systemically.

A third approach, tissue culture, is used to control virus diseases by rapid multiplication of virus-free tissues **in vitro**. Although plant tissue culture dates from the early years of this Century, its application to the production of disease-free plant stocks began in 1949, when Limasset and Cornuet found a decrease in

the concentration of Tobacco mosaic virus (TMV) in tobacco leaves near the plant apex. Based on this discovery, Morel and Martin (1952) succeeded in obtaining virus-free dahlia plants by dissecting apical meristems from diseased plants and culturing them aseptically **in vitro** (Quak, 1972). Since then, the production and propagation of disease-free plants by tissue culture techniques have been widely developed and, at the present time, are used commercially in innumerable plant species, including many important crops.

Meristem culture for the production of virus-free plants consists of dissecting either the apical meristem (apical dome) or the subapical meristematic region (apical dome plus a few subjacent leaf primordia) and culturing them in artificial media under aseptic conditions (Hu and Wang, 1983).

Significance of Potato virus X (PVX) and Potato virus S (PVS)

As noted above, some 23 viruses affect potatoes; also, virus diseases are especially important in potato seed production because they can be transmitted to the next generation through seed-tubers. Among potato viruses, PVX and PVS are of particular concern in potato seed production because they cause latent infections which are difficult to detect visually.

Potato virus X (PVX) is also known as potato latent virus, healthy potato virus, and potato virus 16 (Beemster and Rozendaal, 1972). It is the most widespread of potato viruses, causing yield reductions estimated at up to more than 15% (Munro, 1981); however, necrosis-evoking PVX strains may cause losses of over 50% in some potato varieties.

PVX particles are flexuous thread-like filaments, with dimensions of 515x13 nm and helical substructure. PVX is a RNA virus with a single-stranded nucleic acid. Its thermal inactivation point is between 68° and 76°C, depending on the strain; the dilution end point is between 10^{-5} and 10^{-6}; the stability in desiccated tissue is at least 250 days, and stability **in vitro** is up to several weeks (Munro, 1981).

PVX is difficult to detect visually because it usually produces very mild symptoms. PVX typically causes only a mild mottle, but some strains cause severe or rugose interveinal mosaic, with dwarfing of the plant, reduced leaflet size and, in some cases, even extensive top necrosis depending on varietal susceptibility and environmental conditions. Temperatures between 16°C and 20°C enhance symptoms in most plants and symptoms are mild or masked above 28°C.

Under field conditions, PVX spreads readily through sap inoculation, by contact between foliage or roots of healthy and infected plants, and by transport on farm implements, clothing and animal fur. Aphids do not transmit PVX, but chewing insects can transmit it by contact. Zoospores of the fungus Synchytrium endobioticum are reported to transmit the virus. This virus is so contagious that it can be transmitted by sprout-to-sprout contact during seed storage and handling.

PVX is detectable by either indicator plants or serology. Among the indicator test plants are Gomphrena globosa, Nicotiana tabacum, Datura stramonium, Lycopersicum esculentum, Chenopodium amaranticolor and others. PVX is strongly immunogenic, allowing the

use of serological testing for its detection. PVX antisera are now readily available for use in routine tests such as the enzyme-linked immunosorbent assay (ELISA), agglutination or precipitin methods.

The most effective method of controlling PVX is the use of PVX-free seed. PVX-free stocks are now routinely produced by a combination of thermotherapy and meristem culture. Avoidance of contamination through contact with infected plants or tubers and the use of tolerant or resistant cultivars are also beneficial.

Potato Virus S (PVS) is also found wherever potatoes are grown. PVS was first accidentally detected in the Netherlands, during efforts to produce an antiserum to potato virus A. Yield depression due to PVS varies between 10 and 20%, depending on virus strain and variety, but infected plants normally yield a large number of small tubers (Beemster and Rozendaal, 1972).

PVS particles are straight to lightly curved filaments, approximately 650x12nm, with RNA as the nucleic acid. The PVS inactivation point is 55-60°C; dilution end point in crude potato sap is about 10^{-3}; stability in desiccated tissue is 11 months and stability **in vitro** is about four days (Bagnall, 1981).

Visual diagnosis of PVS is difficult because infection is almost symptomless. However, one characteristic symptom is a deepening of veins on the upper surfaces of leaves, which may become rugose. Also, plant stunting and a more open type of growth can be observed in some varieties, as can a slight or distinct mottle and sometimes a faint banding of veins. Very sensitive varieties turn bronzy, with rugose leaves and necrotic spots on their upper surface. Instead of turning uniformly yellow, lower old leaves in

the shade develop greenish-bronze spots. Symptoms are more evident on cloudy days (Bagnall, 1981).

PVS is readily transmitted by contact. Contact seems to be the only natural mechanism of transmission. However, some evidence suggests that the virus (at least some strains) can be transmitted by aphids. PVS usually spreads slowly in the field (Beemster and Rozendaal, 1972).

In diagnosis, serological tests are preferred over visual symptoms because of the mild, easily confused symptoms of PVS in potatoes or any other host that could be used as an indicator plant.

The use of PVS-free planting stock obtained through meristem culture seems to be the most practical method of control. Roguing seed fields to remove PVS is not effective because infected plants are difficult to detect (Bagnall, 1981).

The role of tissue culture in potato seed production

Plant tissue culture is defined as the aseptic culture of plant cells, tissues or organs (Murashige, 1979). It is one of the most important developments in the last 25 years of plant science. Tissue culture has potential applications ranging from basic research at the molecular level to commercial propagation of crop plants.

In potatoes, tissue culture has revolutionized seed production because viruses can be eliminated through meristem culture, and virus-free stocks can then be maintained and increased through rapid multiplication systems using tissue culture. In the US, all states producing seed potatoes use tissue-cultured plants as stock material

in certification schemes. Tissue culture techniques are being widely adopted in developing countries as well (Wang and Hu, 1982; Uyen and Vander Zaag, 1983).

Meristem culture is now considered the most efficient method available for eliminating viruses from infected plants (Kartha, 1981). Since early work by Morel and Martin (1952), this technique has been improved and used in eliminating viruses from at least 49 plant species (Wang and Hu, 1980).

Meristem culture basically consists of dissecting the apical growing tissue, culturing it in artificial nutrient media under aseptic conditions, and providing the required environment for plantlet regeneration. Various terms including "meristem-tip culture", "tip culture", "culture of shoot apices", "shoot-apex culture", and "shoot-tip culture" have been used in the literature referring to this technique, depending on the amount of dissected tissue used (Wang and Hu, 1980).

Various theories have been proposed to explain the effectiveness of the culture of meristematic tissue in virus eradication. One possible explanation, which prompted the pioneer research of Morel and Martin in 1952, states that there is a gradient in virus concentration from the base of the plant to the top, with very low concentration or total absence of viruses in the meristematic region. Isolation of meristematic tissue from the infected mother plant by dissecting it, does indeed increase the chance of producing virus-free plantlets, therefore supporting the concept of a virus concentration gradient.

There is no clear explanation for the decrease in virus concentration in the meristematic region, but four possible explanations have been offered (Wang and Hu, 1980):

a.- Lack of vascular system. Viruses readily move inside the vascular system, whereas spread from cell to cell through plasmodesmata is very slow. Cell division in the meristematic region occurs faster than virus invasion and replication. In the rapidly dividing meristem, the vascular system is still undifferentiated, therefore, virus infection of meristematic cells is restricted.

b.- High metabolic activity. It is presumably more difficult for viruses to control the metabolism of actively dividing meristematic cells, where the synthesis of RNA needed for viral replication may be suppressed.

c.- Inactivating system. A natural mechanism of defense against virus infection has been suggested. This system may be more active in the apical region, protecting the meristematic cells from infection. Activity of this system might increase when the meristem is detached from the mother plant.

d.- High auxin concentration. High concentrations of 2,4-D in culture media may interfere with nucleic acid metabolism, consequently affecting virus replication. A similar effect possibly could be produced by high endogenous auxin levels in the meristem.

Nevertheless, not all plants generated from meristem-culture are virus-free and neither are all meristems. Some virus particles have been detected in apical domes using electron microscopy or indicator plants (Wang and Hu, 1980). The fact that it is still possible to obtain virus-free plantlets through meristem culture,

even though the virus is present in the meristem, suggests that something related to the meristem culture technique is also involved.

Several authors suggest that some components of the culture media might be responsible for virus elimination. Growth-regulating substances, especially auxins and cytokinins, have been suggested as being responsible for the virus-inhibiting effect of culture media. Quak (1961) reported that treatments with 2,4-D before excision of the meristem increased the chances of producing virus-free potato plants.

This culture media theory is supported by results of experiments not involving tissue culture. In tomato leaf disks inoculated with tobacco mosaic virus (TMV), cytokinins (kinetin and benzyladenine) at a concentration of 20 mg/l reduced virus content. Virus titre, however, was increased at a concentration of 0.002 mg/l, which suggests that only high concentrations of the growth regulators are effective (Bailiss, **et al.**, 1977).

Studies by Antoniw, **et al.** (1981) in tobacco support the theory of virus suppression by growth-regulating substances in tissue culture. They found proteins associated with natural resistance to virus infection in healthy callus, suggesting that a component of the growing media stimulates the production of resistance-associated proteins. By injecting IAA, 2,4-D and BAP (components of the callus-inducing media) into leaves of tobacco, and then inoculating with TMV, they were able to reduce the number and size of virus lesions with all growth regulator treatments.

As another possible explanation of virus elimination through meristem culture, Mellor and Stace-Smith (1977) suggest that virus replication requires the presence of certain enzymes which become unavailable once the meristem is excised from the mother plant. In the absence of these enzymes, the virus is unable to replicate, and viral RNA is degraded and possibly utilized by the plant cells.

Regardless of the mechanism of action, meristem culture is at present considered the most effective method available for eradicating viruses from infected stocks. In potatoes, extensive literature reports virus elimination through meristem culture alone or in combination with thermotherapy and chemotherapy.

High temperatures inactivate viruses, limit virus production or restrict virus movement in the plant; therefore, newly developed plant parts may be virus free. Plants free of viruses have been obtained by exposing plants or tubers to temperatures between 33°C and 40°C for different periods, followed by excision and culture of the shoot-tips.

Stace-Smith and Mellor (1968) eliminated PVX and PVS by maintaining infected plants at air temperatures between 33°and 37°C and dissecting axillary buds of different sizes (0.3-3.0 mm long) at different intervals during heat treatment. The size of the excision was critical in virus eradication after short or no heat treatment but of less importance with longer heat treatment. After 8 weeks of heat treatment, 50% of the developed plants were PVX-free and after 18 weeks, 100% were virtually PVX-free. PVS was more difficult to eradicate: of 155 PVX-free plants obtained, only 35 were PVS-free. The number of PVS-free plants increased by increasing

heat treatment up to 6-8 weeks but longer treatment did not further increase virus-freedom.

Thermotherapy in combination with shoot-tip culture is now widely used with heat treatment intervals of 6 to 10 weeks. Use of alternating temperatures in a four-hour regime increases virus-freedom. Alternating temperatures include a high temperature (40°C) exposure long enough to temporarily inactivate virus replication followed by an optimal temperature (25°C) that allows the plant to recover from heat stress but still short enough to prevent the virus from resuming multiplication. The use of temperature differentials has increased plant survival and effectiveness of the heat treatment in reducing virus content (Lozoya-Saldaña and Dawson, 1982).

Meristem culture has also been used in combination with chemotherapy. The use of antiviral compounds in field conditions is limited because of phytotoxicity and high costs, especially when repeated applications are required as the crop develops. Tissue culture represents an alternative to the use of field chemotherapy in virus elimination since only small amounts of the chemical are required (Cassells and Long, 1980).

Antiviral substances, such as ribavirin (Virazole), have been added to the culture media before or after excision of the meristem. Cassells and Long (1982) took explants and meristems from potato plants infected with PVX, PVY, PVS and PVM in different combinations. Infection in plants grown from adventitious shoots cultured in the presence of 205 μM Virazole varied from 9% to 29%, compared to at least 79% virus infection in the absence of Virazole. Differences among viruses were also detected: in the potato variety

May Queen, infected with PVX, PVY and PVM, only PVX was detected in 13% of the progeny, and PVY and PVM were apparently selectively eliminated. Meristems taken from another variety infected with PVM were cultured in the presence of Virazole at 41 and 205 µM with the results that 58% and 100% of the progeny were virus-free, respectively.

Klein and Livingston (1982) took potato shoot-tips from PVX-infected tubers of the varieties Russet Burbank and Red McClure. Tips were cultured on liquid medium containing 0, 1, 10 or 100 µg/ml ribavirin. PVX could not be detected in more than 80% of the plantlets developed from shoot-tip cultures treated with 10 µg/ml of ribavirin. Plants developed from these cultures were maintained for up to 9 months without showing detectable PVX symptoms. Phytotoxic effects were reported, however. Klein and Livingston (1982) reported that concentrations of 1 and 10 µg/ml of ribavirin were toxic to the potato cultivars Russet Burbank and Red McClure, delaying plantlet development, and that 100 µg/ml was lethal. They also reported that the cultivar Red McClure seemed to be more sensitive to ribavirin than Russet Burbank.

In spite of phytotoxicity, ribavirin in combination with tissue culture offers an alternative to heat therapy for eradicating viruses especially those difficult to eliminate with heat therapy, such as PVX and PVS.

Benomyl is a systemic fungicide that also seems to exhibit some antiviral activity. Klein and Livingston (1983) found that benomyl (25 mg ai/l) had a weak eradicative effect on PVS and no effect on PVX when it was used as a constituent of potato shoot-tip culture

media. However, the authors consider the use of benomyl in combination with thermotherapy appropriate because it could increase the number of PVS-free plantlets, promote shoot-tip growth, and decrease regeneration time.

Factors influencing explant survival and virus elimination through tissue culture

Explant size

The use of small explants in tissue culture favors virus freedom. However, tissue survival decreases with decreasing tissue volume. True apical meristems (0.05-0.1 mm) have rarely been cultured with success because of difficulty in isolation, low survival rate, and slow plant development (Murashige, 1979). Mellor and Stace-Smith (1977) usually rejected potato buds less than 0.3 mm and more than 0.7 mm because small explants did not root readily, either dying or going through a callus phase, and large ones were liable to infection. Tissue survival improves when the first pair of leaf primordia is included along with the meristem dome, possibly because the developing leaves produce hormones required for meristem growth (Murashige, 1979).

Viruses to be eradicated

Some viruses are more readily eliminated than others. In potatoes, PLRV, PVA, PVY, PVX and PVS are listed in increasing order of difficulty (Quak, 1977).

Previous treatment

High temperature and antiviral treatments applied to the mother plant prior to excision not only increase the probability of virus-

free plants but also permit the excision of larger tissue volumes, thereby increasing survival (Stace-Smith and Mellor, 1968).

Culture media

Culture medium composition influences survival and rooting. Several media comparisons suggest that relative to most others, the medium developed by Murashige and Skoog (1962), better known as MS-62, increases both the number of developed plantlets and the rate of development (Mellor and Stace-Smith, 1977). Liquid media appear to be superior to solid media for axillary-bud culture (Stace-Smith and Mellor, 1968). Liquid creates an intimate contact between the explant and the nutrients and allows the diffusion of toxic plant wastes (Hu and Wang, 1983).

Season

Tissue culture success is influenced by the season. In potatoes, tips excised immediately after tuber dormancy (in spring and early summer) root more readily than those taken later in the year (Mellor and Stace-Smith, 1969). Actively growing tissues are preferred due to strong growth potential and low virus concentration (Hu and Wang, 1983).

Potential approaches favoring growth of potato sprouts and plantlets

Virus eradication through culture of meristematic tissue is not well understood. Wang and Hu (1980) suggest that viruses are unable to control the metabolism of actively dividing cells of the meristematic tissue, therefore viruses are either absent or at low concentration in this region. Several authors have also suggested that

growth regulators used as components of the culture medium are responsible for virus eradication because these substances interfere with virus replication (Quak, 1961; Wang and Hu, 1980; Antoniw **et al.** 1981). Based on these reports, it seems possible that promoting growth by exposing infected plant material to growth regulators might not only stimulate cell division but, at the same time, also interfere with virus replication.

Several growth regulators are reported to promote sprout growth. Gibberellic acid (GA_3) promotes sprout elongation when used to break tuber dormancy. Rappaport **et al.** (1957) found that the rest period of newly harvested potatoes of three varieties was terminated by immersing the tubers in GA_3 solutions at concentrations between 50 and 2000 ppm for 5 to 90 minutes. The time required for sprouting was shortened by two to three weeks. However, when GA-treated tubers were planted, undesirable secondary effects were observed, such as excessive stem elongation, delayed leaf development, chlorosis of foliage, yield depression, and changes in tuber shape (Smith, 1977). Bruinsma **et al.** (1967) recommended dipping excised eyes in a solution containing 1 to 5 ppm of GA, for 10 minutes to break dormancy, followed by dipping the treated excised eyes in a solution of the inhibitor N-Dimethylaminosuccinamic acid (B9) to avoid excessive stem elongation.

Gibberellic acid also stimulates growth **in vitro**. GA_3 at moderate to high concentrations (1-25 µM) increases tobacco stem growth **in vitro** (Engelke **et al.**, 1973); these authors recommended concentrations between 0.2-1 µM for optimal overall shoot develop-

ment because, at high concentrations, the plants were spindly with poor leaf development.

Auxins and cytokinins also play important roles in plant growth. High levels of auxins in tissue culture media promote callus formation while high cytokinin levels favor shoot proliferation. Both growth regulators must be present, however, to achieve normal shoot development in culture (Skoog and Miller, 1957). In tissue culture, auxin concentrations range from 0.1 to 10.0 μM and cytokinin concentrations, from 0.1 to 100.0 μM.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most used in tissue culture. 2,4-D is more active than the naturally occurring Indolacetic acid (IAA) because it is less rapidly destroyed by auxin-degrading mechanisms. 2,4-D is also preferred because it exhibits weak polar transport compared to IAA (Moore, 1979).

Growth can be enhanced by means other than application of growth regulating substances. Darkness, for example, also enhances shoot elongation. The meristematic apex is the main site of auxin production in dicots. Light activates the degradation system of the natural occurring auxin IAA (Leopold, 1964). In the absence of light, IAA is not rapidly degraded, therefore stem elongation is enhanced.

MATERIALS AND METHODS

The suggestion has been made that virus elimination through shoot-tip culture is possible because high metabolic activity in the meristematic region prevents virus invasion. During rapid shoot growth meristematic activity proceeds at a faster rate than virus invasion thereby creating virus-free tissues near the plant apex. This research was undertaken to develop procedures that promote growth of potato plantlets **in vitro**, and then, to evaluate relationships among growth rate, shoot-tip size and the incidence of potato viruses X (PVX) and S (PVS).

The variety Norchip was used in all tests. Norchip was released in 1968 by North Dakota State University. It is widely used in the U. S. potato chip processing industry. In order to obtain sufficient plant material for this research, 52 tubers were tested for potato viruses X, S and Y and potato leaf roll virus (PLRV) using the serological ELISA test. Twenty-six tubers infected with both PVX and PVS were selected for the purposes of this research.

Completely randomized designs were used in all experiments.

Experiment 1.- Effect of Gibberellic acid (GA_3) on sprout elongation.

PVX- and PVS-infected tubers were surface-disinfected by immersion in a 0.525% solution of sodium hypochlorite (commercial bleach) for 5 minutes and then rinsed with tap water. Tubers were then cut into small seed pieces weighing approximately 8 gm and

containing one eye each. Representative seed pieces from each tuber were planted in pots in the greenhouse to confirm virus presence through visual symptoms (eye-indexing).

The remaining seed pieces were placed in closed black plastic boxes at room temperature for five days under dark, humid conditions to promote suberization. After suberization, seed pieces were randomly selected for treatment.

Seed pieces were immersed in solutions of GA_3 at four concentrations (0, 2.5, 5.0 and 10.0 ppm) for either 5 or 10 minutes. They were then either held at 25°C constant, or subjected to 21°C and 37°C alternately in 4-hour cycles. Treatments were applied daily for 10 days with each day representing one replication of the treatments. One seed piece per treatment was treated each day.

The treated seed pieces were placed in black plastic boxes to prevent light suppression of sprout growth. Seed pieces were misted with water twice daily to maintain high humidity favorable to sprout growth. Sprout lengths were measured at two-day intervals beginning the first day of treatment.

Most of the seed pieces began to decay four days after treatment and at least one seed piece was subsequently lost in each of four replications. Approximately 50% of the sprouts died within 12 days. Shoot-tips were excised from the remaining live sprouts, but serious microbial contamination was evident.

Since contamination was difficult to avoid using these methods, it was necessary to change procedures to insure that tissues were free of bacteria or fungi before treatment applications.

Experiment 2.- Interaction of 2,4-D and BAP on potato plantlet growth *in vitro*.

Small tubers were harvested from greenhouse-grown plants which showed visual virus symptoms during the eye-indexing test conducted in experiment 1. Presence of PVX and PVS was confirmed using the ELISA test. The virus-infected tubers were sprouted at room temperature in darkness.

In order to obtain plant material uncontaminated by bacteria or fungi, sprouts (1-2 cm long) were removed and surface-disinfected by immersion in a 0.525% solution of sodium hypochlorite (commercial bleach) for 15 minutes. The surfactant Tween-20 was added at 0.5% to improve wetting. After soaking, the sprouts were rinsed three times with sterile distilled water and placed in test tubes containing 10 ml of sterile MS-62 culture medium solidified with agar at 0.6%. The culture medium was prepared using stock solutions according to Mellor and Stace-Smith (1977), but without kinetin. Sprouts showing evidence of bacterial or fungal contamination were discarded after five days in culture. The remaining sprouts were kept as PVX- and PVS-infected stock material for the experiment.

Growth regulator treatments consisted of four concentrations (0, 0.1, 1.0 and 10.0 µM) of the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) factorially combined with five concentrations (0, 0.1, 1.0, 10.0 and 100.0 µM) of the synthetic cytokinin 6-Benzylaminopurine (BAP).

The growth regulators were dissolved in dimethylsulfoxide (DMSO) and added to test tubes containing 10 ml of MS-62 solid medium under a laminar flow hood, immediately after autoclave

sterilization of the medium and before solidification of the agar. The DMSO solvent also acts as a sterilizing agent, allowing the addition of heat sensitive substances after autoclaving (Schmitz and Skoog, 1970). DMSO seems to have no effect on virus eradication (T. Allen, personal communication, 1986), therefore, a treatment without DMSO was not included. The final concentration of DMSO in each tube was 0.1%, a level considered safe. Four replications of each of the twenty treatments were prepared.

Nodal cuttings were taken and transferred to test tubes containing the various growth regulator treatments 10 days after the virus-infected sprouts were established in the normal medium. The cuttings were exposed to a 16-hour photoperiod at a light intensity between 3750 and 4300 lux. Light was supplied by cool-white fluorescent lamps at a distance of approximately 60 cm from the cuttings. Temperatures oscillated between 21° and 32° C.

The rate of plantlet elongation was monitored by marking the level of each plant apex on the test tube wall at two-day intervals. Distances between marks were measured with a caliper. Eight measurements were made per tube.

Experiment 3.- Effect of growth-regulating treatments on plantlet growth *in vitro*

In order to evaluate effects of gibberellins on the growth of potato plantlets ***in vitro***, five different concentrations (0.1, 0.5, 1.0, 5.0, and 10.0 µM) of GA_3 were compared to nine of the most promising treatments resulting from experiment 2, namely 2,4-D at 0, 0.1 and 1.0 µM arranged in all possible combinations with BAP at 0,

1.0 and 10.0 µM. The growth regulators were added to the MS-62 culture medium (without kinetin) as described in experiment 2.

To evaluate the effect of dark-induced etiolation on plantlet growth, light was excluded by wrapping the test tubes with aluminum foil. MS-62 culture medium without growth regulators was used for this treatment.

A total of fifteen treatments were used: nine factorially combined concentrations of 2,4-D and BAP, and five concentrations of GA_3 in light, and one dark control without growth regulators (untreated-dark). Nodal cuttings from plantlets grown **in vitro** were used as plant material. Each treatment was replicated four times, with one nodal cutting per replication. Medium preparation, environmental conditions, and measurements were similar to those described for experiment 2. Stem growth measurements for foil-wrapped tubes were made in a dark room using green light, which is not appreciably absorbed by plants.

Experiment 4.- Effect of growth-regulating treatments on shoot-tip culture and virus eradication.

This experiment was designed to determine the relationship between plantlet growth and eradication of PVX and PVS through shoot-tip culture. Treatments favoring rapid stem elongation in experiment 3 (2,4-D at 0.1 and 1.0 µM, and GA_3 at 0.5 and 1.0 µM) were repeated in this experiment. The dark treatment was included because of ease of application and low cost. The most effective treatment in inducing stem elongation (2,4-D at 0.1 µM) was combined with darkness. The fungicide benomyl was also included at a concen-

tration of 25 mg/l active ingredient (ai). Benomyl has shown growth-promoting and antiviral activity (Bailiss, **et al**, 1977; Klein and Livingston, 1983).

Nodal cuttings from untreated plantlets grown **in vitro** were transferred to test tubes containing either MS-62 medium amended with the growth regulator substances or normal MS-62 medium to be exposed to the dark treatment.

To determine effects of shoot-tip size on survival and virus content, two sizes of shoot-tips (0.2 and 0.5 mm long) were excised from the stem cuttings under the growth-promoting treatments. The smaller shoot-tips (0.2 mm) included the meristem dome and the first pair of leaf primordia; the larger tips (0.5 mm) included one or two pairs of leaf primordia (Figure 1). Shoot-tip measurements were made by use of a scale in the ocular of the stereo-microscope used for dissecting.

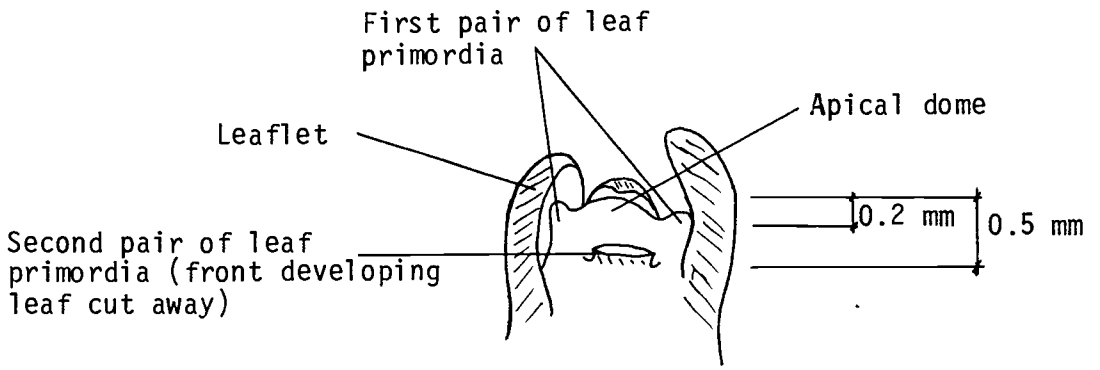

Figure 1.- Shoot-tip showing meristematic apical dome and leaf primordia

Both sizes of shoot-tips were excised from plantlets exposed to each of the eight growth-regulating treatments making up a total of sixteen treatments. Treatments were replicated seven times, with one stem cutting representing each replication. The shoot-tip excision process was programmed over seven days with each day representing one replication.

Experiments 2 and 3 showed that growth rate was generally greatest during the 13th day after the cuttings were placed in test tubes. Therefore, the shoot-tips were excised on day 13 in this experiment. Plantlet heights were measured before removal of the shoot-tips.

The shoot-tips were excised under a laminar flow hood to reduce contamination. The plantlets did not require additional surface-sterilization before excision because they had been grown under aseptic conditions. Sterile needles, forceps and scalpels were used to dissect the shoot-tips. To avoid virus spread by contact, two sets of tools were used: one set to remove excess tissue adjacent to the shoot-tip and the other set to excise the shoot-tip.

The excised shoot-tips were placed in liquid MS-62 medium, prepared according to Mellor and Stace-Smith, 1977. The test tubes were placed in the culture environment described in experiment 2. The explants were transferred to fresh MS-62 liquid medium each 7 to 10 days. After the explants developed into plantlets with leaflets and roots (approx. 10-15 mm length), they were transferred to MS-62 medium solidified with agar at 0.6%.

When the plantlets had five or more nodes, they were multiplied by nodal cutting techniques to produce tissue samples for virus testing and other uses; each plantlet was dissected into sections including at least one leaf node per section. The nodal cuttings were placed in solid MS-62 medium in test tubes, with the basal part of the stem immersed at least 5 mm into the agar medium. Plantlets generated from the two basal nodes (Figure 2, nodes A and B) were analyzed for PVX and PVS using the ELISA test. Plantlets generated from cuttings of the two upper nodes (C and D) were held in reserve as back-up material. Remaining nodes were discarded.

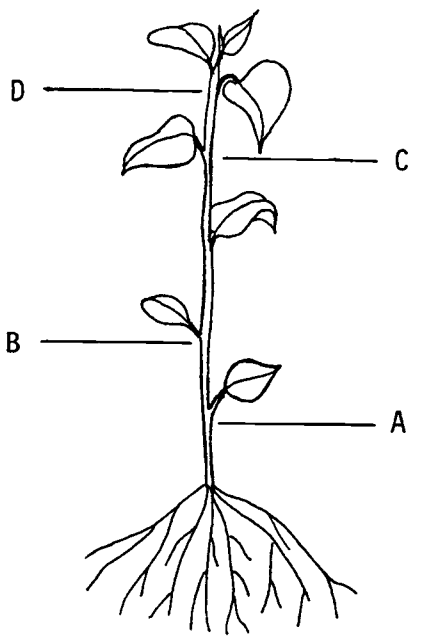

Figure 2.- Potato plantlet generated **in vitro** from shoot-tip culture showing nodes used to produce plantlets for virus testing (A and B) or reserve (C and D).

RESULTS AND DISCUSSION

Experiment 1.- Effect of Gibberellic acid (GA_3) on sprout elongation.

High levels of decay caused by fungi and bacteria reduced the value of this experiment. Four days after treatments were initiated, a high percentage of the sprouts darkened and contaminant microorganisms were evident on the seed piece surface. At least one observation was lost in each of four replications because of contamination. Twelve days after treatment, approximately 50% of the sprouts were dead. Tuber disinfection was not sufficient to control contaminant microorganisms; high humidity favored sprout growth but also favored decay organisms.

Growth rate information was complete only until the sixth of the twelve days of evaluation for six of the ten replications. Statistical analysis showed that all GA_3 treatments increased sprout elongation when compared to the untreated sprouts. GA_3 is known to enhance cell division and elongation (Leopold, 1964; Moore, 1979; Salisbury and Ross, 1978). Sprouts seemed to respond to this effect (Table 1). GA_3 concentrations above 2.5 ppm, however, did not differ significantly.

Table 1.- Effect of GA_3 concentration on the average growth rate of Norchip potato sprouts.

GA_3 CONCENTRATION (ppm)	AVERAGE GROWTH RATE (mm/day)
0	0.40b*
2.5	1.02a
5.0	0.96a
10.0	0.97a

* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 0.40.

Treatment immersion time (5 or 10 minutes) had no significant effect on growth. Although GA_3 is a large molecule, immersion for five minutes was sufficient for tissue penetration and stimulation of growth.

The two temperature regimes imposed after treatment application affected sprout growth significantly differently. Sprouts exposed to a constant temperature of 25°C ±2 grew an average of 1.24 mm/day compared to 0.43 mm/day for those exposed to 21°C and 37°C in alternating 4-hour cycles ($LSD_{.05}$= 0.29). It seems possible that the relatively high temperature of 37°C might have exhausted sprout energy reserves due to increased respiration rate, thereby restricting sprout growth.

Shoot-tips were excised from the remaining sprouts but contaminant microorganisms caused high losses of explants. Further efforts were abandoned pending the development of more satisfactory techniques.

Experiment 2.- Interaction of 2,4-D and BAP on potato plantlet growth in vitro

Plantlet growth rate differed among the various treatment combinations of 2,4-D and BAP (Table 2). Untreated plantlets grew fastest but not significantly faster than plantlets treated with 0.1 and 1.0 µM of 2,4-D or the combination of 2,4-D and BAP at either 0.1 µM and 1.0 µM, or 1.0 µM and 10.0 µM, respectively. All treatments using the highest dosage of both growth regulators showed reduced growth as did treatments including BAP at 0.1 µM.

Table 2.- Average elongation rate (mm/day) of **in vitro** Norchip potato plantlets exposed to four levels of 2,4-D combined with five levels of BAP.

BAP µM	2.4-D µM			
	0	0.1	1.0	10.0
0	2.26 a*	1.70 ab	1.90 ab	0.11 gh
0.1	0.44 fgh	0.91 c-f	0.79 d-g	0.12 gh
1.0	1.41 b-e	1.98 ab	0.72 efg	0.29 fgh
10.0	1.47 bcd	1.42 b-e	1.76 ab	0.00 h
100.0	1.56 bc	0.32 fgh	0.95 c-f	0.02 h

* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 0.7.

2,4-D alone did not affect plantlet elongation **in vitro** except for the highest concentration which inhibited elongation while favoring callus formation. Apparently the 1:10 ratio of 2,4-D:BAP did not affect sprout growth except when both substances were at the highest concentration (Table 2) and callus formation was induced. Compared with the untreated plantlets, all 2,4-D/BAP combinations that included BAP at the lower concentration interacted reducing growth.

The highest growth rate (average of all treatments) occurred during the 13th day after the nodal cuttings were exposed to the different treatments (Table 3).

Table 3.- Average elongation rates of **in vitro** Norchip potato plantlets for all treatments of experiment 2.

DAYS AFTER TREATMENT	AVERAGE GROWTH+ (mm)
3	0.70 d*
5	0.88 cd
7	0.62 d
9	1.42 cd
11	1.71 c
13	4.06 a
15	3.70 ab
17	3.00 b

+ Growth during the preceding two days.
* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 0.88.

Plantlets exposed to 2,4-D at 1.0 µM showed marked root swelling and at 10.0 µM exhibited callus formation. BAP, in the presence of any concentration of 2,4-D, induced branching as the BAP concentration increased. These results agree with the concept that high levels of auxins in tissue culture media promote callus formation while high cytokinin levels favor shoot development. BAP at 1.0 µM caused browning of the roots. Leaflet size decreased and chlorosis increased with increasing concentrations of both growth regulators. When both growth regulators were present at the highest concentration, buds did not develop into new plantlets and callus tissue was formed at the base of the cutting in contact with the medium.

Experiment 3.- Effect of growth-regulating treatments on plantlet growth *in vitro*.

Treatments favoring rapid plantlet growth included 2,4-D at 0.1 and 1.0 µM, and GA_3 at 0.5 and 1.0 µM (Table 4). GA_3 at 0.1 µM and 10.0 µM, and darkness also increased growth rate slightly. Growth rates for remaining treatments did not differ from the control.

Growth promotion by 2,4-D at 0.1 and 1.0 µM was not expected since 2,4-D did not favor plantlet growth relative to the untreated control in experiment 2. Nodal cuttings from experiment 3 came from plantlets developed ***in vitro*** and not from sprouts as in experiment 2. It is possible that nodal cuttings from plantlets might respond more to exogenous auxins because endogenous auxin concentration might be lower than in nodal cuttings from sprouts. Sprouts probably received optimum hormone levels from the mother tubers.

GA_3 at 0.5 and 1.0 µM also increased plantlet growth rate relative to the untreated but illuminated control. Under higher GA_3 concentrations, however, plantlets were brittle and probably difficult to handle for shoot-tip cutting.

Table 4.- Average stem elongation rate **in vitro** of Norchip potato plantlets exposed to selected growth-regulating treatments

TREATMENT+			AVERAGE GROWTH RATE (mm/day)
BAP	2,4-D	GA_3	
0	0	0 (light)	1.56 f*
0	0	0 (dark)	3.21 cde
0	0.1	0	6.91 a
0	1.0	0	4.04 bc
1.0	0	0	1.77 f
1.0	0.1	0	2.56 def
1.0	1.0	0	2.14 ef
10.0	0	0	1.89 ef
10.0	0.1	0	2.45 def
10.0	1.0	0	2.09 ef
0	0	0.1	3.17 cde
0	0	0.5	3.97 bc
0	0	1.0	5.30 b
0	0	5.0	2.82 c-f
0	0	10.0	3.71 cd

+ Growth promoting substance concentrations expressed in µM.
* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 1.37.

As in experiment 2, the maximum growth rate (mean of all treatments) occurred on day 13 after treatments (Table 5).

Table 5.- Average **in vitro** stem elongation rate of Norchip plantlets exposed to various growth regulating treatments. Experiment 3.

DAYS AFTER TREATMENT	AVERAGE GROWTH+ (mm)
3	2.46 d*
5	4.74 c
7	5.84 bc
9	6.63 abc
11	7.63 ab
13	8.28 a
15	7.24 ab
17	7.90 a

+ Growth increment observed at two-day intervals.
* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 2.00.

Characteristics of plantlets exposed to combinations of 2,4-D and BAP in experiment 3 were similar to those described for the same treatments in experiment 2. The effect of BAP on root development was pronounced in this instance, however. In experiment 3, BAP at 10.0 µM alone or in any combination with 2,4-D prevented root development. Plantlets grown in media containing GA_3 at 5.0 and 10.0 µM were spindly (small leaves and thin stems). Plantlets grown in darkness were also spindly and etiolated.

Experiment 4.- Effect of growth-regulating treatments on shoot-tip culture and virus eradication.

a.- Effect of growth promoting treatments on plantlet size and morphology.

Growth regulator treatments strongly affected the size of the plantlets developed from nodal cuttings taken from untreated plantlets. GA_3 at both concentrations (0.5 and 1.0 µM) and darkness favored maximum plantlet height (Table 6). Benomyl at 25 mg ai/l seemed to decrease plantlet size relative to the untreated light control but the reduction was not statistically significant at the 5% level.

Table 6.- Average height of Norchip potato plantlets **in vitro** after 13 days of exposure to various growth-regulating treatments.

TREATMENT			AVERAGE PLANT HEIGHT (mm)
2,4-D	0.1 µM		19.0 cd*
2,4-D	0.1 µM	+ darkness	20.6 bcd
2,4-D	1.0 µM		17.8 d
GA_3	0.5 µM		27.3 abc
GA_3	1.0 µM		33.6 a
Benomyl	25 mg ai/l		13.3 d
Untreated	darkness		28.5 ab
Untreated	light		18.6 d

* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 9.0.

Plantlet morphological characteristics did not differ substantially from those observed in previous experiments. Benomyl did not alter the general appearance of the plantlets.

Etiolated plantlets produced in darkness had fewer and less developed leaf primordia, making excision of shoot-tips relatively easier.

b.- Effect of plantlet growth-regulating treatments and initial shoot-tip size on relative growth of the excised shoot-tips.

Effects of plantlet treatments on subsequent shoot-tip growth were not statistically evident at the fourth week after excision but were at the sixth week. Shoot-tip growth was greatest for the 0.5 μM GA_3 treatment but this value was not statistically significantly larger than those for GA_3 at 1.0 μM, benomyl at 25 mg ai/l, 2,4-D at 0.1 μM or darkness alone (Table 7). Effects of the growth regulators on shoot-tip growth were probably related to the stimulative effect of these substances on tissue differentiation. A high number of shoot-tips excised from plantlets exposed to 1.0 μM of 2,4-D developed only callus or did not grow, hence the relative growth was low.

Table 7.- Effect of growth-regulating treatments on the relative growth of excised Norchip shoot-tips at 6 weeks after excision.

TREATMENT			RELATIVE GROWTH+
2,4-D	0.1 µM		3.50 abc*
2,4-D	0.1 µM	+ darkness	3.36 bc
2,4-D	1.0 µM		2.86 c
GA_3	0.5 µM		4.43 a
GA_3	1.0 µM		3.93 ab
Benomyl	25 mg ai/l		4.21 ab
Untreated	(dark)		3.71 abc
Untreated	(light)		3.29 bc

+ Average of seven replications and two shoot-tip sizes. Relative growth scale: 1= no growth; 2= callus; 3= visible leaflet; 4= visible leaflet and stem (no roots); 5= complete plantlet.

* Values followed by the same letter are not significantly different at the 5% level. $LSD_{.05}$ for means comparisons = 0.97.

The larger shoot-tips (0.5 mm) grew relatively better than small ones (0.2 mm) during weeks four and six after treatments were initiated (Table 8). Several possible reasons can be cited to explain effects of shoot-tip size on growth. For example, large shoot-tips were more differentiated, and probably better withstood the stress of the excision. Large shoot-tips probably did not rely entirely on substances provided by the culture medium because developed leaf primordia produce substances required for shoot-tip growth. In contrast, small shoot-tips must depend heavily on substances in the culture medium. Small shoot-tips are less able to

survive excision and frequently either die or simply develop callus tissue.

Table 8.- Effect of Norchip shoot-tip size on relative growth[+] at four and six weeks after excision

SHOOT-TIP SIZE (mm)	WEEK	
	4	6
0.2	3.05 b*	3.11 b
0.5	4.14 a	4.21 a
$LSD_{.05}$	0.45	0.49

+ Relative growth scale: 1= no growth; 2= callus; 3= visible leaflet; 4= visible leaflet and stem (no roots); 5= complete plantlet.
* Average of all treatments. Within columns, values followed by the same letter are not significantly different at the 5% level.

c.- Effect of growth-regulating treatments and shoot-tip size on plantlet regeneration.

Analysis of the total number of regenerated plantlets (Table 9) using the Chi-square test indicated that the growth-regulating treatments also affected the numbers of plantlets generated. GA_3 at 0.5 µM produced the largest number of regenerated plantlets followed by Benomyl at 25 mg ai/l, GA_3 at 1.0 µM and darkness (Table 9). 2,4-D treatments regenerated low numbers of plantlets and growth was mostly expressed as callus tissue. 2,4-D is routinely added to culture media to induce **in vitro** callus formation on several species (Narayanaswamy, 1977; Yamada, 1977).

Fifty-nine percent of the shoot-tips regenerated plantlets in this experiment. Stace-Smith and Mellor (1968) reported a

regeneration rate of 37% when comparing different durations of heat treatment to eradicate PVX and PVS from excised potato buds. Lozoya-Saldaña and Merlín-Lara (1984) obtained a 42% regeneration rate, when excising axillary buds (0.5 - 1.00 mm) from potato plants of six cultivars treated with thermotherapy to eradicate PVX. The same authors (1982) reported between 20% and 70% bud survival from six potato varieties treated with different thermotherapy regimes. Klein and Livingston (1982) obtained 40% survival of shoot-tips of two potato varieties cultured on a medium containing Ribavirin. Based on these reports, the 59% regeneration rate obtained in these studies seems acceptable.

Table 9.- Effect of growth-regulating treatments on plantlet regeneration from shoot-tips.

TREATMENTS		REGENERATED PLANTLETS*	PERCENT REGENERATION
2,4-D	0.1 µM	7	50
2,4-D	0.1 µM + darkness	7	50
2,4-D	1.0 µM	5	36
GA_3	0.5 µM	12	86
GA_3	1.0 µM	9	64
Benomyl	25 mg ai/l	11	79
Untreated	(dark)	9	64
Untreated	(light)	6	43

* Fourteen shoot-tips were excised in each treatment (seven for each shoot-tip size).

$\bar{x} = 8.25$; $s^2 = 2.44$; $X^2 = 12.25$; $p = 9.27$

The average time required for plantlet regeneration varied from 25 (mean of the explants exposed to GA_3 at 1.0 μM) to 59 days (mean of the explants exposed to darkness) (Table 10). Because the number of plantlets regenerated in each treatment differed, treatment means were compared to the untreated-light control. These comparisons indicate that only the dark treatment differed from the untreated-light control, requiring more time to regenerate plantlets.

The time required to regenerate plantlets in this experiment was considerably shorter than reported by Klein and Livingston (1983) while studying the eradicative effect of Benomyl on PVX- and PVS-infected Russet Burbank potato plants. These authors added Benomyl to the liquid medium used to culture the shoot-tips after excision, and reported means of 103 to 132 days to regenerate plantlets from PVX+PVS-infected material. When the same authors used Rivabirin to eradicate PVX from the potato varieties Russet Burbank and Red McClure (1982), the time required for regeneration ranged from 6 to 8 months. Stace-Smith and Mellor (1968) reported that buds smaller than 1 mm, excised from heat-treated plants, rooted slowly or failed to root within a period of 10 weeks.

Table 10.- Effect of growth-regulating treatments on the average number of days required for regeneration of Norchip potato plantlets from shoot-tips.

TREATMENT	PLANTLETS REGENERATED	DAYS+	LSD VALUES#
2,4-D - 0.1 µM	7	29.9	18.2
2,4-D - 0.1 µM + darkness	7	31.1	18.2
2,4-D - 1.0 µM	5	30.2	19.8
GA_3 - 0.5 µM	12	35.8	16.3
GA_3 - 1.0 µM	9	24.8	17.2
Benomyl - 25 mg ai/l	11	25.7	16.6
Untreated-dark control	9	58.8*	17.2
Untreated-light control	6	25.5	----

+ Mean number of days required to develop plantlets ready for transfer to solid media.
# LSD values depend on the number of replications involved in the comparison with the untreated-light control.
* Significantly different from the untreated-light control at the 5% level.

The size of the excised shoot-tips also affected the number of regenerated plantlets and the time required for plantlet development (Table 11); 0.5-mm shoot-tips produced plantlets in less time than 0.2-mm shoot-tips. Large shoot-tips commonly include two pairs of leaf primordia which produce substances (carbohydrates, hormones, etc.) required by the developing plantlets.

Larger shoot-tips (0.5 mm) produced more plantlets than smaller shoot-tips (0.2 mm) in most of the treatments with the exception of darkness (Table 12).

Table 11.- Effect of shoot-tip size on the number of regenerated plantlets and days required for regeneration.

SHOOT-TIP SIZE mm	TOTAL PLANTLETS REGENERATED+	PERCENT REGENERATION	DAYS# REQUIRED
0.2	25	44.6	42.7
0.5	41	73.2	27.5*

+ Total for all treatments.
# Average for all treatments.
* Different at the 5 % level. $LSD_{.05} = 8.3$.

d.- Effect of growth-regulating treatment and shoot-tip size on the eradication of PVX and PVS.

Treatment effects on eradication of PVX and PVS could not be analyzed statistically because of limited numbers of plants regenerated. Examination of the data (Table 12) suggests that there is no general eradicative treatment effect on both viruses. However, all plantlets regenerated from 0.2-mm shoot-tips excised from plantlets exposed to benomyl at 25 mg ai/l were free of both PVX and PVS. Benomyl has cytokinin-like activity. Cytokinins stimulate cell division (Leopold, 1964, Salisbury and Ross, 1978). Although the virus eradicative effect of the cytokinins is not well understood, it is apparently related to increased cell division since viruses probably have difficulty controlling RNA synthesis of actively dividing cells (Wang and Hu, 1980). Cytokinins may also stimulate the production of substances associated with resistance to virus infection, such as the resistance-associated proteins reported by Antoniw, **et al**. (1981).

PVX was not detected in most of the plantlets regenerated from either 0.5-mm shoot-tips excised from plantlets exposed to 2,4-D at 1.0 µM or from shoot-tips of both sizes from plantlets treated with GA_3 at 0.5 µM. The PVX-eradicative effect of the GA_3 and the 2,4-D at these concentrations probably have a similar explanation to that of the virus eradicative effect of Benomyl.

Table 12.- Number of virus-free Norchip potato plantlets regenerated by two sizes of shoot-tips from plantlets exposed to selected growth-regulating treatments.

TREATMENT		SHOOT-TIP SIZE (mm)	NUMBER OF PLANTLETS		
			REGENERATED+	PVX-free	PVS-free
2.4-D	0.1 µM	0.2	3	2	1
		0.5	4	1	0
2.4-D + darkness	0.1 µM	0.2	2	1	0
		0.5	5	1	0
2.4-D	1.0 µM	0.2	1	1	1
		0.5	4	3	1
GA_3	0.5 µM	0.2	5	3	1
		0.5	7	5	2
GA_3	1.0 µM	0.2	2	0	0
		0.5	7	2	0
Benomyl	25 mg ai/l	0.2	4	4	4
		0.5	7	2	0
Untreated	(dark)	0.2	6	2	3
		0.5	3	1	0
Untreated	(light)	0.2	2	2	2
		0.5	4	2	1

+ Total regenerated plantlets from seven shoot-tips excised for each size.

An average of 48.5% of the total regenerated plantlets were PVX-free while only 24.2% were PVS-free. This indicates that PVX could be eradicated somewhat more easily than PVS, except in the case of plantlets regenerated from 0.2-mm shoot-tips from plantlets either treated with benomyl at 25 mg ai/l or grown in darkness. PVS resistance to eradication has been reported by several authors (Mellor and Stace-Smith, 1968, 1970; Quak, 1977).

Small shoot-tip size (0.2 mm) favored virus elimination, especially for PVS. Out of 25 plantlets regenerated from 0.2-mm shoot-tips, 60% were PVX-free and 48% were PVS-free while 41.5% of 41 plantlets regenerated from 0.5-mm shoot-tips were PVX-free and only 9.8% PVS-free.

Most of the plantlets freed of PVS were also PVX-free, except for two isolated cases. This also indicates that PVS is more difficult to eradicate than PVX.

In spite of the potential of some treaments to eradicate PVX and PVS, it seems necessary to increase the number of replications to assure that eradication of these viruses was not a fortuitous event.

e.- Effect of plantlet size on eradication of PVX and PVS.

In order to evaluate the effect of plantlet size on virus elimination, plantlets were grouped by size classes, indicating the percentage of virus-free plantlets in each size class (Table 13). There is no indication that a relationship exists between plantlet size and virus eradication because the values do not follow a defined pattern. This indicates that the eradicative effect is

probably more related to some metabolic response to the treatments than to treatment effects on plantlet growth rate. In fact, the size of plantlets treated with benomyl at 25 mg ai/l did not differ from that of plantlets exposed to the untreated-light control (Table 6) and yet benomyl was the most effective treatment in virus eradication.

Table 13.- Effect of plant size on virus eradication through shoot-tip culture.

PLANTLET SIZE (mm)	TOTAL PLANTLETS REGENERATED	PERCENTAGE	
		PVX-free	PVS-free
1.00 - 10.99	15	46.6	26.7
11.00 - 20.99	24	58.3	29.2
21.00 - 30.99	10	28.6	30.0
31.00 - 40.99	11	45.5	9.1
41.00 - 50.99	4	50.0	25.0
51.00 - 60.99	1	0.0	0.0
61.00 - 70.99	1	0.0	0.0

CONCLUSIONS

GA_3 promoted growth of potato sprouts attached to seed pieces, regardless of concentration and exposure time. Growth promotion was likely due to increased cell division as well as elongation, and related in part to enhanced breakdown of reserve substances in the seed piece by induced starch-metabolizing enzymes.

An alternating temperature regime of 22° and 37° C reduced sprout growth in comparison to a constant 25° C. The relatively slow growth rate at 37° C might have been due to increased respiration which exhausted energy reserves.

Although treating sprouted seed pieces offers a convenient, rapid method of producing plants for shoot-tip culture, excess decay favored by high humidity made tuber pieces unsuitable for plantlet production in these studies. Seed pieces were therefore abandoned in favor of isolated disinfected sprouts and plantlets produced aseptically **in vitro** by nodal cutting.

Nodal cuttings from plantlets developed **in vitro** responded more obviously to growth-regulating treatments than those taken from sprouts. It seems possible that the growth-regulator balance was different in nodal cuttings from plantlets developed **in vitro**. Another possibility for varied growth response is that endogenous substances supplied by the seed piece to developing sprouts may have satisfied growth requirements, eliminating responses to additional materials supplied **in vitro**. Conversely, nodal cuttings from plantlets would depend heavily on the culture medium for growth-promoting substances.

2,4-D at 0.1 and 1.0 μM, and GA_3 at 0.5 and 1.0 μM promoted plantlet growth. Growth was also enhanced by inducing etiolation with darkness. In the absence of light, IAA is not rapidly degraded, therefore stem elongation is enhanced.

Plantlet growth was reduced when 2,4-D and BAP were added to the culture medium at concentrations of 10.0 μM and 100.0 μM, respectively. At these concentrations, callus growth, excessive branching, or both were observed. Growth was unorganized in the presence of 2,4-D and BAP at high concentrations, resulting mostly in callus tissue.

Except for the dark treatment, 0.5-mm shoot-tips regenerated more plantlets in less time than 0.2-mm shoot-tips, probably because larger shoot-tips were more differentiated and better able to withstand the stress of excision. Small shoot-tips probably relied more than larger ones on the culture medium for growth-regulating substances.

Contrary to the results for other treatments, 0.2-mm shoot-tips from etiolated plantlets survived better than 0.5-mm shoot-tips. It is possible that the concentration of endogenous auxin per tissue volume, the production of phenolic compounds induced by exposure to light, or both were higher when more tissue was excised and these induced development of callus tissue or death of the explant.

Small (0.2 mm) shoot-tips excised from plantlets grown in darkness grew slower than the rest of the treatments, but still survived well. It is probable that endogenous carbohydrate/energy reserves were low in the absence of photosynthesis. Etiolated shoot-tips probably took longer to grow because more time was

required for transformation of etioplasts to chloroplasts and subsequent chlorophyll synthesis.

Shoot-tips excised from plantlets treated with GA_3 at 0.5 and 1.0 µM and benomyl at 25 mg ai/l showed rapid growth and good survival. The stimulative effect of GA_3 and benomyl on cell division and differentiation apparently persisted in excised shoot-tips from treated plantlets. The effect of 2,4-D apparently also persisted, but in this case growth occurred primarily as callus tissue.

Comparison of the results of these studies with published reports on the use of thermotherapy to eradicate viruses shows that survival rate was increased and the time required for regeneration of plantlets was shortened by some treatments used herein. Plantlet stress at high temperature before shoot-tip excision may possibly explain the low and relatively slow rate of plantlet regeneration associated with thermotherapy.

In most cases, PVX was more readily eradicated than PVS. This trend has also been reported when thermotherapy has been used to eradicate these two viruses.

PVX was not detected in a high percentage of the plantlets regenerated from plantlets treated with GA_3 at 0.5 µM, regardless of the size of the excised shoot-tip. This suggests a PVX-eradicative effect of GA_3 at this concentration.

PVX and PVS were not detected in any of the plantlets developed from 0.2-mm shoot-tips excised from plantlets treated with benomyl at 25 mg ai/l; however, the viruses were present in a high percentage of the plantlets generated from 0.5-mm shoot tips. This indicates that benomyl probably has an eradicative effect on both

viruses but the effect depends somewhat on shoot-tip size, compared to the PVX-eradicative effect of GA_3 which seemed to be independent of shoot-tip size.

2,4-D had no effect on PVX or PVS levels in these studies.

PVS was apparently easier to eradicate than PVX with dark-induced etiolation when 0.2-mm shoot-tips were excised; however, no eradicative effect was shown with 0.5-mm shoot-tips. Treatment application (darkness) and excision were comparatively easy. Therefore excision of 0.2-mm shoot-tips from plantlets grown in darkness is potentially a good approach to virus eradication in situations, such as in developing countries, where experience with tissue culture techniques and specialized equipment are limited.

There is no apparent relationship between the size of the plantlet from which the shoot-tip is excised and virus eradication. The virus eradicative effect of these treatments is therefore probably related more to some effect on cell metabolism that interferes with virus replication than to stem elongation.

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