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Yichun Wang

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CHARACTERIZATION OF A PURIFIED EXTRACT FROM TOMATO APICES THAT INCREASED THE GROWTH OF PLANTS

By

Yichun Wang

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF A CHEMICAL(S) FROM TOMATO APICES THAT INCREASES PLANT GROWTH

BY

Yichun Wang

An aqueous methanol extract ($CH_3OH:H_2O$, 60:40 v/v) of tomato (*Lycopersicon esculentum* Mill) apices increased the growth of *Chlamydomonas rheinhardtii*, the algae species used to determine bioactivity throughout this study. The active substance was purified by thin layer (TLC), C_{18} flash column and C_{18} reverse phase high performance liquid chromatography (HPLC). The purified extract increased the growth of tomato seedlings at 100 ug/l and rice (*Oryza sativa* L.) seedlings at 1000 ug/l. In a dose response study with *Chlamydomonas*, the purified extract significantly increased cell division 111% at 100 ug/l and chlorophyll content 23% at 10,000 ug/l in 18 hr.

Nuclear magnetic resonance (NMR) and mass spectroscopy (MS) indicated that the purified fraction is a mixture of compounds with sugar moieties with no aromatic groups or olefinic protons. TLC showed that the extract also contains ninhydrin reactive compounds, suggesting the presence of amino sugars, amino acids or both. These compounds were more polar than known plant hormones and did not have a similar mass, TLC profile, or biological activity, thus indicating the presence of an unknown chemical in tomato apices which increases plant growth at less than 100 ug/l.

Dedication

This work is dedicated to my father Jian Wang, and my husband Yixing Bao, who made it possible in so many ways.

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I have received a great deal of help from many people, and while it is not possible to recognize all of them. I would like to thank specific individuals. I would like to express my appreciation to Dr. Ries, my major advisor, who provided me the opportunity and his experience which helped me to develop as a scientist. I also would like to thank the members of my advisory committee, Dr. Widders and Dr. Branham for their advice and guidance. I would also like to give special thanks to my unofficial committee members, Violet Wert and Dr.Muralee Nair who gave me many helpful suggestions and confidence throughout my research. Without their input, there would be no thesis. I would also like to thank Deborah Thorogood for her friendship, and Jye Chang for his assistance in the preparation of this manuscript. Of course, none of this would have been possible without the love, encouragement and sacrifice of my husband Yixing Bao.

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INTRODUCTION

A simple vegetative shoot consists of an apical meristem, axillary meristems, a stem, and leaves. These structures arise initially from groups of apical meristematic cells which may remain in an embryonic condition for long periods of time (11, 16, 49). The shoot meristem of higher plants has the potential to give rise to a primary stem and leaf, modified leaf and floral bud initials.

Clearly, plant scientists are interested in understanding how similar parenchymatous cells divide and ultimately differentiate in an orderly manner into diverse cell types which constitute the complex tissues and organs of the shoot. The regulation of this process is thought to involve endogenous growth hormones and nutrients (3, 49).

Since Wolff recognized the importance of the shoot apex organ development, two major aspects of organ initiation in the shoot have been investigated. First, the site of organ initiation relative to preexisting organs, and secondly, the cytological and biological events which occur uniquely in the initiating cells to cause them to divide and differentiate into complex organs (30).

In spite of the considerable research on the morphology and histology of shoot apices, chemical investigations are rare because of the extremely small size of the shoot apex as well as the large number of chemicals present in any plant extract (15, 45).

Since the shoot apex is the site for growth initiation, it was postulated that there were unknown growth stimulating and inhibiting chemicals in this tissue besides the known growth hormones.

This study was initiated to discover previous unknown compounds present in the shoot apex, which may stimulate or inhibit cell expansion and/or division. After evaluating several plant species including wheat (Triticum aestivum L.), cabbage (Brassica oleracea capitata L.), carrot (Dancus carota L.), rice (Oryza sativa L.) and tomato (Lycopersicon esculentum Mill) (data not presented), tomato was selected to study the activity of bioactive chemicals in the shoot apex because of the activity of the crude extract and ease of obtaining plant materials.

LITERATURE REVIEW

THE VEGETATIVE SHOOT APEX

In 1759 Wolff (54) termed the apex of the stem the "punctum vegetationis" where he discovered that new leaves and tissues are initiated. Today, the term shoot apex and its synonyms are generally used (16), instead of the somewhat inaccurate term "growing point", since the processes of growth cannot be restricted to the "growing point". In this research, the shoot apex refers to the "promeristem", which comprises the apical meristem and associated cells (24, 25, 47, 48).

MORPHOLOGY AND HISTOLOGY OF THE SHOOT APEX

In the nineteenth century, research workers dealt mainly with the problems of the number and the arrangement of the initials in the apices and the determination of tissues derived from apices. In addition to the problem of initials, modern research also deals with the cyto-histological division of the apex into various zones and the activities of the cells in those zones (16).

The status of certain cells as initials depends on their position in the promeristem (16, 21). To better understand the behavior of the meristem, Newman (34) classified the shoot apices in the apical meristem into three types depending on fundamental differences in structure:

1)Only one initial cell (many cryptogams);

2)Several initials in one cell layer (most gymnosperms);

3)Several initials in more than one layer (most angiosperms);

These three kinds of apices have the same basic pattern of growth. They all consist of a distally located initiating zone (promeristem) and two derivative zones (the outer and inner), in which organogenesis and histogenesis begin.

Higher plants will be primarily considered here. Since the shoot apex was first recognized by Wolff in 1759 as an underdeveloped region from which growth of the plant occurred, the early plant anatomists of the nineteenth century expected to find a "single" apical cell also in gymnosperms and angiosperms like in the lower plants, and indeed described such cells. Later, however, it became apparent that there is no clearly recognizable "single" cell in the apices of higher plants (16, 35).

The vegetative shoot apices of flowering plants are conventionally classified either as layered or zonate (14, 41). In a layered apex, described as the "tunica-corpus", two regions recognized by Schmidt (42) are distinguished by the planes of cell division in them. The tunica is the outmost layer(s) of cells, which divides in a plane principally by anticlinal division, and the corpus, the inner cell mass, whose plane of cell division is in all directions. Recent electron microscope studies of shoot apices reveal that the tunica enlarges in surface area and the corpus in volume (19).

In the zonate apex, suggested by Foster (20) as a general pattern of development in the gymnosperm apex, a number of zones are recognized which included the central and peripheral zones and rib meristem. These zones are considered to be superimposed upon the tunica-corpus organization.

The youngest leaf primordia are the active region in shoot apices (48). Based on morphology and histology, the shoot apex appears to be a self-determining region. The position of leaf primordia is independent of the procambium. This view is the product of surgical studies begun 50 years ago by Snow (44) and continued in particular by Wardlaw and his associates (47). They have found that preexisting leaf primordia close to the shoot apex, in addition to the shoot apex itself, determine the position of future primordia. The best evidence to support this view is provided by the observation that when the apex from surrounding tissues was surgically isolated by four vertical incisions, the apices of both Lupinus and the fern Dryopteris, continued to produce new leaf primordia in the normal phyllotactic sequence.

CYTOLOGY OF THE SHOOT APEX

There have been two lines of research concerning cell division in shoot apices. They are the spatial distribution of mitosis and mitotic rate within the shoot apex (3, 15). These two lines of research were prompted by Buvat's hypothesis (12) that the principal meristematic activity of the apex is seated in the annean initial, the initial or meristem ring, and the central "meristeme d'attente", the reserve meristem, is passive during vegetative growth, but becomes active during the formation of a terminal flower or inflorescence. This hypothesis generated controversy and has led to a considerable amount of research concerning relative cell division rates in various regions of the shoot apex.

Cells in the apex remain of the same order of size, and any displacement of cells from the initiating center must inevitably be accompanied by an increase in the number of divisions occurring in these cells if the shape of the apex is to be retained (22, 23).

Ball (8, 9) photographed the surface layers of shoot apices of Lupinus, Vicia and Asparagus for periods of time, and observed equal frequency in rate of cell division in all parts of the surface layer of the shoot apex.

Using autoradiographic techniques, Gifford & Tepper (26, 27, 28) demonstrated a uniform synthesis of nucleic acids in all cells of the vegetative apex.

However, the point is not whether the central terminal cells of the apex divide, but whether they have a role as initials in the development of the shoot (12, 21). According to Buvat (12) and Lance (33) even when these cells divide they do not function as initials, giving rise to the tissues of the vegetative shoot; this function is fulfilled by the anneau initial.

Recent researchers have paid attentions to cytohistochemical differences between various zones of shoot apex. A very detailed study on this aspect was made of the shoot tip of Brachychiton (Sterculiaceae) by West & Gunckl (52). Starch was reported to be present in the central zone and pith rib meristem, but absent in the peripheral meristem. The types of cell wall polysacharides were determined in 3 transverse zones of the shoot tip. The first 0.5 mm of the apex was characterized by both radial expansion and cell elongation. Cell walls in the central zone were high in pectic substances, extremely low in cellulose and contained about 20% each of hemicellulose and non-cellulosic polysacharides. Cell walls in the second portion of the apex (0.5-3.5 mm), were characterized by radial growth, with approximately 40% non-cellulosic polysacharides, 27% pectic substances, 20% hemicellulose, and 7% cellulose. By using autoradiographic technique, they observed that twice as much labelled RNA was formed per cell in the peripheral zone as in the central zone. In the initial growth zone (0.5 mm segment), characterized by cell expansion and elongation, RNA and protein per cell increased sharply. In the 3.5 mm region, characterized by radical growth, the metabolites were relatively constant. Thus, the central zone deemed "quiescent" by some investigators is metabolically inactive.

Evans & Berg (17) made a semiquantitive histochemical comparison of leaf-initiating and non-primordial cells of the shoot apex of Triticum aestivum and found no differences in the RNA/DNA ratio or the total nuclear protein/DNA ratio. However, the autoradiographic data suggested a higher rate of RNA synthesis and therefore, turnover in the leaf-initiating cells. They also found higher histone /DNA ratio in the leaf-initiating cells when an acid fast-green stain was used.

By using an histoimmunochemical procedure, Pierard (36) observed 3 antigenic proteins in the apical bud of Sinapis alba L., appearing in different stage in transition to flowering. Protein A, present in vegetative meristem, increased in concentration during the first 48 hr following the start of flowering induction treatment. It stayed constant for up to 96 hr and disappeared completely later. Two other proteins called B and C, absent in the vegetative meristem, appeared in the meristem of the induction treatment and accumulated at the floral primordia.

REGULATION OF GROWTH IN THE SHOOT APEX

The leaf initiation site in the shoot apex is described as a "growth center", defined by Wardlaw (47) as a locus of "special metabolism". It is generally assumed that hormones influence the rate of cell division.

Shoot apex growth and development has been mainly attributed to the plant hormones, especially auxins produced in the apical bud.

It was demonstrated by Thimann and Skoog (46) that auxin, probably IAA, was synthesized in growing apical bud. Very soon after that, the same authors also reported that exogenous IAA could substitute for the shoot apex in tissue culture.

A further line of evidence that indicates a primary role for auxin in correlative control of the shoot apex has come from studies with inhibitors of auxin transport. Treatment of plants with TIBA (Triodobenzoic acid), a auxin transport inhibitor, results in either a reduction or inhibition of shoot apical growth (35).

Gibberellins are also synthesized in the shoot apex and young leaves. The enhanced shoot apical growth in response to gibberellin treatment has led to suggestions that apical growth in intact plants treated with gibberellin is an indirect effect due to an increase of endogenous auxin levels (35).

When cytokinin was applied to soybean (Glycine max L.) shoot apices before GA_3, 5-fluorouracil (a cytokinin) did not prevent the effect of GA_3 on cell expansion. However, application of 5-fluorouracil after GA_3 inhibited cell expansion(16). This indicated that the role of cytokinin was to initiate cell division and that gibberellin was required for the subsequent enlargement of newly formed cells in the shoot apex.

There are also regulatory effects of known growth regulators on the distribution of nutrients within the shoot system. Went (50, 51) presented the "nutrient-diversion theory" which proposed that metabolites move towards regions of highest auxin concentration, in growing apical shoots. Metabolites such as sugars, phosphates, and cytokinins, are translocated to and accumulated in the growing shoot apex. The term hormone-directed transport (HDT) has been applied to this phenomenon (35).

Nutrients also have a major effect on shoot apex growth. The original nutritive theory, proposed by Goldsmith (28), suggested that since the shoot apex is normally present in the embryo, it will continue to control supplies of nutrients from roots and leaves by virtue of constituting a metabolic sink. Little research has been done on this aspect (18, 31, 35, 37, 39, 55).

Babenko & Mai Suan (7) investigated the carbohydrate content in apical growth and upper leaves during ontogenesis of winter wheat during the V-VII phases of generative organogenesis. They revealed that during plant development the content of carbohydrates increased both in the growing point and in the upper leaves. This was shown as an increase in oligosaccharide and sucrose synthesis respectively.

MATERIALS AND METHODS

Collection of shoot apices from tomato plants: Shoot apices were obtained from 25-day-old tomato (*Lycopersicon esculentum* 'Ohio 7870') plants grown in the greenhouse of the MSU Pesticide Research Center, East Lansing. Plants were grown in 30 x 30 cm styrofoam flats under metal halide lights, with a photosynthetic photon flux density of 425 uMol/m^2/Sec measured at the top of canopy. The greenhouse was maintained with a 14-hr-light-10-hr-dark photoperiod at 28°C and 22°C during the light and dark period respectively. Flats were fertilized weekly with 500 ml soluble 20-20-20 fertilizer at a rate of 1 g/liter of water.

Whole plant shoots were harvested and lyophilized at 5°C for 24 hr. Terminal cuttings 5-10 mm long were collected from the dried plant shoots, and stored in tightly closed glass containers. Prior to extraction, shoot apices were ground in a Wiley mill (40u mesh screen).

For fresh plant tissue extraction, unexpanded 1 cm apical sections were removed as needed, and immediately extracted as follows.

Extraction of apices: Homogenized powder from shoot apices was placed on cotton located at the base of a column (2.5 x 45.0 cm).

This material was sequentially partitioned with the following solvent series ranging from least polar to most polar (53): hexane, chroloform, methanol and water (Fig. 1.). Twenty ml of solvent was used for each 0.1 mg of the dry plant material.

Chlorophyll was removed from all fractions (38), except the hexane fraction, with activated (C-170) decolorized neutral charcoal, which was refluxed by Soxhlet at 60°C for 20 min. One g of charcoal was used for every 0.4 g of dry plant material. A clear solution was collected through Whatman #1 filter paper containing celite (0.5 g) in the bottom of the hand-folded filter paper. The solvent was removed from each fraction by rotary evaporation at 35°C. Each fraction was redissolved in the solution used for extraction (1.0 mg / 100 ul). Serial dilutions were made to obtain treatments for bioassaying.

For fresh material extraction, fresh shoot apices were homogenized by an automatic mortar with 1 g /10 ml (w/v) water. Extracts were filtered through Whatman #1 filter paper with vacuum. Chlorophyll was removed as previously described. The clear, aqueous extract was lyophilized at 5°C for 24 hr. The dry extract was redissolved in water (1 mg/100 ul), and serially diluted to obtain treatments.

The youngest fully expanded leaves were collected and the same sequential partitioning procedure used for the apices was used for the leaves.

The _Chlamydomonas_ bioassay was used to test for plant growth regulatory properties in all fractions from both dry and fresh shoot

Tomato shoots harvested in the greenhouse

1.Lyophilized at 5°C
2.Apices separated from shoots
3.Homogenized

Loaded into 25x30 mm glass column

Eluted with Hexane (0.1 mg apices/20ml)

Hexane soluble — Dried at reduced pressure — Bioassay

Residues — Eluted with chloroform

Chloroform soluble — Dried at reduced pressure — Bioassay

Residues — Eluted with methanol

Methanol soluble — Dried at reduced pressure — Bioassay

Residues — Eluted with water

Water soluble — Dried at reduced pressure — Bioassay

Fig. 1 Flow diagram for extraction of dried tomato shoot apices with different solvents.

apices and leaves.

Since most activity was present in the water and methanol fractions of the shoot apices, a methanol:water (60:40) solution was used for further extractions. The powdered shoot apices (6.08 g) were stirred at a low speed at room temperature for 60 hr with 200 ml of methanol:water (60:40). During stirring, the solution was replenished every 6-10 hr to account for evaporation. The mixture was filtered using vacuum through a fritted glass filter (40-60 u mesh). Chlorophyll was removed with charcoal-celite (0.5 g each) by Soxhlet for 20 min at 60°C. Solvent in the clear extract was filtered with #1 Whatman filter paper prior to condensation at 35°C. The aqueous residue was lyophilized at 5°C for 24 hr. This material was considered the crude extract for all further tests until the purified compounds were obtained by HPLC. Thus the crude extract equivalent for bioassays during purification was based on the amount of dried material from the tomato apices that was soluble in CH_3OH: H_2O (60:40) in the initial extraction procedure.

Isolation and purification: Further separation of the methanol:water (60:40) extract was conducted using silica gel thin-layer chromatography (TLC) plates (Merck, 0.25 mm, F_{254}). Plates were run in chloroform:methanol:formic acid (5:4:1). Three distinct bands (Rf=0.28, 0.63, 0.71) were visible using ultraviolet (UV) light at 254 and 366nm.

Five areas were scraped for bioassay. These included the area before band 1, band 1, the area between band 1 and 2, bands 2 and 3, and the area after 3. The scrapings were eluted with methanol:chloroform (9:1) through a fritted glass filter (4.0-5.5 u) by vacuum filtration. The solvent was removed by rotary evaporation, and all 5 fractions were assayed with algae at 1, 10, and 100 mg/l.

Liquid flash column chromatography with silica and C_{18} gels was utilized with the crude extract to obtain larger amounts of the active chemicals. For the silica gel procedure, the crude material (295 mg) was partially dissolved in chloroform:methanol: formic acid (5:4:1), and loaded on a flash column containing 12.5 g silica gel (Baker, particle size 10-40 um) after activation of the silica gel in a forced-air oven at 130°C for 12 hr. Pressure for the column was provided with a laboratory air line at a rate of 2.0 ml solvent/min, and eluted with 80 ml chloroform:methanol: formic acid (5:4:1). Thirty vials each containing 2 ml were collected, examined by TLC (silica gel) and run with the same solvent system as for the flash column. These were combined into 6 fractions: Fraction 1, vials 1-5; fraction 2, vials 6-9; fraction 3, vials 10-17; fraction 4, vials 18-20; fraction 5, vials 21-24; and fraction 6, vials 25-30. The 6 fractions were bioassayed using concentrations of 10 and 100 mg/l.

For the C_{18} flash column procedure, the crude extract (173 mg) was dissolved in methanol:water (30:70) and loaded onto a C_{18} flash column (Baker, particle size 40 um, column diam 35/20 mm) with a gel height of 18 cm, previously prepared in absolute methanol. Air pressure for the column was provided with a laboratory air line to give a rate of 2.0 ml solvent/min and the column was eluted with 115 ml of methanol:water (30:70). Twenty-three vials, each containing 5 ml, were collected, and examined by high performance liquid chromatography (HPLC) before grouping them into 3 fractions. One hundred ml of absolute methanol was used to wash the column, and also collected as one fraction. After the solvent was removed by rotoevaporation, all fractions were redissolved in distilled water (1 mg/100 ul), only fraction 4 precipitated and this was soluble in absolute methanol. All of the fractions were bioassayed with algae at 10 and 100 mg/l.

The active fractions collected from both the silica gel and C_{18} flash columns were further separated by preparative HPLC, using a ChemcoPak, Chemcosorb 5-ODS-H C_{18}, 20x250 mm column. The UV detector (Hatachi, model L-4200) measured absorbance at 254 nm. The solvent system used was methanol:water (30:70) with a flow rate of 4.0 ml/min. Fraction 2 from the silica gel flash column separated into 9 fractions, while the active fraction from C_{18} flash column separated into 6 fractions. All fractions from C_{18} flash column were bioassayed with a crude equivalent of 100 mg/l after the removal of the solvents by rotary evaporation. Strong stimulatory activity was associated only with one fraction, which appeared as one sharp HPLC peak after cleaning twice with $CH_3OH:H_2O$ (30:70) at pH 6 and once at pH 2, which was adjusted by using 600 ug/l of trifluoroacetic acid. Eight mg of purified extract was obtained from 6.8 g of lyophilized shoot apices. The average shoot apex weighed 250 ug, thus, this purified extract was obtained from about

27,000 shoot apices. Portions of this purified extract were subjected to spectral analysis by MS and NMR.

TLC and algae bioassays were conducted to determine if the purified extract of tomato apices was chemically or bioactively similar to known plant hormones. Indoleacetic acid (IAA) (Calbiochem, Los angeles), gibberellic acid-3 (GA_3) (Velsicol, Chicago), zeatin (Sigma, St. Louis), benzylaminopurine (BA) and kinetin (Ardrich, Milwaukee) were used.

Bioassays: Three bioassay systems were employed for the detection of compounds from tomato shoot apices which may stimulate or inhibit growth. *Chlamydomonas reinhardtii* was used as the primary bioassay throughout the isolation and identification procedure to screen the growth promotion or inhibition of different fractions. Rice seedlings in solution culture and tomato seedlings in soil culture were treated with foliar applications stimulating purified extract from tomato shoot apices.

Chlamydomonas : This assay proved useful for screening growth stimulating and inhibiting substances throughout all the extraction and purification procedures. The assay is rapid and it is possible to compare many treatments in the same test.

Cultures of *Chlamydomonas reinhardtii* Dangard, (-)strain (N.90), a unicellular green alga, were grown in 2.8 l Fernbach flasks containing 2.0 l of nutrient solution (Table 1.), continually aerated under sterile conditions. Cultures were grown at 30°C, and received 300 umol/m^2/sec light from fluorescent bulbs during 12 hr

Table 1. Nutrient solution for Chlamydomonas reinhardtii

Stock solutions	Composition of stock solution (g/l)	Amount of stock for nutrient solution (ml/l)
KPi Solution		10.0
K_2HPO_4	14.38	
KH_2PO_4	7.26	
Beijerinck's Solution		50.0
NH_4Cl	8.00	
$CaCl_2 \cdot 2H_2O$	1.00	
$MgSO_4 \cdot 7H_2O$	2.00	
Tris Cl Solution		10.0
Tris basic (to pH 7.5 with HCl)	242.00	
Hunter's Trace Elements		1.0
EDTA (Ethylenediamine tetracetic acid)	50.00	
$ZnSO_4$	22.00	
H_3BO_4	11.00	
$MnCl_2 \cdot 4H_2O$	5.10	
$FeSO_4 \cdot 7H_2O$	5.00	
$CaCl_2 \cdot 6H_2O$	1.60	
$CuSO_4 \cdot 5H_2O$	1.60	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (to pH 6.5-6.8 with KOH)	1.10	

photoperiod. The cultures were diluted with fresh nutrient solution weekly.

The algae bioassay was started 0.5-2.0 hr after initiation of the light period. A portion of the algae stock solution was diluted in nutrient solution to obtain a density of $6x10^5$ cells/ml, (0.5-0.6 OD at 652 nm). Before the initiation of each experiment, 100 ml CO_2/l of culture was bubbled through the cell suspension for 30 sec. Ten ml aliquots of the suspension were pipette into disposable culture tubes (16x100 mm) containing the extracts or fractions to be tested. Cells were allowed to grow in these tubes under continuous light of 150 umol/m^2/sec at 30°C. After 18 hr, the algae suspensions were centrifuged at 2800 rpm for 15 min, the pellet resuspended in 5 ml of 80% acetone, and tubes centrifuged at 2800 rpm for 7 min. Chlorophyll readings were taken at 652 nm, and the zero time optical density was subtracted from these readings. Chlorophyll was determined by Arnon's method (6), i.e., the total chlorophyll equals the OD at 652 nm x 28.986(ug/ml) x 5 ug chlorophyll per milliliter Chlamydomonas culture. In tests where cell density was determined, representative samples of the cell suspension were removed from bioassay tubes prior to centrifugation. Cells from the sample were counted using a hemacytometer, and the number of cells per ml calculated.

Tomato seedlings: "Ohio 7870" tomato seedlings were grown in 15-cm clay pots in the greenhouse under the conditions previously describes. Ten to 15 seeds were sown per pot and after 19 days when the fourth leaf just emerged, the seedlings were thinned to the 2 most uniform per pot. The pots were then grouped according to plant size into blocks. Pots were fertilized with 250 ml of a solution containing 1.0 g/l of a soluble 20-20-20-fertilizer 6 hr before the treatment. Foliar applications of purified extract from tomato shoot apices with concentrations of 0.01-10.00 mg/l were made with adjustable, hand-held, aerosol sprayers (Science Products Company Inc., Chicago, Ill.) made from high-density, linear polyethylene. The plants were sprayed until the liquid dripped from the plants. The tomato shoots were harvested 10 days after treatment by cutting at the soil level and dried in a forced-air oven at 70°C for 24 hr. Shoot dry weight was used as the primary measure of growth.

Rice seedlings: A modified version of rice seedling bioassay (40) was used for assaying growth. One week after transplanting, or just before the emergence of the fourth leaf, the plants were sorted visually for size on consecutive days and grouped into blocks. Each cup contained 4 plants placed in 170 ml of fresh nutrient solution. Six replications were employed. Every 40-60 hr, water uptake was measured by weighing the nutrient solution remaining in the cups and subtracting this amount from the initial amount minus water evaporating from cups without a plant. Six days after treatment the plants were harvested, and placed in thin, dense paper (glassine) envelopes for drying over night at 70°C in a forced-air oven. Oven-dried samples were weighed after equilibration with the laboratory environment and the dry weight of whole plant was used as a measure of growth.

Statistical procedures: All experiments were conducted utilizing randomized complete block designs, with 3 blocks for the algae tests and 6 blocks in the higher plant bioassays. The observable variance due to plant size was assigned to blocks during the plant thinning and sorting process. All treatments were randomized within blocks utilizing a random-number table. Analysis of variance was conducted and the means separated using the LSD when the F test was significant. Trend effects were noted when the F value was significant.

RESULTS AND DISCUSSION

Extraction of tomato shoot apices: Extracts from tomatoes were used not only because of their activity in the algae bioassay but also because of the uniform size of the apices, their availability, and because they could be grown in the greenhouse throughout the year.

The best method for the initial extraction of the active compounds in tomato apices was by using a series of solvents. The most efficient solvents in extracting dry weight were methanol and water, as indicated by the extraction index (Table 2.). The most stimulatory activity, as measured by the promotion of the algae growth after 18 hr, occurred in extracts from apices made with either methanol or water (Table 3.). The algae responded equally well to both water extracts from dried or fresh tomato apices (data not presented). Since the most activity was concentrated in the methanol and water fractions, the extract of methanol:water (60:40, v/v) was used in an attempt to isolate the chemicals responsible for both the increase and decrease of algae growth.

To avoid the effect of the chlorophyll on spectrophotometric measurements, the chlorophyll was removed before purification and assaying with Chlamydomonas. This was accomplished by refluxing 60% methanol extract with neutral charcoal at 60°C for 20 min or directly loading this extract onto a C_{18} flash column before elution

Table 2. Quantity of extract (mg) from 4.0 g of dried apices and 15.3 g of dried leaves.

Treatment			
Solvent	Tissue	Quantity extracted (mg)	Extraction index[z] (mg/g)
Hexane	Apex	31	7.8
	Leaf	73	4.8
Chloroform	Apex	8	2.0
	Leaf	68	4.4
Methanol	Apex	282	70.5
	Leaf	2499	163.3
Water	Apex	753	188.2
	Leaf	2408	157.4

[z]Proportion of extract to dried dried apices or leaves.

Table 3. Response of <u>Chlamydomonas</u> in the 18 hr to extracts obtained by different solvents from tomato leaves and apices. A sequential system of hexane, chloroform, methanol, and water was used for extraction.

Treatment		ug chlorophyll/ml Chlamydomonas			
Solvent	Tissue	0.1 mg/l	1.0 mg/l	10 mg/l	100 mg/l
Hexane	Apex	2.23	2.39	2.04	2.25
	Leaf	2.27	2.39	2.09	2.20
Chloroform	Apex	2.23	2.36	2.06	1.75
	Leaf	2.28	2.39	2.20	2.45
Methanol	Apex	2.25	2.43	2.64	4.39
	Leaf	2.28	2.42	2.14	0.94
Water	Apex	2.28	2.45	3.01	1.97
	Leaf	2.26	2.38	2.29	2.46
Control		2.23	2.33	1.98	2.09
LSD 5%		NS	0.06	0.12	0.16
LSD 1%		NS	0.08	0.16	0.22
C.V.(%)		1.60	1.50	3.05	3.92

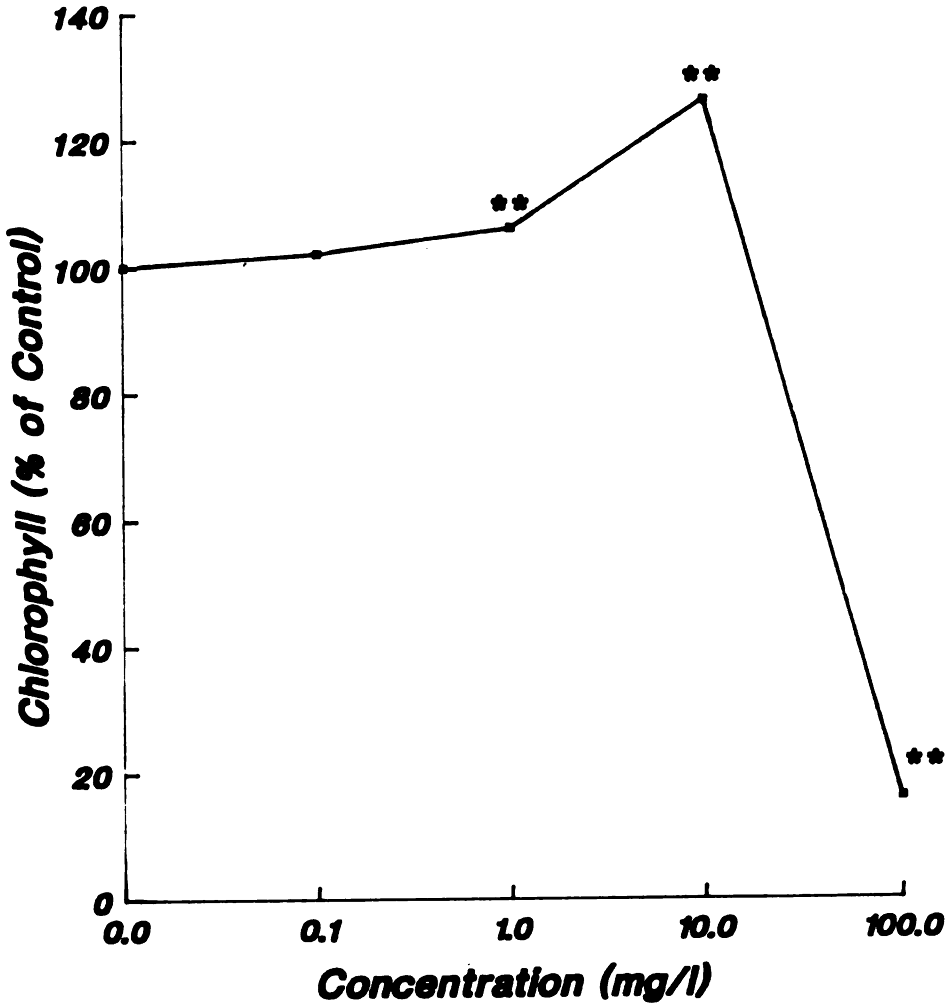
with methanol:water (30:70, v/v). Passing the crude extract through a C_{18} Sep-pak or stirring the extract with neutral charcoal at room temperature was ineffective because both the Sep-pak and charcoal adsorbed active compounds.

To extract the active chemicals for identification, dried tomato apices were stirred for 60 hr at room temperature in methanol:water (60:40, v/v). At 10 mg/l this extract increased growth 26%. One hundred mg/l completely inhibited the new growth of algae compared to a control of distilled water (Fig. 2.). There are at least two possible explanations for this quadratic trend of algae growth with concentration of extract. First, one of the chemicals may have demonstrated the super-optimal property of hormones (16), where low doses stimulate growth and high doses inhibit the growth. Second, the extract may contain compounds that both inhibit and stimulate algae growth.

To answer this question, the extract was further separated by TLC and six distinct areas were detected. The Rf values for bioassaying from the TLC plates were determined by using visible and UV light (366 nm and 254 nm) (Table 4.). Active compounds were extracted from two areas which stimulated (Rf 0.48) and inhibited (Rf 0.68-0.74) algae growth (Fig. 3.).

The TLC procedure showed that the crude extract contained both a chemical that inhibited algae growth as well as one that stimulated growth. The compound from the TLC plate which stimulated growth was more active (37% more than control) than the crude extract (24% more than control). This may be due to removal of

Fig. 2. Response of Chlamydomonas in 18 hr to different concentrations of $CH_3OH:H_2O$ (60:40) extracts of tomato apices. ** LSD significantly different from control at 1% level.

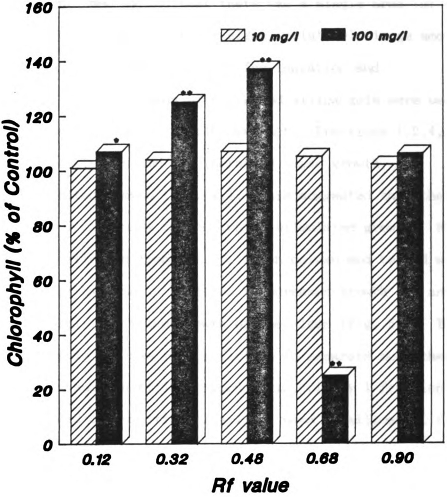


140
120
100
80
60
40
20
0
Chlorophyll (% of Control)
**
**
**
0.0
0.1
1.0
10.0
100.0
Concentration (mg/l)

Table 4. Visibility under different types of light of the $CH_3OH:H_2O$ (60:40) extract from tomato apices on a silica gel $60F_{254}$ plate run in $CHCL_3:CH_3OH:HCOOH$ (5:4:1).

Rf value	Type of light		
	Visible	UV(366nm)	UV(254)
0.12			
0.32		*	*
0.48	*		
0.62	*	*	*
0.74		*	
0.90			

Fig. 3. Biological activity of extracts from tomato apices as determined by the Chlamydomonas bioassay. The extracts were separated on a silica gel $60F_{254}$ plate using a solvent system of $CHCl_3:CH_3OH:H_2O$ (5:4:1). *, ** LSD significantly different from control at 5% and 1% levels, respectively.


10 mg/l
100 mg/l
Chlorophyll (% of Control)
0
20
40
60
80
100
120
140
160
*
**
**
**
0.12
0.32
0.48
0.68
0.90
Rf value

inhibitor. Since the solvent system for TLC contained 10% formic acid, it indicated that the purified extract was stable to acid treatment.

Isolation and characterization of compounds in purified extract

After it was confirmed by TLC that there was a single area (Rf 0,32-0.48) that stimulated the growth of algae, relatively large amounts of dried tomato apices were extracted for isolation and identification. Flash columns with C_{18} and silica gels were used to separated the methanol:water (60:40) extract. Fractions 1,2,4,5 and 6 from the silica gel column all increased algae growth, and fraction 3 inhibited growth (Fig. 4.). This suggested that there may be at least 2 compounds present which stimulated growth. Five fractions were collected from the C_{18} flash column and tested with the Chlamydomonas bioassay. Fraction 2 increased growth 39% and fraction 5 severely inhibited growth of the algae (Fig. 5.). This indicated that this C_{18} was more efficient to separate both the growth promoting and inhibiting compounds in primary 1 fraction. Concern about acidic properties of solvent used on silica gel flash column and future extract separation by C_{18} HPLC, this C_{18} flash column was used for further research in isolation and identification.

Fraction 2 from C_{18} was resolved into 6 peaks (Fig. 6a.) by HPLC. The peaks with a retention time of 11.5 to 12.6 min appreciably increased the growth of algae (Table 5.). After all the purification procedures on HPLC, as described previously, a single

Fig. 4. Response of Chlamydomonas to fractions (900 mg/l crude equivalent) from a silica gel flash column. Fractions were obtained by passing crude extract of tomato apices over silica gel flash column eluted with $CH_3Cl:CH_3OH:H_2O$ (5:4:1). ** LSD was significantly different from control at 1% level.

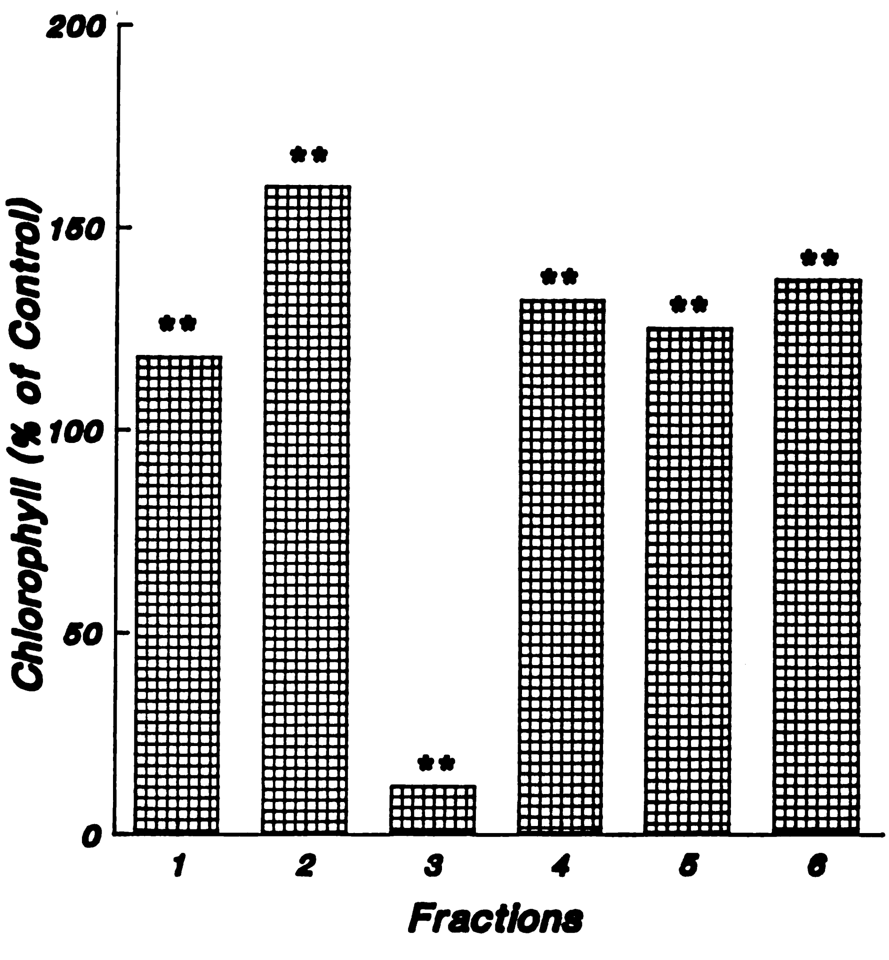

200
150
100
50
0
Chlorophyll (% of Control)
**
**
**
**
**
**
1
2
3
4
5
6
Fractions

Fig. 5. Biological activity of extract of tomato apices (100 mg/l crude equivalent) as determined by the Chlamydomonas bioassay. Extracts were separated on C_{18} flash column eluted with methanol: water (30:70). *, ** LSD significantly different from control at 5% and 1% levels, respectively.

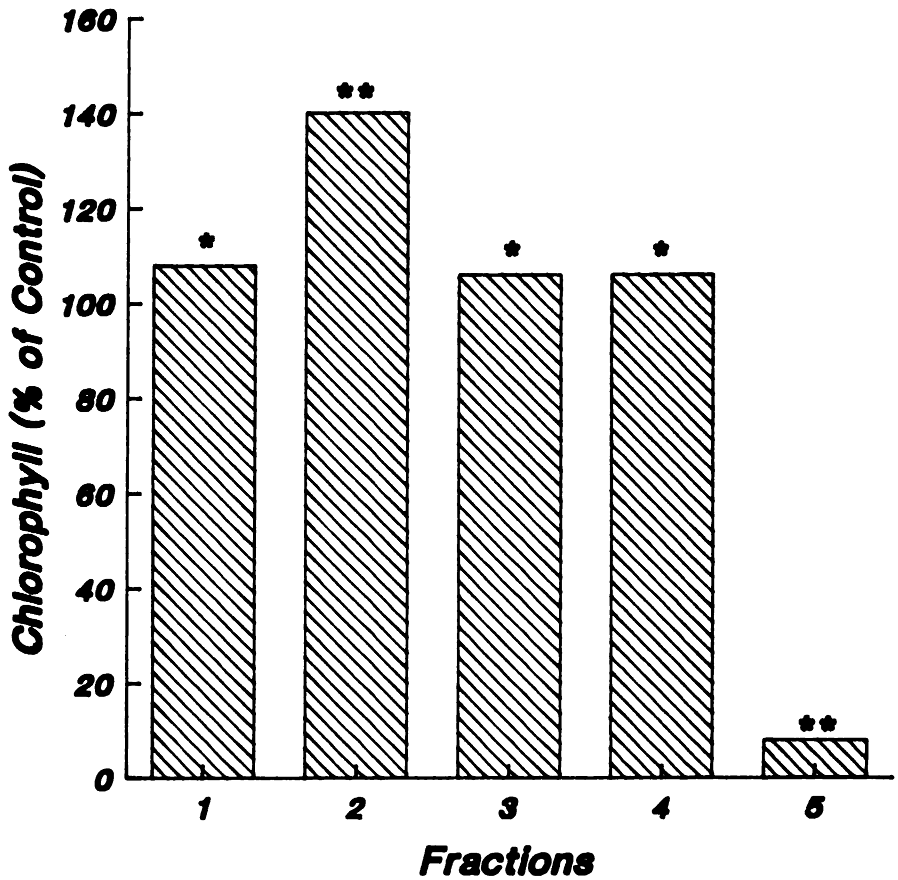

160
140
120
100
80
60
40
20
0
Chlorophyll (% of Control)
*
**
*
*
**
1
2
3
4
5
Fractions

Fig. 6. Retention time by HPLC [CH_3OH:H_2O (30:70), 4 ml/min] when sample passed through HPLC C_{18} column at pH 6 two times (a) and finally at pH 2 (b).

Table 5. Chlamydomonas bioassay of HPLC fraction (Fig. 6a) of fraction 2 from C_{18} flash column of the crude extract of tomato apices. Each fraction was applied at a concentration equivalent to 100 mg/l of the crude extract.

Retention time (min)	Algae growth ug chlorophyll/ml	% over control
5.0-10.0	0.72	14
11.5	1.15	80
12.6	1.26	98
13.6	0.70	9
14.2	0.64	0
14.7	0.65	2
Water control	0.64	-
LSD 5%	0.07	--
LSD 1%	0.11	--
C.V.(%)	2.14	--

sharp peak was obtained (Fig 6b.).

Analysis of the [^{1}H]nuclear magnetic resonance (NMR) spectrum (Fig. 7.) of this single peak in deuterated methanol indicated that it was not pure, but contained substituted amino sugars, amino acids or both. This was substantiated by TLC of the purified extract followed by developing the plate with ninhydrin (Fig. 9b). Four areas on the plate gave a positive test for ninhydrin.

Chemical ionization mass spectroscopy (MS) was used with CH_4 with a scan range from 1-467 (Fig. 8.). At scan 9, 15-74, 76, and 97 the masses were 424, 163, 149,and 216, respectively. Thus, there were at least 4 compounds in that HPLC purified extract of tomato apices. This was also substantiated by TLC (Fig. 9.).

<u>Comparison of purified extract of tomato apices with plant hormones by TLC and effect on algae growth</u>: Tests were conducted to determine if the purified extract of tomato apices contained a compound(s) similar to known plant hormones. This was established by TLC and the algae bioassay.

None of the plant hormones tested significantly increased the growth of algae (Table 6.), as measured by chlorophyll content, at the concentration of 0.1-10.0 mg/l. However, GA and IAA decreased the chlorophyll content of the algae at 100 mg/l. BA increased the chlorophyll content of algae 6% at the highest concentration (100 mg/l). The purified extract from tomato apices increased the chlorophyll content 9 and 43% more than control at 10 and 100 mg/l, respectively. Other tests (Wert and Ries, unpublished data) have

Fig. 7. [^{1}H] nuclear magnetic resonance spectrum of purified extract from tomato shoot apices dissolved in deuterated methanol.

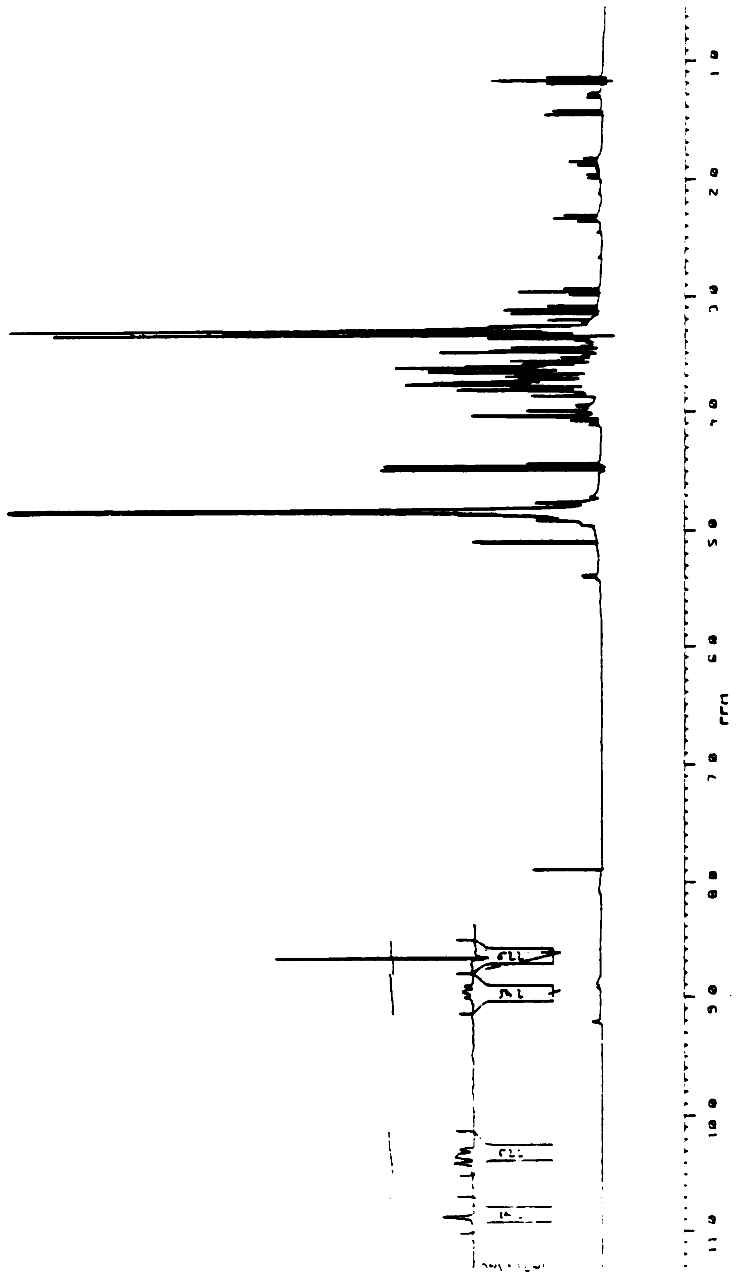
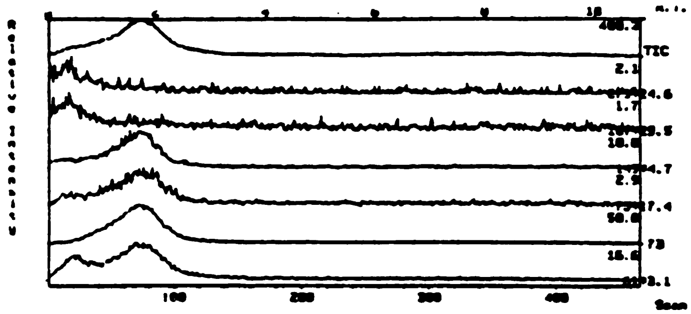
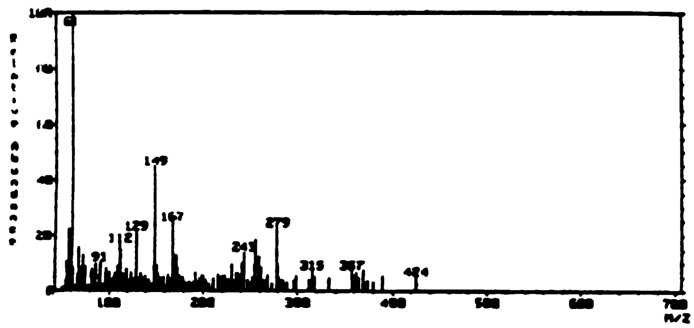


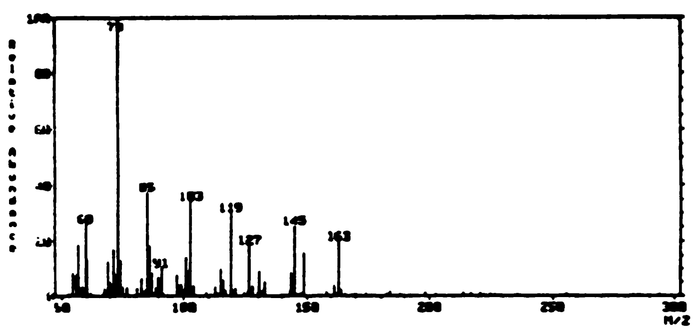
Fig. 8. Chemical ionization MS (CH_4) of purified extract of tomato apices. (a) scan range 1-469, (b) scan range 9, (c) scan range 15-74, (d) scan range 76, (e) scan range 97.



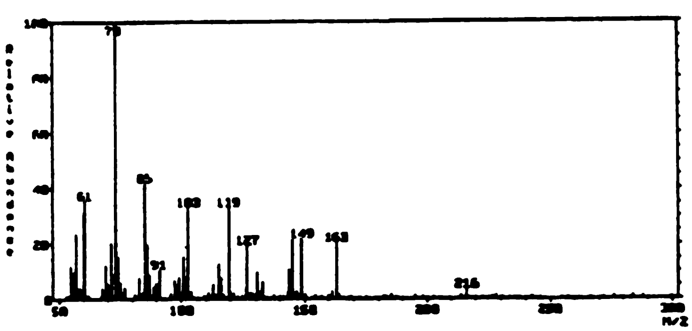
(a)



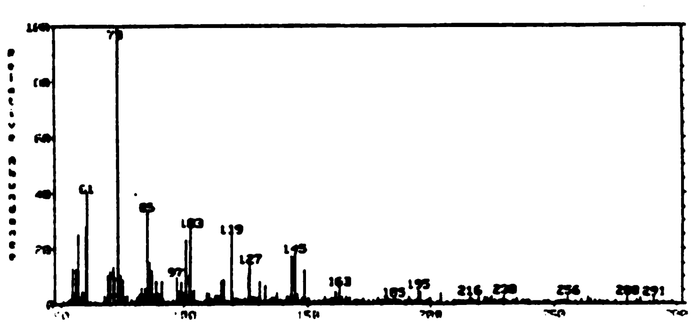
(b)



(c)

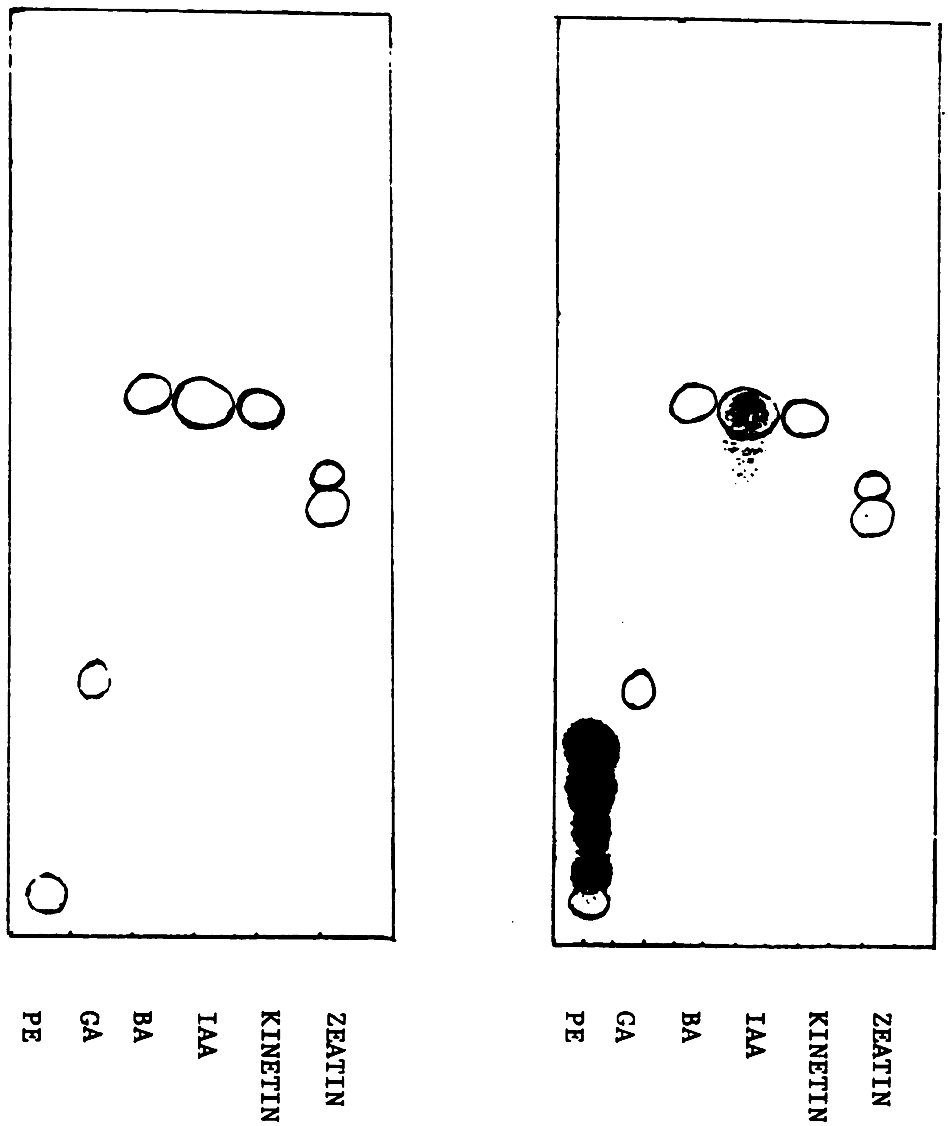


(d)



(e)

Fig. 9. TLC plate (silica gel, $60F_{254}$) comparing the purified extract of tomato apices with several known plant hormones. The plate was run in $CHCl_3:CH_3OH:CF_3CO_2H$ (15:5:0.1). (a) Before spraying with ninhydrin, all of the spots were visible UV light at 254 and 366 nm. (b) After spraying with ninhydrin, 4 of the spots from the purified extrcat gave a positive reaction.



(a) (b)

Table 6. Response of Chlamydomonas to purified extract from tomato apices and plant hormones.

Concentration (mg/l)	ug chlorophyll/ml			
	GA	IAA	BA	PE[z]
Control	2.19	2.23	2.25	1.32
0.1	2.23	2.35	2.27	1.34
1.0	2.27	2.29	2.28	1.37
10.0	2.27	2.29	2.28	1.44
100.0	1.30	1.31	2.38	1.89
LSD 5%	0.13	0.19	0.07	0.04
LSD 1%	0.19	0.30	0.12	0.07
C.V.(%)	3.47	5.41	1.80	1.62

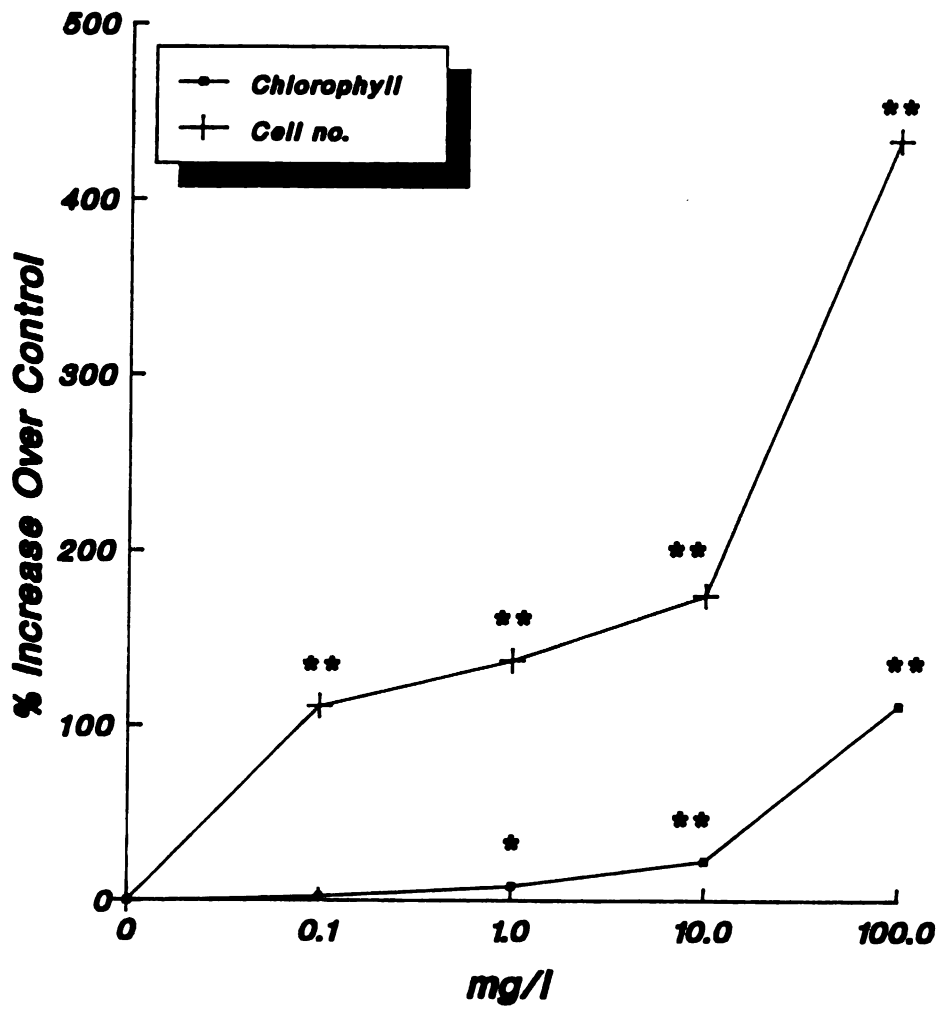
[z]PE: purified extract from tomato apices.

shown that GA_3, ABA, kinetin, IAA, ethylene (ethaphon) and glucose do not increase the chlorophyll content of algae. The same Chlamydomonas assay was used as in this study with concentrations of these chemicals up to 10 mg/l. TLC analysis of the HPLC purified extract of tomato apices indicated that it was more polar than other known plant hormones (Fig. 9.). The NMR spectrum of the purified extract also showed that there were no aromatic groups or olefinic protons. This is further evidence that the purified extract did not contain any known plant hormones. Thus, the NMR analysis, algae bioassay and TLC test indicated that the purified extract from tomato apices was not similar to known plant hormones.

The effect of the purified extract of tomato shoot apices on growth of algae and higher plants: The number of algae cells were counted and the chlorophyll content was determined after 18 hr incubation with the HPLC purified extract from tomato shoot apices. As the concentration of purified extract increased, the chlorophyll content and cell density of Chlamydomonas cultures increased (Fig. 10.). The cell density was increased dramatically (111% in 18 hr) at a much lower concentration (0.1 mg/l) than the chlorophyll content. At 100 mg/l, the purified extract increased both cell division and chlorophyll content in 18 hr 433 and 111%, respectively. Clearly, cell division was affected at lower concentrations than chlorophyll content.

The same cultivar of tomatoes as used to obtain the purified extract was tested to determine if this purified extract would

Fig. 10. Effect of purified extract from tomato apices on cell number and chlorophyll content of Chlamydomonas. The cell number and chlorophyll content for the control were 8.8×10^5 cells/ml and 1.33 ug/ml, respectively. The F value for the linear trend with concentration for both chlorophyll concentration and cell number was significant at 1% level. *, ** LSD was significantly different from control at 1% level.



Chlorophyll
Cell no.
500
400
300
200
100
0
% Increase Over Control
0
0.1
1.0
10.0
100.0
mg/l

increase the growth of tomato plant. In two tests with the same treatments under greenhouse conditions, the purified extract increased growth with all concentrations ranging from 0.01 to 10.0 mg/l (Figs. 11&12.). The effect of the purified extract was not visible until 4 days after treatment. However, after 10 days 100 ug/l increased the total dry weight of the tomato shoots 35 and 24 % in Test 1 and 2, respectively. Both tests showed a quadratic trend of growth increase with concentration. This is typical of many plant growth regulators (16), including triacontanol (40). The most effective concentration which increased the growth of tomato plants was much lower (100 ug/l) than needed to increase the chlorophyll content of algae (10,000 ug/l).

The purified extract of tomato apices increased both water uptake and dry weight of rice seedlings (Table 7.). Thus, this plant extract has been shown to affect the growth of algae, monocotyledonous (rice), and dicotyledonous (tomato) plants.

An efficient extraction procedure was developed for isolation and characterization of a growth promoting substance from tomato apices. It is postulated that this purified extract from tomato shoot apices is a natural product of the plant, and an unknown plant growth regulator.

Fig. 11. Response of 29-day-old tomato seedlings grown in the greenhouse 10 days after application of the purified extract from tomato shoot apices at different concentrations. (1) Water control, (2) 10 ug/l, (3) 100 ug/l, (4) 1,000 ug/l, (5) 10,000 ug/l.


1
2
3
4
5

Fig. 12. Increase in dry weight over control of 29-day-old tomatoes 10 days after application of different concentrations of the purified extract from tomato apices. In Test 1 and 2, the control plants (0 mg/l) weighed 1.74 and 2.02 g respectively at the end of test. The F value for the quadradic trend with increase in concentration was significant at the 1% level for both tests. *, ** significantly different from control at 5% and 1% levels, respectively.

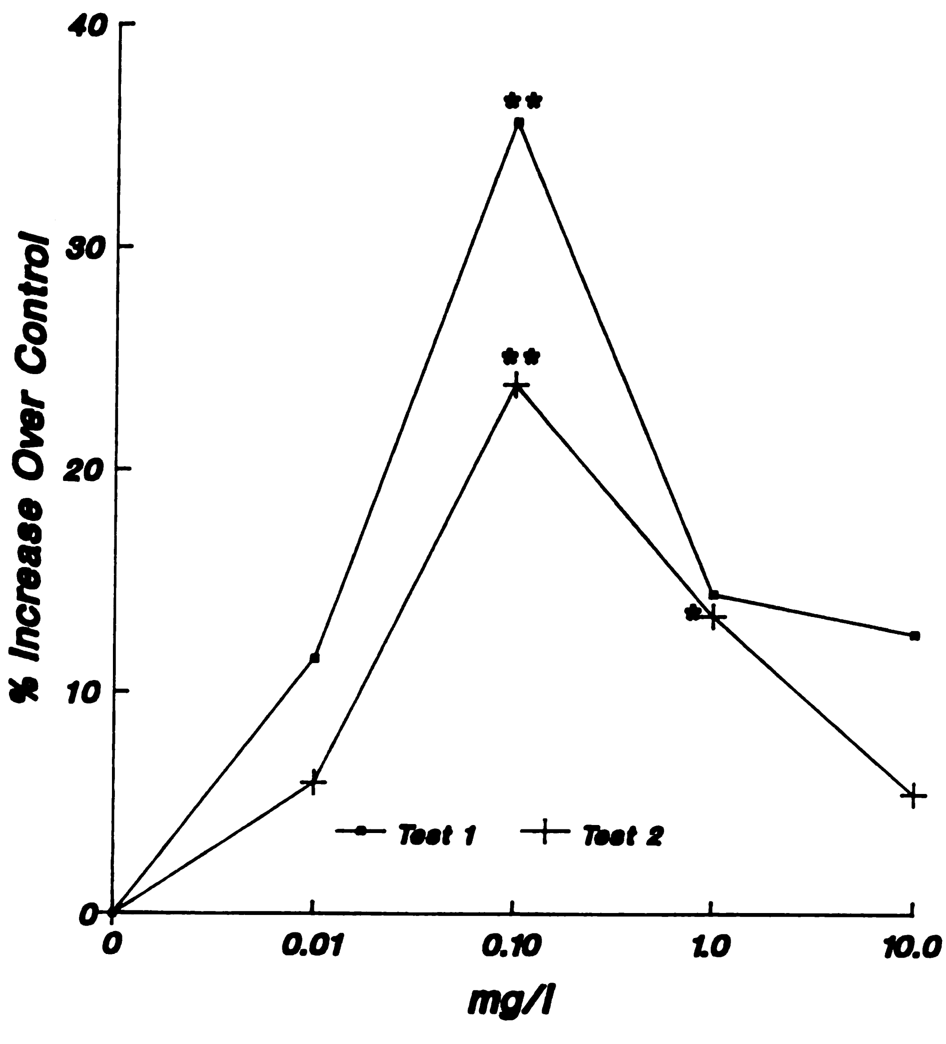

% Increase Over Control
40
30
20
10
0
**
**
*
Test 1
Test 2
0
0.01
0.10
1.0
10.0
mg/l

Table 7. Response of 18-day-old rice seedlings to purified extract isolated from tomato apices.

Treatment (ug/l)	DW (mg/plant)	Water uptake (ul/hr/plant)[z] I	II	III
Control	129.2	229	378	393
100	132.5	231	390	401
1000	136.2	240	400	406
LSD 5%	5.1	NS	16	NS
LSD 1%	7.3	NS	23	NS
C.V.(%)	3.01	4.47	2.89	5.59

[z] I 1st 40 hr after treatment

II 40-102 hr after treatment

III 102-142 hr after treatment

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