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GROWTH AND ROOT INHIBITION

IN AXENIC HYDRILLA VERTICILLATA CULTURES

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ABSTRACT

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IN AXENIC <u>HYDRILLA</u> <u>VERTICILLATA</u> CULTURES

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<u>Hydrilla</u> <u>verticillata</u> Royle is a submersed aquatic weed infesting waterways throughout the world. <u>Hydrilla</u> was introduced into the United States in 1960 and has spread across the southern states. <u>Hydrilla</u> has extensive reproductive capacity, spreading by seeds, axillary and subterranean vegetative buds, fragmentation, and stolons. Subterranean buds, called tubers, are the predominant form of reproduction. This reproductive potential has made mechanical, chemical, and biological control largely ineffective.

To study the life cycle of <u>Hydrilla</u>, Klaine and Ward (1981) developed a method for producing axenic cultures from tubers. The process by which axenic cultures were produced involves surface treatment of the tuber with sodium hypochlorite and removal of outer bud scales. Tubers were grown in a mineral salts media containing 150 ug/ml penicillin and 100 ug/ml streptomycin to prevent bacterial growth. Two percent glucose and 0.5 per cent casein were added to facilitate detection of bacterial contamination. Axenic cultures produced by this method have inhibited shoot and root growth. In the present study, different variables in the process were analyzed for inhibitory effects.

Addition of yeast extract, IAA, IBA, GA_3, and kinetin failed to overcome stem or root growth inhibition.

Neither NaOCl treatment nor dissection as separate factors affected growth or root development. Sodium hypochlorite treatment followed by dissection resulted in retarded growth at times, although mature plants did develop.

Addition of penicillin and streptomycin or penicillin, streptomycin, glucose, and casein resulted in inhibited growth and root production. Casein and streptomycin both inhibited stem and root growth in all cases. Glucose slightly enhanced growth. Penicillin had no significant effects on shoot growth or root formation and growth. Axenic cultures with penicillin and glucose exhibited no inhibitions.

Streptomycin inhibits growth of roots at 15, 50, and 100 ug/ml. Penicillin did not affect growth of stems or roots at 25, 75, or 150 ug/ml but did not effectively control bacteria.

Neomycin, ampicillin, tetracycline, and chloramphenicol were tested separately at 25, 75, and 150 ug/ml and in combinations at 75 ug/ml as possible alternatives to streptomycin. All were inhibitory to shoot growth. Root formation was significantly inhibited by all concentrations of chloramphenicol, and the higher concentrations of neomycin and tetracycline. Several of the antibiotic combinations, tested in conjunction with 25 ug/ml penicillin, were effective against bacteria but were inhibitory to hydrilla growth at tested concentrations.

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1.0 INTRODUCTION

1.1 Background and Problem

An axenic culture is one that contains only one living species. Axenic cultures are used to study nutrient requirements, understand environmental influences on growth, investigate the impact of an organism on the environment, and study the biochemical processes and interactions of the organism. These factors cannot be fully understood if any other organisms are present in the culture.

Hydrilla verticillata Royle is an aquatic weed that causes severe problems in waterways around the world. _Hydrilla_ produces numerous subterranean vegetative buds, called tubers, as its major means of reproduction. The buds are protected by the hydrosoil from mechanical and chemical control methods and thus serve to reinfest an area.

A step in controlling the production of tubers is to understand the environmental conditions conducive to tuber development. In studying this, Klaine and Ward (1981) developed a technique to produce axenic cultures of _Hydrilla_ from tubers. However, after germination, plant growth was stunted and roots did not form. The objective of this research was to determine the cause of stem growth and root formation inhibition in axenic cultures of _Hydrilla_.

The procedure developed for obtaining axenic cultures can be divided into three steps: disinfestation, dissection, and antibiotic treatment. Tubers are surface-treated with 5

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per cent sodium hypochlorite. Bud scales covering the tuber are removed by aseptic dissection. Tubers are grown in 10 per cent modified Hoagland's medium augmented with 200 mg/l sodium bicarbonate, 150 ug/ml penicillin, 100 ug/ml streptomycin, 2 per cent glucose, and 0.5 per cent pancreatic digest of casein. Tubers treated by this method are generally free of contaminants but stop growing by the end of three weeks and become senescent.

All three steps in the procedure could lead to or contribute to growth inhibition. Multiple factors in the medium could influence stem growth and/or root formation: glucose, casein, penicillin, and streptomycin. Not all tubers subjected to the procedure are axenic, but even contaminated tubers exhibit shoot growth and root formation inhibition.

To appreciate the importance of Hydrilla and the necessity of learning more about it, some knowledge of its background and the aquatic weed problem is needed. The origins of Hydrilla, its taxonomy, reproduction, and favored growing conditions will be presented. The problems created by aquatic weeds and the current techniques of aquatic weed control will be reviewed with emphasis placed on Hydrilla. As this work deals specifically with Hydrilla tubers, a detailed description of the tuber and information regarding vegetative bud formation and germination will be given. The method for producing axenic cultures will be detailed and

factors identified as potential inhibitors will be enumerated. Methods used for experiments, their results, and a discussion will follow.

1.2 Hydrilla verticillata Royle

Hydrilla verticillata Royle, commonly called hydrilla, is a submersed hydrophyte of the caulescent type belonging to the family Hydrocharitaceae. It was first introduced into the United States in Florida about 1960. By 1969 it infested over 60,000 ha of water in the southeastern United States (Blackburn and Weldon, 1969). Hydrilla is thought to be native to the warm regions of Asia. It is found throughout Asia, Europe, Africa, Australia, New Zealand, North America, and the South Pacific Islands (Cook and Luond, 1982). Reports vary as to its presence in South America. Holm et al. (1969) report that hydrilla was introduced to the United States from South America, but Cook and Luond (1982) say the plant does not occur in South America.

Hydrilla grows in dense mats that clog waterways, interfering with navigation, recreation, and flood control (Reid, Martin, and Young, 1975). Hydrilla is capable of spreading by several methods. This makes control of its populations difficult. The primary means of reproduction is through vegetative buds. Seeds are produced although this is not thought to occur in the U.S. It can also survive when fragmented or uprooted (Martin, Doig, and Millard, 1971).

Hydrilla also spreads by growth of stolons above and rhizomes below the surface of the hydrosoil (Steward, 1969).

1.2.1 Taxonomic Characteristics

The species _Hydrilla verticillata_ shows a great deal of anatomical variation. This variation often results in it being misclassified. Leaves can be in whorls of 2 to 12. Leaf length varies from 2 to 25 mm. Leaf width ranges from 1 to 4 mm. Leaves may be thick and dark green or reddish, or thin and pale green. Nodes may be densely spaced or up to 80 mm apart (Cook and Luond, 1982). Leaves of hydrilla are verticillate and narrowly lanceolate. Lower leaves are oppositely arranged while upper leaves are in whorls of 4 or 8 (Blackburn and Weldon, 1969). Hydrilla observed at Lake Conroe, Texas, the collection site for this research, has a mean internodal length of 24 mm. Each node carrys from 2 to 6 leaves. Leaf length varies from 2 to 17 mm.

Hydrilla is frequently found as either diploid or triploid. In the normal diploid, 2n = 16. Triploid plants are 2n = 24 (Larsen, 1963). Cook and Luond (1982) report the presence of tetraploid plants (2n = 32) in Alabama.

1.2.2 Means of Reproduction

Hydrilla reproduces both sexually and asexually. However, even in areas in which both pistillate and staminate flowers occur, asexual reproduction predominates (Basiouny et

al., 1978). Hydrilla reproduces asexually through vegetative buds and fragmentation. When fragmented, hydrilla produces adventitious roots at stem nodes enabling it to reroot. Vegetative buds are produced both above and below the hydrosoil. Buds formed in the leaf axis are referred to in the literature as axillary turions or turions. Buds formed on the apices of positively geotropic stems are called tubers (Pancho and Soerjani, 1978). It should be noted that these are not true tubers but are dormant vegetative buds. In an infested area, 3×10^6 tubers and turions may be produced per ha. Tubers form up to 18 cm deep in the hydrosoil. These propagules are a major means of reinfestation after chemical treatment as well as a means of overwintering (Basiouny et al., 1978).

Hydrilla is monecious, staminate flowers and pistillate flowers are borne on separate plants. The small flowers have three petals and three sepals streaked with white or red. Pistillate flowers may develop up to five brown, fusiform seeds about 2.5 mm long (Cook and Luond, 1982).

1.2.3 Growth and Physiology

Water quality seems to have little influence on hydrilla growth. It is found in brackish, oligotrophic, eutrophic, and strongly alkaline waters (Cook and Luond, 1982). Hydrilla growth does decrease with increasing salinity of the water. Salinities of greater than 6.66 per cent are toxic to hydrilla (Haller et al., 1974).

Water transparency has proved to be a more important factor in hydrilla growth than temperature, O_2, CO_2, total hardness, alkalinity, total nitrogen or phosphate concentration. Increased water transparency, and the corresponding increased light, is positively related to hydrilla growth (Blackburn et al., 1968). Hydrilla responds to low light levels or high temperature with shoot elongation and subsequent canopy formation. With increased shade, shoot number decreases. Temperature affects biomass production and carbon metabolism more than light, with low growth occurring at low temperatures. Depth distribution may be predominately influenced by light; however, the increased shoot elongation at low light levels may decrease its significance (Barko and Smart, 1981).

Hydrilla does not grow in some waters that support other aquatic macrophytes. A factor that inhibits hydrilla growth has been isolated from the sediment extracts of a Florida lake that does not support hydrilla growth. The inhibitor has been broadly characterized as an anionic moiety with physical and chemical characteristics similar to humic substances (Dooris and Martin, 1980). In another Florida lake the presence of cypress sawdust apparently prevents the growth of hydrilla. Addition of sawdust or cationic exchange resin to hydrilla cultures causes death of the plants (Martin et al., 1971).

Nutritional requirements for hydrilla growth under laboratory conditions have been studied. Most of the

researchers have used Hoagland's medium (Hoagland and Arnon, 1938) or modified Hoagland's medium (Table 2.1). Steward and Elliston (1973) found that 10 per cent Hoagland's medium augmented with sodium bicarbonate gave optimal hydrilla growth.

1.3 Aquatic Weeds

Hydrilla is only one of a number of aquatic plants whose large populations cause problems for man. To understand the importance of hydrilla it is necessary to look at it in terms of the entire aquatic weed problem. The factors leading to excessive growth, both natural and man-made, competitive advantages of certain weeds, and current control methods must be considered.

1.3.1 Role of Aquatic Plants in the Environment

Aquatic plants are a necessary part of the ecosystem. They provide food and concealment for insects, fish, and other wildlife (Andres and Bennett, 1975). However, large populations of aquatic weeds can block or hinder navigation in infested waterways. They can interfere with irrigation by retarding water flow, intensifying evaporation and water loss through transpiration (Holm et al., 1969).

Aquatic weeds may be divided into six groups based on size, shape, and habitat. Two groups, plankton and filamentous, pertain to algae. The higher plants make up the other four groups. Emersed weeds are rooted in the hydrosoil

but most of the plant is above the water surface. Submersed weeds are rooted in the muddy bottom and most of the plant is below the water surface. Floating plants drift on the water's surface. These plants have roots but are not anchored to the bottom (Lawrence and Weldon, 1965). Some researchers class aquatic plants as either submersed or emersed. Floating weeds are then referred to as emersed (Gallagher, 1970).

Aquatic growth is influenced by physical factors such as light intensity, water quality, temperature, water depth, flow velocity, and wave action. Size, shape, type of substratum, and slope of bottom also affect the types and numbers of aquatic plants present. Submersed aquatic vegetation usually grows prolifically in shallow, nutrient-rich waters (MacKenthum and Ingram, 1967).

Man's activities have affected aquatic weed growth. Nutrient enrichment of waters from industrial and municipal wastes and agricultural run-off may increase vascular plant populations (Boyd, 1970). Man has also aggravated the aquatic weed problem by building dams, canals, and ditches. Dams supply calm water for weed multiplication and prevent rain surges from washing weeds seaward. Canals and ditches link bodies of water that were previously secluded providing avenues for spreading. Many plants that are imported as ornamentals or aquarium plants end up as problem aquatic weeds (Zettler and Freeman, 1972). Often in being moved from their native environment they are separated from natural

competition, disease, and predation that may control their population.

Submersed and free-floating weeds are considered to cause the most problems although it is debated which of these two classes is the most serious threat (Andres and Bennett, 1975). Sculthorpe (1967) sees floating weeds as the major problem on a world-wide basis. Holm et al. (1969) view submersed weeds as more important as they are more difficult to control.

1.3.2 Competitive Advantages of Hydrilla

Problem aquatic weeds are usually those which are adapted to rapid growth and efficient reproduction. Often other plants are unable to compete in growth rate or the growth of the weed inhibits their growth. Hydrilla is such an aquatic weed. Much of hydrilla's success over other submersed plants is due to the formation of a dense canopy near the water surface and efficient utilization of light. Nearly 20 per cent of hydrilla's biomass is in the top 10 cm of water. This canopy limits light penetration inhibiting the growth of most other plants. Hydrilla is able to take advantage of the lowered light levels as 87 per cent of its biomass is photosynthetic standing crop (Haller and Sutton, 1975). In addition, the photosynthetic light saturation level is lower than for many other aquatic plants. Van, Haller, and Bowes (1976) found the photosynthetic light saturation level to be between 600 and 700 uEinsteins/m^2s

while Barko and Smart (1981) report a level of 1050
uEinsteins/m^{2}s. In addition Van, Haller, and Bowes (1976)
discovered hydrilla has a lower light compensation point than
aquatic plants such as <u>Ceratophyllum</u> <u>demersum</u> and
<u>Myriophyllum</u> <u>spicatum</u>. Light and CO_2 saturated photo-
synthetic rates for all three species are from 50 to 60
umoles O_2/mg chlorophyll at 30 $^\circ$C.

Dark respiration, photorespiration, and net and
apparent photosynthesis of hydrilla, <u>Potamogeton</u> <u>pectinatus</u>,
and <u>Vallisnera</u> <u>spiralis</u> were compared. Hydrilla had the
lowest dark and photorespiration rates and greatest apparent
photosynthetic rate. The low respiration rates and the high
photosynthetic rate are thought to contribute to the
dominance of hydrilla in lakes where the three species occur
(Jana and Choudhuri, 1979).

1.3.3 Methods of Aquatic Weed Control

Ideally four steps should go into the solving of an
aquatic weed problem: 1) determination of the amount of
vegetation needed to maintain the ecosystem, 2) calculation
of the minimum amount that interferes with man's use of the
water, 3) selection of an amount of aquatic vegetation that
can satisfy wildlife and man, 4) and choosing of a method of
weed control that is economical and satisfies the first three
steps. Generally too little is known to consider all four
steps completely (Sculthorpe, 1967).

There are three basic types of aquatic weed control; mechanical, chemical, and biological. Mechanical control includes such processes as cutting or mowing of weeds. Application of herbicides is the usual means of chemical control. Biological control, or biocontrol, involves the use of either animals that feed upon aquatic plants or plant pathogens. Each method has advantages and disadvantages.

Mechanical methods remove the plant from the water preventing nutrient recycling and eutrophication but generally leave enough plant behind for reinfestation. Mechanical control is limited by weed disposal problems as well as increasing maintenance and operating costs. Control is often inefficient with only limited success.

A possible solution to the weed disposal problem is the use of plants as cattle forage or fertilizers. However, water content and harvest method may be a constraint. Only 8 per cent of hydrilla is dry matter. Hydrilla has enough crude protein, essential amino acids, and total available carbohydrates on a dry weight basis to potentially serve as a forage source. It does not have a high enough nitrogen and potash concentration on a fresh weight basis to use as a green manure (Boyd, 1969).

Currently most aquatic weed control is done chemically. Large amounts of herbicides are needed for aquatic weeds because of dilution. The amount of herbicides used can cause problems economically and environmentally. Rising cost of chemicals can make treatment of a large area cost-exorbitant.

Toxicity to fish and other wildlife is also a concern. If the water to be treated is a drinking water supply, chemical residues may be particularly undesirable.

Experimentation has been done to test herbicide application methods that give more efficient hydrilla control. Treatment of a portion of a body of water decreases the amount of herbicide used and gives temporary control (Bitting, 1970). Use of oil emulsions aids absorbance of the herbicide through the plant cuticule (Gates, 1972). Application of the herbicide to lake bottoms gives effective control with an 85 per cent reduction in the amount of herbicide needed for control (McClintock, Frye, and Hogan, 1974). Lake draw down in combination with herbicide treatment has been used to control hydrilla in Louisiana (Johnson and Manning, 1974).

Blackburn and Weldon (1969) did laboratory tests on 75 herbicides and found a combination of diquat and copper sulfate to be most effective for hydrilla control. In the field, acrolein, endothall salts, aromatic solvents, and copper sulfate proved to be effective. Haller and Sutton (1973) found that the addition of low copper concentrations, 0.4 to 2.0 uM, increased endothall uptake by hydrilla.

Blackburn, Boyer, and Timmer (1971) showed that herbicide form influences its efficiency. Controlled-release pellets of hydrothall are more effective for hydrilla control than liquid, technical, or granular hydrothall or commercial

endothall. The controlled-release pellets also give longer control with reduced fish toxicity and residue levels.

Biological control has low overhead with low costs and little or no maintenance. The major concern with biological control agents is population control. Control agents may either overpopulate or not reproduce requiring frequent restocking. Another potential problem is the possibility of eradication of all vegetation in an area or of a desired species. Classical biocontrol is used for large populations of one species. Polyphagous organisms, such as herbivorous fish or snails, may be used for control of several species (Andres, 1977). Manatees and snails have both been used for control agents. Manatees, Trichechus manatus, rapidly clear weeds but are slow to reproduce and are restricted to warm waters (Gallagher, 1970). The snail, Marisa cornvarietis, is also limited to use in warm climates. In addition it eats virtually anything, including aquatic crops such as rice (Andres and Bennett, 1975).

One of the more prominent and controversial biocontrol agents is the grass carp, Ctenopharyngodon idella. An herbivorous fish native to China, the carp eats a number of aquatic weeds including submersed, emersed, and floating weeds. The carp will live in waters of both high and low temperatures (Andres and Bennett, 1975). Introduced to the U.S. in 1963 for aquatic weed control, controversy over use of the carp generally surrounds the possibility of its natural reproduction and the effects of this on the aquatic environment (Sutton, 1977).

Tests have shown carp to be effective in hydrilla control. Small carp consume 100 to 200 per cent of their own body weight in hydrilla per day. Large carp, weighing 6 kg or greater, consume 25 to 28 per cent of their body weight. Hydrilla control is effective with a carp density of 130 kg fish/ha (Shireman and Maceina, 1981).

The use of plant pathogens as a means of biological control of aquatic weeds has largely been overlooked. Pathogens are diverse, numerous, host-specific, self-maintaining, and nonpathogenic to animals (Freeman, 1977). Potential pathogens for hydrilla have been isolated from hydrilla in India. These pathogens include six fungi and one type of bacteria (Charudattan, 1973).

1.4 Dormant Vegetative Buds of Aquatic Plants

Axenic cultures are obtained by the treatment of hydrilla tubers. Therefore, a knowledge of tubers is needed. An understanding of factors affecting germination is also essential if cultures are to sprout and grow. This section will give a detailed description of hydrilla tubers and will review vegetative bud formation and germination in hydrilla and other aquatic plants.

1.4.1 Description of Hydrilla Tubers

The tuber is a condensed axis consisting of 12 to 15 internodes with thick, fleshy leaves at each node. At the distal end is a meristematic area completely surrounded by

leaves or bud scales (Lakshmanan, 1951). During tuber formation the meristem of the positively geotropic stem stops growing. Starch accumulates in the bud scales and in the cells just behind the meristem. This meristematic and starch-rich area of the stem is the tuber (Steward, 1969). Starch is the major component of tubers (46.80 per cent dry weight). Sucrose is also found along with trace amounts of reducing sugars, lipids, and nitrogen. Calcium and potassium ions are the major inorganic constituents (Miller et al., 1976).

Tubers are relatively resistant to drying. Tubers sprout even with moisture losses of up to 50 per cent. A 50 per cent moisture loss is equivalent to removal from water for periods of up to 64 hours at 30°C and 40 per cent relative humidity. Resistance to drying may contribute to the spread of hydrilla via boats, boat trailers, etc. (Basiouny et al., 1978a).

1.4.2 Vegetative Bud Formation

In growth chambers hydrilla forms tubers with photoperiods of less than 13 hours and at temperatures from 14 to 33°C. Lowering temperatures to 9°C prevents tuber formation even if photoperiods are shortened to 10 hours. Addition of abscisic acid, ABA, to plants results in tuber formation even if plants have not been subjected to tuber formation conditions (Van, Haller, and Garrard, 1978). Klaine and Ward (1983) found that with short photoperiods

tuber formation can be prevented by night interruption with red light. This inhibition is overcome if the red light exposure is followed by far-red light. Tubers were also produced with long photoperiods if exposed only to blue or green light.

Turion formation in Spirodela polyrrhiza is prompted not only by changing photoperiods but also by variation of light intensities, night and day temperatures, and the nitrate concentration of the media (Perry, 1968). Turion formation in Myriophyllum verticillatum is also controlled by photoperiod. Three conditions must be met to initiate formation of its turions. Short photoperiods that have been preceded by long photoperiods are needed in conjunction with temperatures of $15^{\circ}C$ or lower. The combination of these three conditions insures that the turion, which is for overwintering, forms during the fall rather than in the cool, short days of spring. Abscisic acid treatment slightly stimulates turion formation under these conditions (Weber and Nooden, 1976).

1.4.3 Vegetative Buds Germination

Vegetative bud dormancy is usually broken by cold treatment or hormone application rather than change in photoperiod. Cold treatment alone breaks dormancy in both hydrilla tubers and turions (Sastroutomo, 1980, and Basiouny, et al., 1978b). There is an apparent physiological difference between tubers collected in the winter and summer.

Winter tubers require chilling at 5 °C for a minimum of 288 h before 100 per cent germination is obtained, even in conjunction with other treatments. Summer tubers will sprout without chilling if treated with the proper hormones (Basiouny et al., 1978b).

Dormancy in noncold-treated turions is broken by photoperiods of 16 h. Continuous lighting or photoperiods of 8 to 12 h do not induce sprouting. Cold-treated turions subjected to red or far-red light will germinate but will not if exposed to blue or green light (Sastroutomo, 1980). Tubers sprout in continuous light and grow normally (Basiouny et al., 1978a).

Steward (1969) investigated the influence of several herbicides on hydrilla tuber and turion germination. Diquat, paraquat, endothall, and 2,4-D amine were tested. Herbicides had a positive effect on noncold-treated winter tubers although none increased germination above 55 per cent. Tests were also performed with tubers and turions collected in April. Germination and growth were both inhibited by 2,4-D amine. Growth but not germination of turions was retarded by diquat. Endothall had no significant effects. Differences between winter and spring collected propagules were attributed to the degree of dormancy.

Combinations of chilling and hormonal treatment are required for germination of turions of Spirodela polyrrhiza and Potamogeton sp. S. polyrrhiza turions germinate in response to either chilling or gibberellic acid treatment and

long photoperiods (Perry, 1968). Sastroutomo (1981a) has shown that various combinations of temperatures and plant hormones influence germination of turions of the pondweed species, Potamogeton. Germination of nondormant turions is influenced by water temperature but not by light intensity. Dormant turions are induced to germinate by exposure to warm temperatures for two weeks or by cold treatment for one week. Treatment of the turions with gibberellic acid, GA_3, and indoleacetic acid, IAA, also breaks dormancy. Sastroutomo (1981b) found that turions of P. berchtoldii require cold treatment for 35 days to break dormancy. Varying photoperiods will not induce turions to germinate.

1.5 Analysis of Axenic Treatment for Possible Inhibitory Effects

Each part of the axenic treatment was evaluated to determine its effects on hydrilla growth. Antibiotic presence, dissection, NaOCl treatment, and axenicity were all considered as possible inhibitory agents. Glucose and casein were thought to be more likely beneficial than inhibitory unless they were interacting with some other inhibitory agent.

1.5.1 Commensal Relationships with Bacteria

Some plants have commensal relationships with bacteria. It was theorized that if hydrilla had such a relationship, the elimination of bacteria from the cultures might inhibit

growth. Nonaxenic tubers from treated cultures would also be inhibited if commensal bacteria were susceptible to NaOCl treatment, penicillin, or streptomycin.

Microorganisms may produce and release into the water vitamins or enzymes that can be utilized by plants. The genera Pseudomonas, Aeromonas, Vibrio, Bacillus, Micrococcus, and Azotobacter synthesize and apparently excrete vitamin B_{12}. Thiamine, biotin, riboflavin, pantothenic acid, nicotinic acid, and folic acid may also be released into water by microorganisms. Phosphatases, saccharases, and amylase are synthesized by algae and bacteria and are discharged into the water upon death of the organism (Rheinheimer, 1980).

Epiphytic bacteria that produce IAA have been isolated. Addition of these bacteria to sterile plants increases plant growth by raising auxin levels in the plant (Libbert et al., 1968). Wichner and Libbert (1968) found that IAA is produced from tryptophan by both the homogenate and washings from non-sterile parts of Pisum sativum while preparations from sterile plants produce no measurable IAA. Sterile filtration and addition of some bactericidal agents stop IAA production indicating epiphytic bacteria associated with the plant do to the actual conversion of trytophan to IAA.

1.5.2 Sodium Hypochlorite Treatment and Tuber Dissection

It was thought that sodium hypochlorite, a powerful oxidant, could damage tubers causing inhibition. While tubers subjected only to sodium hypochlorite (NaOCl) treatment show no indication of retarded growth, NaOCl treatment in conjunction with some other factor might inhibit growth. Meristematic areas could be affected directly or the hormonal or enzymatic balance of the germinating tuber could be upset.

Tuber dissection was also examined as a possible cause of stem and root growth inhibitions. Dissections could damage meristematic areas preventing normal growth. Disrupted hormonal or enzymatic balances might also lead to inhibition. Removal of bud scales and portions of the axis could reduce starch and nutrient supplies to a critical level. Remaining nutrient supplies may not be large enough to sustain the plant until it becomes self-sufficient.

1.5.3 Presence of Penicillin and Streptomycin

Antibiotics are known to affect plant growth in various, often inhibitory, ways. Much of the variation is due to the use of different plants, concentrations, and application methods in testing. These known inhibitory effects made the presence of two antibiotics, penicillin and streptomycin, very suspect in the inhibition of axenic

hydrilla cultures. The literature review showed streptomycin to be particularly inhibitory to plant growth.

Effective levels of streptomycin sulfate vary with plant species. While streptomycin concentrations greater than 50 units/ml (70 ug/ml) are toxic to tomato and radish seeds, wheat can tolerate concentrations of 200 units/ml (280 ug/ml) with no apparent injury (Anderson and Nienow, 1947).

Some of streptomycin's effects are the result of its action on chloroplasts and mitochondria. Brian (1957) proposed that streptomycin affects chloroplast development as mature chloroplasts (containing chlorophyll) are generally not affected by streptomycin. However unpigmented chloroplasts, as in etiolated plants, do not produce chlorophyll when placed in light if streptomycin is present. Zamski and Umiel (1982) found that when sensitive tobacco strains are exposed to streptomycin, chloroplasts have reduced thykaloid, starch grain, and lipid droplet formation. In the mitochondria, there is general degradation and swelling of the cristae.

Both penicillin and streptomycin influence photosynthesis in hydrilla. Penicillin inhibited photosynthesis by 35 to 40 per cent at 100 ppm but had no effect at 0.01 ppm or lower. Streptomycin inhibited photosynthesis by 12 to 20 per cent at 10 ppm. At 0.01 ppm streptomycin stimulated photosynthesis by 8 to 12 per cent (Dubash and Zutshi, 1959).

1.6 Research Objectives and Plan

Axenic cultures are needed to study the physiology, life cycle, and environment interactions of hydrilla. A method of obtaining axenic cultures has been developed; however, axenic hydrilla produced by this method exhibit stem growth and root formation inhibition. The primary objective of this research is to determine the cause or causes of these inhibitions. A secondary objective is to find a way to produce axenic hydrilla that is not inhibited. Uninhibited axenic hydrilla cultures could be obtained by avoiding procedural steps that cause inhibition, finding an alternative way to produce axenic cultures, or determining a way to overcome inhibitory effects.

The procedure for obtaining axenic cultures was analyzed. Three components of the procedure were determined to potentially have inhibitory effects. These were NaOCl treatment, dissection, and presence of penicillin and streptomycin. The possibility of a necessary commensal relationship with a bacterium was also considered. At this time, casein was not considered as a possible inhibitory agent unless it was interacting with some other factor.

A series of experiments was designed to test each of these components, their effects on hydrilla growth, and their interactions with each other. In addition, two series of experiments were done to attempt to overcome inhibition. Yeast extract was added to axenic cultures to determine if

inhibition was due to a nutrient deficit. Various plant growth hormones were tested. It was thought that the hormones might correct inhibition if it were caused by a hormonal imbalance or override the effects of the inhibitory agent. No tests were ever done that actually tested the relations of tuber growth and indigenous bacteria. This experiment was deemed unnecessary by the production of axenic cultures that grew normally.

2.0 MATERIALS AND METHODS

2.1 Tuber Collection and Storage

Tubers were collected from two sites. Most were collected from Lake Conroe, Texas, off the Walden golf course. This area was not subjected to herbicidal treatment for hydrilla control. When this site was inaccessible due to high water, tubers were collected from a small pond at Rice University. This pond was originally stocked with hydrilla from Lake Conroe. Incoming pond water was run through an activated carbon column to remove chlorine. Flow was approximately 2 to 3 ml/s.

For collection, hydrosoil was removed and sieved to find tubers. Tubers from Lake Conroe were collected approximately 0.3 to 5.0 m from shore. Hydrosoil was removed with post-hole diggers and was placed on a wire screen with a wood and styrofoam frame for sieving. Pond hydrosoil was in aluminum pans. Pans were removed and soil sieved for tuber

collection. Tubers were washed in deionized water. Clean tubers were wrapped in moist paper towels and placed in plastic bags. Tubers were stored at 5 °C for two to three weeks prior to use.

2.2 Growth Medium

A mineral salts solution, modified Hoagland's medium, was used at 10 per cent full strength. The medium was made using reagent grade chemicals and deionized water run through an activated carbon column to remove residual chlorine. Inorganic carbon was supplied by 200 mg/l sodium bicarbonate, $NaHCO_3$ (Table 2.1).

2.3 Culture System

Tubers were grown in Pyrex storage jars, 100 x 60 mm, with glass covers. Continuous lighting was provided by light banks of cool white fluorescent lamps. Intensity was 120 to 130 uEinsteins/m^2s as measured by a Lambda LI-170 quantum radiometer photometer. Temperature was not controlled but remained in the 26 to 30 °C range.

2.4 Production of Axenic Cultures

Tubers were treated with 5 per cent NaOCl for 20 minutes. Outer bud scales surrounding the meristematic area and axis were removed using sterile forceps and scalpels. Dissected tubers were placed in sterile modified Hoagland's

TABLE 2.1

CONSTITUENTS OF HOAGLAND'S MEDIUM

SALT	g/l	ELEMENT	PPM
$Ca(NO_3)_2 \cdot 4H_2O$	1.1811	Ca	200.17
KNO_3	0.5055	N	154.19
KH_2PO_4	0.1361	K	234.39
$MgSO_4 \cdot 7H_2O$	0.4930	Mg	48.10
NaCl	0.5850	S	64.45
Fe(EDTA)	0.5040	P	31.00
H_3BO_3	0.0029	Na	23.40
$MnSO_4 \cdot H_2O$	0.0015	Cl	35.60
$ZnSO_4 \cdot 7H_2O$	0.0002	Fe	5.00
$CuSO_4 \cdot 5H_2O$	0.0001	B	8.52
H_2MoO_4 (85%)	0.0001	Mn	0.48
		Zn	0.05
		Cu	0.02
		Mo	0.01

medium augmented with 200 mg/l $NaHCO_3$, 150 ug/ml penicillin, 100 ug/ml streptomycin, 2 per cent glucose, and 0.5 per cent pancreatic digest of casein. Gentamycin and mycostatin, used in the original procedure, were not used in this study. The pH of the medium was adjusted to 6 using sterile 1.2 N hydrochloric acid.

All transfers and treatments were done in an ultraviolet light box (Sylvania-Germicidal G 30+8). Glassware was washed in phosphate-free detergent and rinsed in dilute hydrochloric acid followed by deionized water. All equipment and glassware was autoclaved for 20 minutes at 18 psi and $121\,^{\circ}C$.

Generally, 70 per cent of tubers treated in this manner are axenic. Treated tubers refers to tubers that have undergone this process but are not necessarily axenic. As an additional check of axenicity, two 1 ml aliquots were taken from each culture at the end of an experiment. Aliquots were placed in thioglycolate broth and incubated at $23.9\,^{\circ}C$ for three days.

2.5 Analysis of Plant Growth

Total stem length was measured in millimeters (mm) on alternate days for 21 days. Presence of roots was noted. Final length was based on the mean stem length of hydrilla in an experimental set on the twenty-first day. Non-germinated tubers were not considered in calculating length or fraction that produce roots.

Differences in final length were analyzed using Analysis of Variance for Means (ANOVA). Chi square analysis was used for differences in root presence and germination (Zar, 1974). Analysis was based on actual number in a group and not the fraction that germinate or fraction that produce roots. The 5 per cent level of probability was considered to represent statistical significance.

2.6 Chemical Acquisition

Benzyl penicillin G, tetracycline, chloramphenicol, ampicillin, indoleacetic acid, indolebutyric acid, gibberellic acid, and kinetin were obtained from Sigma. Streptomycin sulfate was a Pfizer product. Neomycin was obtained from Dr. D. Moore, University of Texas Medical Center. Casitone, pancreatic digest of casein, was from Difco. Thioglycolate broth was a Baltimore Biological Laboratories product. Glucose was from Matheson, Coleman and Bell. No purity or characterization tests were made.

3.0 EXPERIMENTAL PROCEDURES

3.1 Addition of Yeast Extract

Control tubers were prepared following the procedure for axenic cultures. Experimental tubers were prepared in the same manner except for the addition of 0.5 per cent yeast extract to the media prior to sterilization.

3.2 Hormonal Treatments: IAA, IBA, GA_3, and Kinetin

Indoleacetic acid (IAA), indolebutyric acid (IBA), gibberellic acid (GA_3), and kinetin were tested. Stock solutions for each were made, filter-sterilized, and added to sterile media. With this exception the procedure for axenic cultures was followed. As a check for general vigor and viability, a background set of tubers treated only with NaOCl was grown. Concentrations tested were: 10^{-5}, 10^{-8}, 10^{-10} M IAA; 10^{-7} and 10^{-10} M IBA; 10^{-6} and 10^{-9} M GA_3; and 10^{-7} M kinetin. Only IBA and GA_3 tubers were collected the same day. Experiments were not run simultaneously.

3.3 Effects of NaOCl Treatment and Tuber Dissection

One set of tubers was rinsed in sterile water, dissected, and placed in 10 per cent modified Hoagland's medium. Another set was treated with NaOCl for 20 minutes before being rinsed and placed in media. No organics or antibiotics were used in either set.

3.4 Effects of Progressive Steps of Axenic Treatment

Four sets of tubers were prepared. One set was treated with NaOCl and was grown without antibiotics or organics. The remaining tubers were treated with NaOCl and dissected. The second set was also grown without antibiotics or organics. Set three had 150 ug/ml penicillin and 100 ug/ml streptomycin. The fourth set was grown with 2 per cent

glucose and 0.5 per cent casein in addition to penicillin and streptomycin.

3.5 Interactions of Antibiotics and Organics

A two-tiered experiment was designed. The first tier tested antibiotic presence. Groups had either 150 ug/ml penicillin, 100 ug/ml streptomycin, or both. The second tier tested glucose at 2 per cent and casein at 0.5 per cent. Each antibiotic group was tested with glucose, casein, or both. A control set was grown with no organics or antibiotics present. All tubers were subjected to NaOCl treatment and dissection. A complete factoral experiment was not done due to a lack of tubers.

3.6 Effects of Penicillin and Streptomycin
Concentrations

A complete factoral experiment was designed to test the effects of different antibiotic concentrations. Penicillin was tested at 0, 25, 75, and 150 ug/ml. Streptomycin was used at 0, 15, 50, and 100 ug/ml. No organics were used. All tubers were first treated with NaOCl and dissected. The set with no antibiotics present was considered the control.

3.7 Toxicity Tests of Four Alternative Antibiotics

Toxicity tests of neomycin, ampicillin, tetracycline, and chloramphenicol were done. Each was tested at 0, 25, 75, and 150 ug/ml. Tubers were treated with NaOCl and dissected.

Media was augmented with 0.2 per cent glucose in addition to the antibiotic. A control set with no antibiotic or glucose was grown.

3.8 Effects of Antibiotic Combinations on Growth and Axenicity

Simultaneous to the toxicity tests, a complete factoral experiment of all combinations of neomycin, ampicillin, tetracycline, and chloramphenicol was done. All were tested at 75 ug/ml. All cultures contained 0.2 per cent glucose and 25 ug/ml penicillin. A set with penicillin and glucose only was the control. All tubers were treated with NaOCl and dissected.

4.0 EXPERIMENTAL RESULTS

4.1 Yeast Extract and Hormones

Addition of yeast extract to axenic cultures did not significantly alter the inhibited growth and root formation of hydrilla (Table 4.1).

None of the hormones tested overcame growth inhibition at the concentrations tested. Length was slightly increased by GA_3 (Table 4.2).

4.2 NaOCl Treatment and Dissection

Growth of hydrilla was not retarded by either NaOCl treatment or dissection. Root formation was 100 per cent in

TABLE 4.1

EFFECTS OF YEAST EXTRACT ON GROWTH OF AXENIC HYDRILLA

CULTURES. GROWTH INDICATORS ARE GIVEN IN TERMS OF LENGTH

(mm) ± STANDARD DEVIATION, FRACTION THAT GERMINATE, AND

FRACTION THAT PRODUCE ROOTS.

TREATMENT	LENGTH	GERMINATION	ROOTS
Axenic Cultures	24 ± 8(14)[a]	0.79	0.00
Axenic Cultures with Yeast Extract	15 ± 13(22)	0.68	0.00

[a]
    Number of replicates

TABLE 4.2

EFFECTS OF HORMONAL TREATMENTS ON GROWTH OF AXENIC HYDRILLA CULTURES. GROWTH INDICATORS ARE GIVEN IN TERMS OF LENGTH (mm) $\pm$ STANDARD DEVIATION AND FRACTION THAT GERMINATE.

TREATMENT	LENGTH	GERMINATION
Indoleacetic Acid		
10^{-5} M	$17 \pm 11(15)^{a}$	1.00
10^{-8} M	$17 \pm 4(15)$	0.71
10^{-10} M	$17 \pm 7(15)$	0.83
Background Set	$212 \pm 97(10)^{*}$	0.83
Indolebutyric Acid		
10^{-7} M	$20 \pm 2(15)$	1.00
10^{-10} M	$17 \pm 7(15)$	1.00
Background Set	$152 \pm 38(10)^{*}$	1.00
Gibberellic Acid		
10^{-6} M	$33 \pm 12(15)$	1.00
10^{-9} M	$27 \pm 8(15)$	1.00
Background Set	$152 \pm 38(10)^{*}$	1.00
Kinetin		
10^{-7} M	$7 \pm 6(11)$	0.67
Background Set	$50 \pm 40(10)^{*}$	0.90

[a] number of replications

* difference from all others in set is significant at the 5% level

both sets (Table 4.3). Dissection of tubers following NaOCl treatment may slightly retard growth but does not totally inhibit it. Root formation is not affected (Table 4.4).

4.3 Effects of Organics and Antibiotics on Hydrilla Growth

Addition of penicillin and streptomycin or penicillin, streptomycin, glucose, and casein significantly inhibit growth of NaOCl-treated, dissected tubers. Root formation in cultures containing both antibiotics and organics is significantly lower than in cultures with antibiotics alone (Table 4.4).

Presence of glucose significantly enhances the growth of hydrilla regardless of antibiotic or casein presence although inhibition is not overcome. Root formation does not appear to be affected. However, casein inhibits root formation. It also appears to have an inhibitory effect on growth (Table 4.5).

Growth and root formation of cultures with penicillin and/or streptomycin are statistically the same when analyzed without regard to organic content or axenicity (Table 4.6). When data are broken down by organic content and bacterial contamination differences between cultures with penicillin and streptomycin are apparent. Penicillin in combination with glucose shows no inhibitory effects on hydrilla stem or root growth in uncontaminated cultures. Streptomycin inhibits stem and root growth in all cases. Bacterial

TABLE 4.3

EFFECTS OF NaOCl TREATMENT AND DISSECTION ON HYDRILLA GROWTH.
GROWTH INDICATORS ARE GIVEN IN TERMS OF LENGTH (mm) $\pm$
STANDARD DEVIATION, FRACTION THAT GERMINATE, AND
FRACTION THAT PRODUCE ROOTS.

TREATMENT	LENGTH	GERMINATION	ROOTS
NaOCl Treatment	125 $\pm$ 64(5)[a]	1.00	1.00
Dissection	112 $\pm$ 61(23)	1.00	1.00

[a]
  number of replicates

TABLE 4.4

EFFECTS OF PROGRESSIVE STEPS OF AXENIC TREATMENT ON HYDRILLA

GROWTH.   GROWTH INDICATORS ARE GIVEN IN TERMS OF LENGTH

(mm) ± STANDARD DEVIATION, FRACTION THAT GERMINATES,

AND FRACTION THAT PRODUCE ROOTS.

TREATMENT	LENGTH	GERMINATION	ROOTS
NaOCl	73 ± 66(9)[a]	1.00	0.89
NaOCl + Dissection	67 ± 41(11) *	0.73	1.00
NaOCl + Dissection + Antibiotics	28 ± 25(11)	0.82	0.67
NaOCl + Dissection + Antibiotics + Organics	15 ± 9(14) *	1.00	0.00 *

[a]
    number of replicates
*
    difference from treatments with no antibiotics is
    significant at the 5% level

TABLE 4.5

EFFECTS OF ORGANICS ON HYDRILLA GROWTH WITH ANTIBIOTICS
PRESENT. GROWTH INDICATORS ARE GIVEN IN TERMS OF LENGTH
(mm) $\pm$ STANDARD DEVIATION, FRACTION THAT GERMINATE, AND
FRACTION THAT PRODUCE ROOTS.

ORGANIC	LENGTH	GERMINATION	ROOTS
Glucose	38 $\pm$ 28(51)[a]	0.82	0.52[*]
Casein	17 $\pm$ 10(50)[*]	0.80	0.00
Glucose + Casein	34 $\pm$ 19(51)	0.73	0.08[*]
Control	130 $\pm$ 52(15)[*]	0.93	0.93

a
  number of replicates
*
  difference for all other sets is significant at the
  5% level

TABLE 4.6

EFFECTS OF ANTIBIOTICS ON HYDRILLA GROWTH WITH ORGANICS

PRESENT. GROWTH INDICATORS ARE GIVEN IN TERMS OF LENGTH

(mm) $\pm$ STANDARD DEVIATION, FRACTION THAT GERMINATE,

AND FRACTION THAT PRODUCE ROOTS.

ANTIBIOTIC	LENGTH	GERMINATION	ROOTS
Penicillin	32 $\pm$ 29(50)[a]	0.74	0.19
Streptomycin	28 $\pm$ 20(51)	0.78	0.20
Penicillin + Streptomycin	29 $\pm$ 17(51)	0.82	0.24
Control	130 $\pm$ 52(15)[*]	0.93	0.93[*]

[a]
  number of replicates
[*]
  difference from all other sets is significant
  at the 5% level

contamination can interfere with hydrilla growth. In every group except two, axenic cultures had a greater length than nonaxenic cultures. Only in the penicillin-glucose culture was this difference significant. Axenic penicillin-glucose cultures were comparable to tubers grown with no organics or antibiotics (Table 4.7).

Streptomycin inhibits stem and root growth at 15, 50, and 100 ug/ml. Penicillin at 25, 75, and 150 ug/ml has no significant effect on growth or root formation. Streptomycin is more effective than penicillin at controlling bacterial growth. Neither showed significant differences in control at the high and low concentration (Tables 4.8 and 4.9).

Neomycin, ampicillin, tetracycline, and chloramphenicol were all inhibitory to hydrilla growth at the tested concentrations. Root formation was significantly inhibited by all concentrations of chloramphenicol, and the higher concentrations of neomycin and tetracycline (Table 4.10). Several combinations of these four antibiotics plus penicillin were effective at preventing bacterial growth. However, all were inhibitory to growth (Table 4.11).

5.0 DISCUSSION

5.1 Factors of Treatment Noninhibitory to Hydrilla Growth

Neither NaOCl treatment and/or tuber dissection cause growth inhibition in hydrilla. In some preliminary

TABLE 4.7

EFFECTS OF COMBINATIONS OF PENICILLIN (PEN), STREPTOMYCIN (STR), GLUCOSE (GLU), AND CASEIN (CAS) ON GROWTH OF AXENIC AND NONAXENIC HYDRILLA CULTURES. GROWTH INDICATOR IS LENGTH (mm) $\pm$ STANDARD DEVIATION.

TREATMENT	AXENIC LENGTH	NONAXENIC LENGTH	TOTAL LENGTH
Pen + Glu	113 $\pm$ 17(3)[a*]	23 $\pm$ 14(12)	42 $\pm$ 40(15)
Pen + Cas	26 $\pm$ 10(4)	18 $\pm$ 13(7)	21 $\pm$ 12(11)
Pen + Glu + Cas	32 $\pm$ 26(3)	31 $\pm$ 23(8)	32 $\pm$ 22(11)
Str + Glu	31 $\pm$ 20(11)	37 $\pm$ 13(2)	32 $\pm$ 19(13)
Str + Cas	19 $\pm$ 10(5)	13 $\pm$ 11(10)	15 $\pm$ 11(15)
Str + Glu + Cas	40 $\pm$ 11(3)	39 $\pm$ 24(9)	39 $\pm$ 24(12)
Pen + Str + Glu	63 $\pm$ 15(3)	34 $\pm$ 13(11)	40 $\pm$ 18(14)
Pen + Str + Cas	20 $\pm$ 7(5)	13 $\pm$ 5(9)	16 $\pm$ 6(14)
Pen + Str + Glu + Cas	26 $\pm$ 3(3)	33 $\pm$ 14(11)	32 $\pm$ 13(14)
Control	[b]	[b]	130 $\pm$ 52(15)[*]

[a] number of replicates- does not include nongerminated tubers

[b] controls were not treated to be axenic

[*] difference from all other sets is significant at the 5% level

TABLE 4.8

EFFECTS OF PENICILLIN AND STREPTOMYCIN CONCENTRATION ON

GROWTH AND AXENICITY OF HYDRILLA CULTURES. GROWTH INDICATORS

ARE GIVEN AS LENGTH (mm) $\pm$ STANDARD DEVIATION AND FRACTION

THAT GERMINATE. FRACTION OF AXENIC CULTURES IS GIVEN.

ANTIBIOTIC	CONCENTRATION	LENGTH	GERMINATION	AXENIC
Penicillin				
	150 ug/ml	$64\pm35(32)$[a]	0.81	0.42
	75 ug/ml	$69\pm42(32)$	0.91	0.41
	25 ug/ml	$86\pm53(32)$	0.84	0.47
	0 ug/ml	$86\pm60(32)$	0.91	0.31
Streptomycin				
	100 ug/ml	$67\pm46(32)$	0.81	0.56
	50 ug/ml	$60\pm28(32)$	0.94	0.50
	15 ug/ml	$63\pm25(32)$	0.84	0.39
	0 ug/ml	$116\pm66(32)$*	0.88	0.16*

[a] number of replicates

* difference from all other sets is significant
   at the 5% level

TABLE 4.9

EFFECTS OF PENICILLIN AND STREPTOMYCIN CONCENTRATION ON
ROOT LENGTH. VALUES GIVEN AS FRACTION OF SET IN ROOT LENGTH
(mm) CLASS. N = 32.

ANTIBIOTIC	CONC. (ug/ml)	0	0-10	ROOT LENGTH CLASS 10-15	15-25	25-35	>35
Penicillin							
	150	0.08	0.31	0.27	0.12	0.04	0.19
	75	0.07	0.28	0.21	0.12	0.00	0.24
	25	0.00	0.15	0.41	0.11	0.07	0.26
	0	0.03	0.28	0.17	0.28	0.07	0.17
Streptomycin							
	100	0.12	0.38	0.23	0.23	0.04	0.00
	50	0.00	0.37	0.40	0.20	0.03	0.00
	15	0.00	0.26	0.37	0.27	0.07	0.00
	0	0.07	0.00*	0.04*	0.00	0.04	0.86*

* difference from all others in root length class for
  tested antibiotic is significant at the 5% level

TABLE 4.10

EFFECTS OF NEOMYCIN (NEO), AMPICILLIN (AMP), TETRACYCLINE
(TET), AND CHLORAMPHENICOL (CHL) ON HYDRILLA GROWTH. GROWTH
INDICATORS ARE GIVEN AS LENGTH (mm) ± STANDARD DEVIATION,
FRACTION THAT GERMINATE, AND FRACTION THAT PRODUCE ROOTS.
ANTIBIOTICS WERE TESTED AT 150, 75, AND 25 ug/ml.
N = 8.

ANTIBIOTIC	CONC. (ug/ml)	LENGTH	GERMINATION	ROOTS
NEO	150	8 ± 3	1.00	0.00
	75	20 ± 6	0.88	0.00
	25	40 ± 9	0.88	0.43 *
AMP	150	34 ± 27	0.75	0.50
	75	44 ± 53	0.75	0.50
	25	32 ± 24	0.88	0.86 *
TET	150	22 ± 7	1.00	0.13
	75	34 ± 7	0.88	0.57
	25	34 ± 14	1.00	0.75
CHL	150	14 ± 6	0.50	0.00
	75	13 ± 5	0.75	0.00
	25	17 ± 7	1.00	0.13
CONTROL		90 ± 46	0.75	1.00

*
  difference from others in same antibiotic group
  is significant at the 5% level

TABLE 4.11

EFFECTS OF COMBINATIONS OF PENICILLIN (PEN), NEOMYCIN (NEO),
AMPICILLIN (AMP), TETRACYCLINE (TET), AND CHLORAMPHENICOL
(CHL) ON GROWTH AND AXENICITY OF HYDRILLA CULTURES. GROWTH
INDICATORS ARE GIVEN AS LENGTH (mm) ± STANDARD DEVIATION AND
FRACTION THAT GERMINATE. FRACTION OF AXENIC CULTURES IS
GIVEN. PEN CONCENTRATION WAS 25 ug/ml. ALL OTHERS WERE
75 ug/ml. N = 8.

ANTIBIOTIC COMBINATION	LENGTH	GERMINATION	AXENIC
Pen + Neo + Amp + Tet + Chl	10 ± 3	0.75	0.88
Pen + Neo + Amp + Tet	31 ± 11	0.86	0.88
Pen + Neo + Amp + Chl	12 ± 4	0.75	0.38
Pen + Neo + Amp	23 ± 8	1.00	0.50
Pen + Neo + Tet + Chl	12 ± 4	1.00	1.00
Pen + Neo + Tet	29 ± 8	0.88	0.75
Pen + Neo + Chl	11 ± 4	0.88	0.63
Pen + Neo	13 ± 4	0.88	0.25
Pen + Amp + Tet + Chl	10 ± 3	0.88	0.00
Pen + Amp + Tet	36 ± 25	0.88	0.25
Pen + Amp + Chl	10 ± 3	0.88	0.50
Pen + Amp	39 ± 37	0.75	0.00
Pen + Tet + Chl	14 ± 2	0.63	0.13
Pen + Tet	44 ± 14	0.63	0.25
Pen + Chl	15 ± 6	0.63	0.50
Pen	41 ± 41	0.88	0.00

experiments a degree of stem growth retardation was exhibited by tubers subjected to both. However, the algal-free plants continued to grow, unlike axenic plants which become senescent.

Glucose had a positive effect on growth of hydrilla cultures. In all cases addition of glucose enhanced the growth of the cultures (Tables 4.5 and 4.7). Growth enhancement by glucose was anticipated. It is frequently used as a carbon source in plant cultures. Street and Henshaw (1965) reported the use of glucose as a carbon source in culture systems increased the growth of monocotyledonous plants (such as hydrilla). When used as a carbon source in a mineral salts medium, glucose addition enables sterile Marsilea cultures to continue to grow and develop normally in complete darkness (Allsop, 1952).

Penicillin had no visible effect on hydrilla growth; however, it proved to be very ineffective at controlling bacterial growth. Hydrilla growth does drop when the penicillin concentration is increased from 25 to 75 ug/ml but the decrease is not siginificant (Tables 4.6 to 4.9). Bacterial contamination interference gave the superficial appearance of growth inhibition by a penicillin-glucose interaction. The statistical equivalence of axenic penicillin-glucose cultures and hydrilla not exposed to antibiotics or organics repudiates this. The continued growth of axenic cultures also disproves the theory of a

necessary commensal relationship between hydrilla and bacteria.

Excessive bacterial growth in a number of cultures seemed to interfere with hydrilla growth (Table 4.7). Heavy bacterial growth could interfere with hydrilla growth by blocking transfer of gases or nutrients, excretion of acidic or toxic substances, or the restriction of light transmission due to turbidity.

5.2 Inhibitory Effects of Casein and Streptomycin

Stem growth and root formation and growth in axenic cultures are inhibited by a combination of casein and streptomycin. Casein inhibits stem growth and root formation. Streptomycin inhibits stem growth and root elongation. Casein is usually used in plant cultures to increase growth. Street et al. (1961) found casein enhanced growth of excised wheat root tips and prolonged the survival of the roots. However, when casein is present in a hydrilla culture, root formation does not appear to be initiated. The only exception is the occasional formation of roots in the presence of glucose and the absence of streptomycin (Table 4.5).

Casein also has an inhibitory effect on stem growth. In cultures where only penicillin and casein are present, growth of the entire plant is stunted. In no instances were plants comparable to the controls produced in the presence of casein (Table 4.7).

In contrast to casein, streptomycin does not appear to interfere with root formation but rather with root elongation. Roots are commonly formed on hydrilla in the presence of streptomycin if casein is not present. However, root length is much less than that of controls or cultures with penicillin only (Table 4.9).

Streptomycin is known to cause root growth inhibition in several plant species. Rosen (1954) found that roots, as well as stems and leaves, are stunted in germinating peas, beans, and corn after exposure to 0.12 to 1.0 per cent streptomycin for 48 h. Gray (1955) showed that exposure of tomato seeds to 20 ppm streptomycin did not inhibit germination but root growth was inhibited by 50 per cent. Streptomycin added to nutrient solutions of seedlings also inhibited root growth. Streptomycin also inhibited stem growth in axenic hydrilla cultures (Tables 4.7 and 4.8). Growth inhibition may be a result of root inhibition rather than a direct effect. However, streptomycin is known to affect plant parts other than roots as was cited previously.

Gray (1955) tested a number of substances in an attempt to overcome the inhibitory effect of streptomycin on tomato seedlings. Thiamine, pyridoxine, nicotinic acid, yeast extract, riboflavin, folic acid, citric acid, and pantothenic acid were tried but none were effective. Klaine (unpublished data) tried the addition of similar substances to increase growth of axenic hydrilla cultures and also had negative results. Nutrients were added in combinations. The

combinations were sucrose, glycine, nicotinic acid, pyridoxine, and thiamine; and pantothenic acid, folic acid, biotin, vitamin B_{12}, riboflavin, and glycerol. Data from the present study also show that the addition of yeast extract does not overcome the inhibitions caused by streptomycin and casein.

Addition of hormones also failed to overcome growth inhibitions at the concentrations tested. Neither IAA or IBA, growth substances that generally induce root formation and promote root elongation, increased root formation in axenic hydrilla cultures. None of the hormones tested had significant effects on germination. In other works, IAA and GA_3 have influenced tuber germination. Steward (1969) found GA_3 and IAA increased germination at 1, 100, and 1000 ppm. Basiouny et al. (1978b) had similar results for GA_3 at 250 and 1000 ppm but found IAA to inhibit germination at 1 and 4 ppm. There were two major differences between the literature procedures and the procedures used in the present study. Tubers used by Steward and Basiouny et al. were not chilled to break dormancy. Also tubers were exposed to the hormones for 24 to 36 h rather than 21 days.

The inhibitory effects of casein and streptomycin combined are greater than the separate effects. Cultures in which both were present had shorter lengths than cultures with just one or the other. It is possible that the shorter lengths are due to an interaction between streptomycin and casein. However, as the differences between the lengths are

not significantly different this is not determinable.

These experiments do not determine if stem growth inhibition is due to root growth inhibition or is the result of direct interference by casein and streptomycin. Plants usually stop growing at the approximate length that root formation is initiated in untreated plants. As roots of aquatic plants are known to play a crucial role in nutrient absorption, root growth inhibition is very likely a contributor to, if not the cause of, stem growth inhibition.

Most of the work done on nutrient absorption by aquatic plant roots has centered on nutrient uptake from the hydrosoil rather than the ambient water. Denny (1972) hypothesized that aquatic plant leaves cannot absorb all needed nutrients directly from the water. He found that being rooted in a nutrient-rich substrate rather than in sand increases hydrilla growth by 7.4 times. However, Denny was working with well water rather than a mineral salts solution. Bristow and Whitcombe (1976) found that labeled phosphorous, ^{32}P, was transported from rooted stem sections upward in three aquatic plant species. The ambient medium rather than the substrate contained the ^{32}P; these results indicate nutrient uptake by the roots directly from the medium.

5.3 Alternative Procedures for Obtaining Axenic
     Hydrilla Cultures

None of the antibiotics or antibiotic combinations tested as alternatives to streptomycin are acceptable. All

inhibited stem growth. Many were ineffective at preventing bacterial contamination (Tables 4.10 and 4.11).

This study illustrates the necessity of testing antibiotics for toxicity as well as efficiency of bacterial control. Various concentrations should also be tested, as inhibitory effects may not be present at lower concentrations. Pramer (1953) found that chlortetracycline, chloramphenicol, neomycin, streptomycin, and oxytetracycline caused stunting of cucumber seedling roots and shoots at 500 ug/ml. When tested at 50 ug/ml growth was comparable to the controls.

A procedure for obtaining axenic hydrilla cultures that grew well was found. Tubers subjected to NaOCl treatment followed by dissection and grown in 10 per cent modified Hoagland's medium with penicillin grow well and produce roots. Addition of glucose to facilitate detection of bacterial contamination will not inhibit growth of axenic cultures. However, the efficiency of this procedure is very low. Few axenic cultures, approximately 20 per cent, are produced.

If large numbers of axenic plants are needed it would be advantageous to find another noninhibitory antibiotic(s) that, when used in combination with penicillin, would give a larger percentage of axenic cultures. Another possibility is to use hydrilla's ability to produce mature plants from plant fragments. Axenic plants could be grown to a set size, cut

into pieces, and transferred aseptically. Plant pieces would develop into individual, axenic plants.

5.4 Conclusions

Casein and streptomycin both contribute to stem and root growth inhibition in axenic cultures of <u>Hydrilla verticillata</u>. Casein inhibits stem growth and root formation. Streptomycin inhibits stem growth and root elongation. Mechanisms for these inhibitions are not known. No other parts of the axenic treatment inhibit hydrilla growth. Axenic cultures of hydrilla can be produced using penicillin as the only antibiotic. Although growth in these cultures is normal, few axenic cultures are obtained.

More work needs to be done to find an antibiotic or a combination of antibiotics to use in conjunction with penicillin to produce axenic cultures. The antibiotics tested should be well-screened for toxicity.

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