1975

Cytokinin-virus interactions in tobacco ringspot virus infected cowpea plants.

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CYTOKININ-VIRUS INTERACTIONS IN TOBACCO RINGSPOT VIRUS INFECTED COWPEA PLANTS

A Thesis Presented

By

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

MASTER OF SCIENCE

September 1975

Plant Pathology

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ACKNOWLEDGEMENTS

Sincere thanks to Dr. George N. Agrios for direction, patience and inspiration throughout my stay at UMass.

I am grateful to Dr. Duane W. Greene and Dr. Mark S. Mount for helpful discussions, encouragement and materials given during the course of this investigation.

Grateful thanks to my mother and father for their love, support and assistance during my academic career.

Best wishes to everyone close and amicable; love to Sigrid for keeping me happy; and thank you too Paul for fun.

CONTENTS

INTRODUCTION

Cytokinins have been isolated and identified from various organs of higher plants. They are known to be responsible for cell division in plant tissue cultures, shoot development and differentiation, release of buds from apical dominance and the delay of senescence in leaf lamina.

Changes in cytokinin concentration are known to occur in a number of plant diseases. These changes are usually associated with symptom expression by the host as a result of infection.

Plant viruses are known to be affected by exogenously applied cytokinins. When leaves of certain plants are floated on cytokinin solutions immediately after inoculation with a virus, local lesion formation is inhibited along with virus production, while under other circumstances local lesion formation and virus production are stimulated. Inhibition or stimulation of plant virus multiplication has been shown to be related to the timing of cytokinin treatment and virus inoculation. All investigations of cytokinin-plant virus interactions have dealt with the effects of cytokinins applied to inoculated leaf tissue.

The effect of virus infection on the production of naturally occurring cytokinins in plant organs has not been examined.

The apical meristems of roots are the source of cytokinins in young plants and tobacco ringspot virus (TRSV) is known to invade and multiply in root meristems. It was considered important, therefore, to determine whether TRSV infection has an effect on cytokinin levels since both virus and cytokinins are being produced in the same tissue in infected plants.

This study was initiated to determine the effects of virus infection and multiplication in roots on cytokinin levels; the effects of leaf treatment with kinetin on virus multiplication in the leaves and in the roots; and the effects of leaf treatment with kinetin on symptom development in TRSV-infected cowpea plants. By determining cytokinin levels in virus infected plants and the effects of kinetin on virus production in and symptoms of inoculated plants we may be able to relate virus-cytokinin interactions in root meristems to symptoms observed in inoculated plants.

LITERATURE REVIEW

Cytokinins, one of the groups of compounds that act as growth regulators in plants, have been isolated from roots, stems and leaves of higher plants. Cytokinin activity in root exudates of many plant species have indicated that these growth regulators are translocated to the shoot from the roots. Flooding the root system of sunflower plants for more than 24 hours reduced the concentration of cytokinins in xylem sap (Burrows and Carr, 1969). After 96 hours of flooding, root apices were dead and cytokinin content of the exuded sap was drastically reduced, indicating that the root apex is responsible for cytokinins found in xylem sap. Kende (1965) demonstrated that two factors contained in root exudate collected from xylem tissue of sunflower plants could induce division in the soybean callus bioassay. Yoshida et al., (1971) identified a nucleotide of zeatin, zeatin [6-(4-hydroxy-3-methyl-2-butenylamino)purine] and a riboside of zeatin in the root exudate of rice plants.

In two-day-old sunflower seedlings, cytokinin activity was greatest in the terminal millimeter of the radicle, while tissue behind the radicle apical meristem showed

slight activity (Weiss and Vaadia, 1965). Similar results were obtained with two-day-old seedling roots of pea (Short and Torrey, 1972). Of three compounds isolated, the compound exhibiting the greatest activity was determined to be zeatin. A cytokinin was also isolated in small concentrations from the transfer RNA of pea root apices.

Isolation and purification of cytokinins from plant tissues commonly involves the use of organic solvents, precipitation with silver salts, ion exchange chromatography and paper chromatography. Variations in isolation procedures abound and much conflicting evidence can be found in the literature as to the amount of active material lost during such procedures.

Hemberg and Westlin (1973) performed model experiments with kinetin, 6-furfurylamino purine, to determine the quantitative yield in purification of cytokinin extracts. Using recrystallized kinetin and redistilled solvents, they determined that when kinetin in a water solution of pH 3.0 was shaken three times with equal volumes of ethyl ether about 50% of the kinetin could be found in the ether. In similar experiments with ethyl acetate an average of 92.3% of the kinetin was found in the ethyl acetate fractions. Both of these solvents are routinely used to eliminate inhibitors in extraction procedures and are subsequently discarded. Experiments with petroleum

ether and hexane showed no loss of cytokinins into these solvents. When 1-butanol was used to wash water solutions containing kinetin, 95% of the kinetin dissolved into the 1-butanol. Butanol is therefore commonly employed to recover cytokinins in extractions.

Letham (1974) performed similar experiments but included naturally occurring cytokinins in his work for comparison. The results demonstrated that kinetin was readily lost into ethyl ether or ethyl acetate, as Hemberg and Westlin had shown, but zeatin and zeatin riboside, which are cytokinins commonly occurring in higher plants, remained primarily in the water phase after washing. Losses of zeatin and of zeatin riboside into the solvents were 3.2% and 1.2%, respectively, into ethyl ether and 14.4% and 11.1%, respectively, into ethyl acetate after 3 fractionations. Another naturally occurring cytokinin, 2iP, 6-(3-Methyl-2-butenylamino) purine, showed considerable loss, exceeding the loss of kinetin in the ethyl ether and ethyl acetate phases. Letham states that 2iP contributes little, if at all, to the cytokinin activity of extracts of many higher plants. Since it is found in a strain of tobacco callus tissue that does not require an exogenous supply of cytokinin for growth in culture, Letham cautions the use of these solvents in some instances. Petroleum ether and n-butanol proved to be valuable solvents with all cytokinins tested for isolation.

To determine cytokinin activity in plant extracts some form of a bioassay system is commonly used. Two of the most frequently utilized bioassays are the soybean callus tissue and the tobacco stem pith tissue bioassays. Both are specific and sensitive (Letham, 1967).

The soybean callus bioassay (Miller, 1963), exhibits a linear relationship with response over a range of 4-10,000 µg/liter of kinetin. No other substances are active in this assay although the activity of gibberellins appears to have not been determined (Letham, 1967). The tobacco stem pith tissue bioassay shows a linear relationship to response over a range of 1-15 µg/liter of kinetin. Gibberellic acid has been shown to have slight activity in this assay compared to that of cytokinins (Letham, 1967). Gibberellin added to culture medium at a standard dose (1 mg/liter), provides a background response to these compounds eliminating further response by gibberellins in extracts (Murashige and Skoog, 1962).

Cytokinin-plant virus interactions have been studied with respect to cytokinin effects on virus multiplication when applied to leaves or leaf-disks prior to or after inoculation with the virus.

Daft (1963) floated leaf sections of tobacco, Nicotiana glutinosa on kinetin solutions before inoculation

with tomato aucuba mosaic virus and found a slight increase or decrease in the number of local lesions produced in this type of experiment. Spraying intact N. glutinosa leaves with kinetin 4 days before and 5 days after inoculation significantly increased lesion production.

Kiraly and Szirmai (1964) found that leaf disks of N. glutinosa inoculated with tobacco mosaic virus (TMV) and immediately floated on kinetin solutions showed a significant decrease in virus multiplication of 70% as measured by the numbers of local lesions produced on the leaf disks. The diameter of the lesions produced on kinetin treated leaf disks was reduced and the appearance of the lesions was delayed 30-35 hours. The kinetin treated leaf disks were also 77% less infective when homogenized and assayed on N. glutinosa half-leaves. Forty to 50 ppm kinetin gave the highest inhibition of the virus in these experiments. The combined effect of 128 ppm thiouracil and 50 ppm kinetin resulted in the complete inhibition of virus production on N. glutinosa leaf disks. Treatment of detached leaves via petioles placed in a 30 ppm kinetin solution for 48 hours before inoculation followed by cutting leaf disks from the leaves and floating them on a 40 ppm kinetin solution immediately after inoculation resulted in an 80-90% reduction in virus production. The daily spraying of

leaves of intact plants for 5 days with 50 ppm kinetin before inoculation did not influence virus multiplication on the treated leaves.

Aldwinckle and Selman (1967) found that N-6-benzyladenine (BA) applied 24 hours before and 72 hours after inoculation was as effective as kinetin in decreasing numbers of local lesions and the infectivity of tomato spotted wilt virus. Numbers of local lesions and infectivity were also decreased in attached leaves when BA was applied 2 days before inoculation. When BA was supplied to the lower leaves of tomato plants before inoculation with TSWV, the infectivity of systemically infected, unsprayed tip leaves used as inoculum was reduced. BA supplied to attached leaves after inoculation increased infectivity, and increased the infectivity of unsprayed tip leaves used as inoculum.

Selman (1964) found that when kinetin was supplied to petunia leaf strips 4 hours before and 3 days after inoculation with TSWV, both the lesion size and numbers were reduced. Kinetin treatment immediately after inoculation reduced by half the number of local lesions produced. Selman postulated that, because kinetin causes an increase in RNase in tissue, the increased RNase may be responsible for reduced virus production. Aldwinckle and Selman (1967), felt that more mRNA was produced with kinetin treatment

and the demand for ribonucleotide triphosphates and their precursors would restrict the capacity of the newly introduced viral RNA to replicate and initiate production of virus protein. Therefore, protein and virus concentration would remain low so long as the kinetin supply was maintained. This hypothesis was challenged when Daft (1965) found that treatments producing more tomato aucuba mosaic virus in tobacco were associated with increased RNase activity.

As mentioned earlier, in seedlings cytokinins are produced in the terminal millimeter of the radicle (Weiss and Vaadia, 1965; Short and Torrey, 1972). Certain plant viruses are unable to infect the meristematic region of roots, which lies within the distal millimeter of the root (White, 1943; Crowley and Hanson, 1960). The lack of multiplication of plant viruses in apical meristems of the root and shoot has been of interest because of the importance of culturing of virus-free clones of commercially important plants and because of the relationship of meristematic infection and the ability of several plant viruses to be seed-transmitted (Crowley and Hanson, 1960).

White (1943) showed that the root meristems of tomato plants infected with TMV were virus free when grown in culture medium. Crowley and Hanson (1960) confirmed White's results and found that the distal 4 mm of roots

of TMV-infected tomato plants grown in water culture were also virus free. When ethylenediamine-tetraacetate (EDTA) was added to the culture medium, virus was recovered from the distal 2 mm sections of the roots. Smith and Schlegel (1964) were unable to detect clover yellow mosaic virus in the terminal 0.4 mm sections of broad bean root tips.

By employing infectivity assays, Crowley et al., (1969) showed that tobacco ringspot virus (TRSV) but not TMV was capable of infecting the terminal 0.5 mm of root tips of French bean plants. TMV was never recovered from the distal 0.5 mm region but was found in regions 0.5-1.0 mm and 1.0-1.5 mm from the root tip. Both viruses were found in root tips 4-5 days after leaf inoculation. TRSV reached much higher concentrations in root tips than in leaf tissue while TMV reached higher concentrations in leaf tissue than in roots. Crowley reported that roots of TRSV-infected bean plants were severely stunted, whereas the root growth of TMV-infected beans was not affected. The presence of TRSV in root tips was confirmed by electron microscope detection of virus particles in the dividing cells of root tips and in the cells of the inner root cap.

Atchison and Francki (1972) found that TRSV invaded the terminal millimeter of French bean roots about 3 days after inoculation of primary leaves. They, too, noticed root stunting and determined that virus invasion of the

terminal millimeter of roots always preceded the reduction in growth rate of the roots. Virus synthesis occurred in root tip cells for about one day following invasion, after which it appeared to cease. The virus concentration in the terminal 5 millimeters of the roots, however, remained constant during the course of the experiment, suggesting that it was due to virus translocated to the roots from other parts of the plant.

Atchison (1973) determined that DNA synthesis decreased in TRSV-infected French bean root tips, the greatest drop occurring 3 or 5 days after inoculation, depending on the technique used to determine amounts of DNA. The decrease in DNA was closely followed by a decrease in the mitotic index of the root tips, for about 3 days before returning to normal. Cell elongation was unaffected by virus infection using the methods applied.

Since TRSV is capable of invading and multiplying in the apical meristem of roots and since cytokinins are known to be produced in the root apical meristem also, knowledge of TRSV affects on cytokinin production in infected root tissue will help in understanding why certain viruses are capable of infecting apical meristematic tissue and what happens when they are present. Also, the nature of symptoms caused by TRSV in cowpeas and the relationship between virus infection or replication, cytokinin

levels and symptom development warrant the present investigation of TRSV-cytokinin interaction.

MATERIALS AND METHODS

The isolate of tobacco ringspot virus used in this investigation, strain ATCC PV 157, was kindly supplied by Dr. J. K. Uyemoto of the Plant Pathology Department, New York State Agricultural Experiment Station, Geneva, N.Y.

Maintenance of the Virus

Tobacco ringspot virus (TRSV), strain ATCC PV 157, was maintained on tobacco, Nicotiana tabacum, variety "Samsun NN." Inoculum was prepared by collecting 10 grams of tobacco leaves showing ringspot symptoms and grinding them in a mortar with a pestil in 100 ml of 0.1M phosphate buffer pH 7.0. The homogenate was then strained through 4 layers of cheesecloth and the sap, to which 1.5% Celite was added, was kept in an ice bath until it was used for all inoculations of cowpea, Vigna sinensis, variety "Early Ramshorn" and tobacco plants. Plants were inoculated by rubbing the leaves with fingers previously dipped into the infected tobacco sap. The time of symptom appearance after inoculation varied with environmental conditions, but first symptoms usually occurred on tobacco plants 5 days after inoculation and on cowpea plants 3 days after inoculation.

Collection of Root Material

Cowpea seeds dusted with captan were planted in the greenhouse in 20 X 12.5 X 8.75 cm plastic flats containing a steam sterilized mixture of equal parts of soil and sand. The plants were fertilized once during the growing period. Plants were inoculated with virus-infected tobacco sap when the primary leaves were fully expanded and the first trifoliate leaves were beginning to expand, i.e., about 10-14 days after planting. Seven days after inoculation, when the plants were showing symptoms consisting of local lesions, petiole necrosis and the beginning of stem necrosis and wilt, the soil was allowed to become somewhat dry until the roots could be easily separated from it and could be harvested. The flats containing the plants were turned upside down over a bucket of water and the roots were freed of the soil by shaking followed by two rinses with water. After the second rinse the roots were clean and free from soil. The root systems were separated from the stems and leaves and both healthy control plants and TRSV-infected plants were blot dried with paper towels. The fresh weights of shoots and roots were measured.

Collection of Sap

Cowpea seeds dusted with captan were planted in the greenhouse in 15 cm pots in a 1:1:1 mixture of soil,

sand, and peat moss. During the experiment the plants were fertilized once and the soil was drenched once with a captan solution. The plants were inoculated as described above. Sap was collected 3, 5, and 7 days after inoculation. For each collection 300-350 TRSV-infected and an equal number of healthy plants were used. The plants were water-saturated at 12 hours and at 2 hours before their tops were cut with a razor blade at 2.5 cm above the soil line. Sap droplets were collected from each cut stem with a pasteur pipette over a 5 hour period by alternately collecting sap from the healthy plants then from the TRSV-infected plants, and so on. An equal number of collections were made. The collected sap was kept in an ice bath until measured and then it was frozen at -20°C in a freezer where it was stored up to 8 months.

Cytokinin Extraction from Root Material

Eighty grams (fresh weight) of healthy and of TRSV-infected root tissue was ground in a Waring blender for 2 minutes in 200 ml of 80% ethanol and was further washed with an additional 100 ml of 80% ethanol. The extract was allowed to set in a refrigerator at 5°C for 6-12 hours. The extract was then filtered through a Buchner funnel fitted with 3 sheets of Whatman No. 1 filter paper and through a course millipore funnel. The residue on the filter paper was washed with 100 ml of 80% ethanol. The

extract and washing were combined and were evaporated *in vacuo* at 50°C in a flash evaporator. The original volume of 400 ml was reduced to 60 ml and was emptied into a 250 ml beaker. The evaporatory flask was washed with 40 ml of distilled water in 2 aliquots and this was added to the extract. The 100 ml extracts of healthy and TRSV-infected plants were brought to pH 9.0 with 1.0 N NaOH and were shaken 3 times, each time for two minutes, with equal volumes of petroleum ether to remove lipids. After each shaking, the phases were allowed to separate and clear before they were fractionated. The petroleum ether fractions were discarded and the aqueous fractions saved. The pH of the aqueous fraction was adjusted to pH 2.5 with 1.0 N HCl and shaken 3 times, each time for 2 minutes with ethyl acetate to remove acid gibberellins, auxins, abscisic acid and phenolics. The phases were separated as before and the aqueous fractions were again saved while the ethyl acetate fractions were discarded. The pH of the aqueous fractions was adjusted to pH 2.3 with HCl. Ten ml of cold 50% saturated $AgNO_3$ was added to each aqueous fraction and the fractions were stirred in the coldroom for 12 hours. The precipitate that formed was collected by centrifugation at 7,000g for 15 minutes. The supernatant was poured off and the pellet was mixed with 40 ml of 0.2 N HCl at 50°C for 30 minutes. This resulted in the removal of the

free purines from the silver ions. The reacted precipitate was collected by centrifugation at 7,000g for 15 minutes and the supernatant was saved. This procedures was repeated and the two 0.2 N HCl fractions were combined. The pH of the HCl fractions was adjusted to pH 8.0 with 1.0 N NaOH and these fractions were shaken 4 times, for 2 minutes each time, with equal volumes of n-butanol. The phases were separated as before and the n-butanol fractions were combined. The n-butanol was evaporated to dryness in vacuo at 50°C. The residue on the evaporation flask was washed twice with 10 ml aliquots of 80% ethanol. The washings were combined and transferred to a small evaporatory flask and again evaporated to dryness. The residue on the flask was dissolved in 2 ml of 80% ethanol and streaked in a 1 cm wide band on a 9 X 57 cm sheet of Whatman 3 MM chromatography paper. The chromatogram was run in descending fashion in a 12:3:5 solution of n-butanol, acetic acid, and water overnight (30 cm) and then removed from the tank, air dired, and prepared for bioassay.

Bioassay.--Maintenance of Tobacco Stem Pith Callus Tissue.

Tobacco stem pith callus tissue was obtained from greenhouse grown "Samsun NN" tobacco plants. Stem sections 10 cm long obtained from the stem area 10 cm below the apex of 40 cm tall plants, were used as source material

for pith. The 10 cm long stem sections were cut into 3 equal sections and surface sterilized with 25% ethanol. The pith from each section was then removed aseptically with a number 1 cork borer. Slabs of tissue were cut from the core of the pith and individual slabs of 30 mg were placed on Linsmaeir and Skoog's medium (1965), which included thiamin at a concentration of 0.4 mg/l, kinetin at 0.075 mg/l, and naphthalene acetic acid at 2 mg/l. After 4 weeks at 27°C in the dark, callus tissue had formed on the slabs and this tissue was used for subculturing on new medium. The callus was stored in an incubator at 27°C in the dark until needed for bioassays.

Bioassay Procedure

After paper chromatography of the purified root extracts, the dried paper chromatograms were cut longitudinally into 3 equal sections, each section corresponding to 26.6 grams of root tissue fresh weight. Each longitudinal section of the paper was then divided into ten equal sections corresponding to the 10 Rf values from the origin to the front. The individual sections corresponding to the Rf values were cut out and placed in individual 110 ml wide-mouthed jars. Each section in each jar was eluted overnight with 5 ml of distilled water. The next day 20 ml of medium lacking kinetin was added to the jars. The jars were then covered with aluminum foil and autoclaved.

The medium used for all bioassays was that described by Linsmaeir and Skoog (1965). The medium contained 0.4 mg/l thiamin HCl and 2 mg/l NAA, but it did not contain kinetin. The medium used for sap bioassays also contained 1 mg/l gibberellic acid.

Kinetin standards were prepared at concentrations of 1.0, 5.0, 10.0, and 25.0 µg/l and added to 20 ml of medium as a 5 ml aliquot. Medium for the standards for purified extracts and for sap samples was prepared as described above.

When the autoclaved medium had cooled off, 3 pieces of tobacco stem pith callus tissue, each weighing approximately 10 mg, were added to each jar under sterile conditions. All jars for each experiment were incubated together for 28 days at 27°C. At the end of the incubation, the 3 tissue pieces were taken out of each jar and their fresh weight was determined on a Mettler balance.

Detection and Measurement of TRSV in the Terminal Millimeter of Cowpea Roots

Cowpea seeds were germinated between paper towels kept moist with a water solution containing streptomycin (25 mg/l) and were maintained in a Percival growth chamber. After 4 days the seedlings were placed on a wire grid support and suspended into a 12 liter tank containing a commercial nutrient solution of major and minor elements

required for plant growth (General Biological Inc., Chicago). An iron sodium salt of ethylenediamine-tetraacetate (FeNaEDTA) was supplied at 71.0 mg/12 liters for the iron requirement. The tanks were aerated with an air pump during the experiment. The plants were grown at 27°C with a 14 hour light period. When the plants had produced fully expanded primary leaves and their first trifoliate leaves were expanding, they were inoculated with TRSV-infected tobacco sap. Beginning with the third day after inoculation, terminal root tips were assayed daily for virus. The assay procedure involved the harvesting of two plants from the tanks each day and washing their root systems with distilled water. The terminal millimeters of 4 washed roots were cut off and arranged on a flat glass spatula. Two drops of 0.1M phosphate buffer pH 7.0 and a small amount of Celite were placed on the root tips. A flat porcelain homogenizer was used to grind the root tips on the glass spatula and the homogenate was rubbed onto individual half-leaves of assay plants. Cowpea plants were used as assay plants. Cowpeas were planted daily beginning 3 days after the experimental plants began to germinate. The cowpeas were planted in a 1:1:1 mixture of soil, sand, and peat in 10 cm pots. They were grown in the same growth chamber with the experimental plants. Eight half-leaves were inoculated each day and local lesion counts were made 4 days after inoculation.

Symptom development was recorded daily beginning on the day after inoculation of the experimental plants.

Kinetin Treatment and TRSV Inoculation

Cowpea seeds dusted with captan were planted 3 per pot in 10 cm pots containing a steam-sterilized mixture of equal parts soil, sand and peat in the greenhouse. In each experiment 12 pots were used for each treatment. Treatments included plants not treated with kinetin and either inoculated or not inoculated with virus and plants treated with kinetin and either inoculated or not inoculated with virus. Cowpea primary leaves were treated with kinetin by soaking a pad of cheesecloth with a 10^{-4}M solution of kinetin and rubbing the pad onto the leaves once a day. Kinetin treatment of leaves began 6 or 7 days after planting and was repeated daily for 4 days before inoculation with TRSV. On the day of inoculation kinetin was applied to treated plants 2 hours after inoculation. Kinetin treatment continued daily following inoculation. Symptom development was recorded daily.

Fresh Weight Determinations

Beginning 3 days after inoculation, plants in 1 pot from each treatment were harvested daily for fresh weight measurement of shoots and roots.

TRSV Multiplication in Root Tissue

On each day, a portion of the weighed root material from virus-inoculated plants was used for local lesion assay to determine the concentration of infective virus in the roots. One gram of washed and blotter-dried root tissue from kinetin-treated, inoculated plants and from untreated, inoculated plants was ground in 3 ml of 0.1 M phosphate buffer pH 7.0. Celite (1.5%) was added to the sap. This inoculum was applied to eight cowpea half-leaves and local lesions were counted 4 days after inoculation.

Statistical Analysis

Statistical analysis of the experimental data was accomplished by the use of the Student's *t* test. In experiments in which the comparison was between the effects of tobacco ringspot virus on growth and on cytokinin levels in cowpea plants, and of kinetin treatment on virus multiplication in cowpea primary leaves, all comparisons involved two sample means. In experiments comparing fresh weight of shoots and roots from kinetin-treated, virus-inoculated plants, the means of the 2 treatments were compared in each test.

Two tests were used in comparisons. When unequal numbers of observations made up the sample mean of the experiments tested, a test for two sample means, unpaired

observations, equal variances was used (Steel and Torrie, 1960). The linear additive model of the t test was employed when an equal number of observations made up the sample mean of the experiments under comparison (Steel and Torrie, 1960).

RESULTS

Detection and Measurement of TRSV in Root Tips

Virus was detected in the terminal millimeter of root tips of cowpea plants beginning 3 days after inoculation of the primary and trifoliate leaves (Table 1). Symptoms at that time consisted of red necrotic local lesions on the inoculated leaves. Not all root tips sampled at 3 days contained detectable virus. Root tip samples obtained 4 days after inoculation produced similar results. Five days after inoculation, when symptoms consisted of necrotic local lesions, yellowing of primary leaves and petiole necrosis, virus concentration in root tips, as measured by local lesion assay, reached the highest levels of all the daily samplings. At day 5 all root tips sampled produced local lesions on assay half-leaves. Seven days after inoculation, shoot symptoms consisted of petiole and stem necrosis and the majority of plants began to wilt, while the roots showed a lack of new root growth as compared to healthy control plants. The virus concentration in root tips assayed on the seventh day was only about 40% that of root tips assayed two days earlier, i.e., 5 days after inoculation. At that time the average shoot weight of TRSV-infected plants was only half that of the average weight of healthy plant shoots (Table 2). The

average root weight of TRSV-infected plants was 71% of the root weight of healthy plants. On day 9, when the shoots of all assayed plants were completely necrotic, there was a further marked reduction in virus concentration in root tips. On days 9 and 10 virus was not detectable in all root tips assayed and was present in rather small quantities in those root tips that yielded virus (Table 1).

Sap collection

During the 5-hour period in which sap was collected on the third, fifth, and seventh days after inoculation, approximately 20 ml of sap were collected from healthy and another 20 ml from TRSV-infected plants. Equal amounts of sap were collected from healthy and from TRSV-infected plants 3 and 5 days after inoculation. On the seventh day after inoculation, the amount of sap collected from TRSV-infected plants was consistently smaller than that collected from healthy plants (Table 3). The average reduction was 15.7%, but the difference was not statistically significant. When TRSV-infected plants were detopped later than 7 days after inoculation, much less sap could be collected from infected plants compared to that from healthy control plants.

Table 1. Detection and measurement of tobacco ringspot virus (TRSV) in the terminal millimeter of roots from leaf-inoculated cowpea plants as determined by daily local lesion assays on cowpea half-leaves.

Experiment Number	Days after leaf inoculation with TRSV								
	2	3	4	5	6	7	8	9	10
1.	0[a]	1.5	7.5	191.5	84.0	58.0	45.0	0	0
2.	0	1.3	0	186.5	134.2	95.6	58.1	2.1	0.3
Average	0	1.4	3.7	189.0	109.1	76.8	51.5	1.0	0.1

[a]Each number represents the average number of local lesions produced on each of 8 half-leaves inoculated with sap from the terminal millimeter of roots of leaf-inoculated cowpea plants.

Table 2. Fresh weight of shoots and roots of healthy and TRSV-infected cowpea plants 7 days after inoculation.

Experiment Number	Shoot Weight		Root Weight	
	Healthy	TRSV-infected	Healthy	TRSV-infected
1.	2.58[a]	1.75	0.49	0.35
2.	2.96	1.07	0.38	0.30
3.	2.55	1.07	0.58	0.33
4.	3.02	1.82	0.67	0.57
Average	2.84	1.42	0.53	0.38

[a]Average weight per plant in grams, 12 plants per treatment.

Table 3. Amounts of sap in milliliters collected from healthy (H) and tobacco ringspot virus (TRSV)-infected cowpea plants cut above the soil line at various times after inoculation.

Experiment Number	Days after inoculation					
	3		5		7	
	H	TRSV	H	TRSV	H	TRSV
1.	18.0	19.5	21.5	19.5	19.0	16.7
2.	20.7	19.0	25.2	22.0	24.0	22.2
3.	19.2	19.5	18.3	20.0	27.5	18.3
4.	26.3	25.6	19.9	21.0	23.6	22.1
Average	21.0	20.9	21.2	20.6	23.5	19.8

Bioassay of Sap

In preliminary experiments aimed at measuring the increase in growth of tobacco stem pith callus tissue produced by various amounts of crude, centrifuged sap, the various amounts of sap were added to 20 ml of Linsmaier and Skoog medium, which changed the final volume. An increase in the final fresh weight of callus pieces was produced and was almost proportional to the amount of sap added. Thus, addition of 0.5 ml of sap produced 62% more fresh weight than did control pieces of tissue; 1.0 ml produced a 170% increase, 2.0 ml a 281% increase and 5.0 ml a 672% increase in fresh weight over control pieces (Table 4).

Cytokinin activity from 2 ml of crude centrifuged sap placed in 10 ml of medium did not correlate exactly with the other results of the preliminary experiments but it did produce a reproducible increase in growth of callus tissue placed on the medium that corresponded to the increase produced by 1.0 ml of sap in the preliminary experiments. The fresh weight increases produced by sap collected from healthy and TRSV-infected plants 3 days and 5 days after inoculation produced slight differences in cytokinin activity which, however, were not statistically significant even at the 0.1 probability level. Healthy sap collected 7 days after inoculation had 43% more

Table 4. Detection and measurement of cytokinins in crude, centrifuged sap from healthy cowpea plants as determined with the tobacco stem pith callus tissue bioassay.[a]

Flask Number	Callus Piece Number	Ml of sap/20 ml of medium				
		0.0	0.5	1.0	2.0	5.0
1.	1.	40[b]	82	100	165	360
	2.	53	57	126	135	415
	3.	53	81	155	135	315
2.	1.	48	72	85	225	396
	2.	45	96	135	180	370
	3.	36	60	143	209	268
	Average	45.8	74.6	124.0	174.8	354.0

[a]Complete tissue culture medium minus kinetin

[b]Each number represents the fresh weight increase of the individual pieces of callus tissue at the end of 28 days of growth.

cytokinin activity than sap collected from the TRSV-infected plants at the same time (Table 5). This difference was statistically significant at the 0.1 probability level.

Sap obtained from both healthy and TRSV-infected plants 3 days after inoculation showed cytokinin activity corresponding to kinetin concentrations ranging between 1 and 5 µg/l, as judged by the response of standards (Table 5, Fig. 1). Healthy and TRSV-infected sap obtained from plants 5 days after inoculation showed cytokinin activity that was slightly less than that shown by 1 µg/l kinetin. Healthy plant sap collected 7 days after inoculation showed cytokinin activity corresponding to responses obtained between 1 and 5 µg/l kinetin, while sap from TRSV-infected plants showed cytokinin activity that was smaller than the activity shown by 1 µg/l kinetin.

Cytokinin Extraction from Root Tissue

The purification of ethanolic extracts of cowpea plant roots with petroleum ether, ethyl acetate and n-butanol, precipitation with $AgNO_3$ and paper chromatography provided extracts that gave reproducible results in the tobacco stem pith tissue bioassay.

Bioassay of Root Extracts

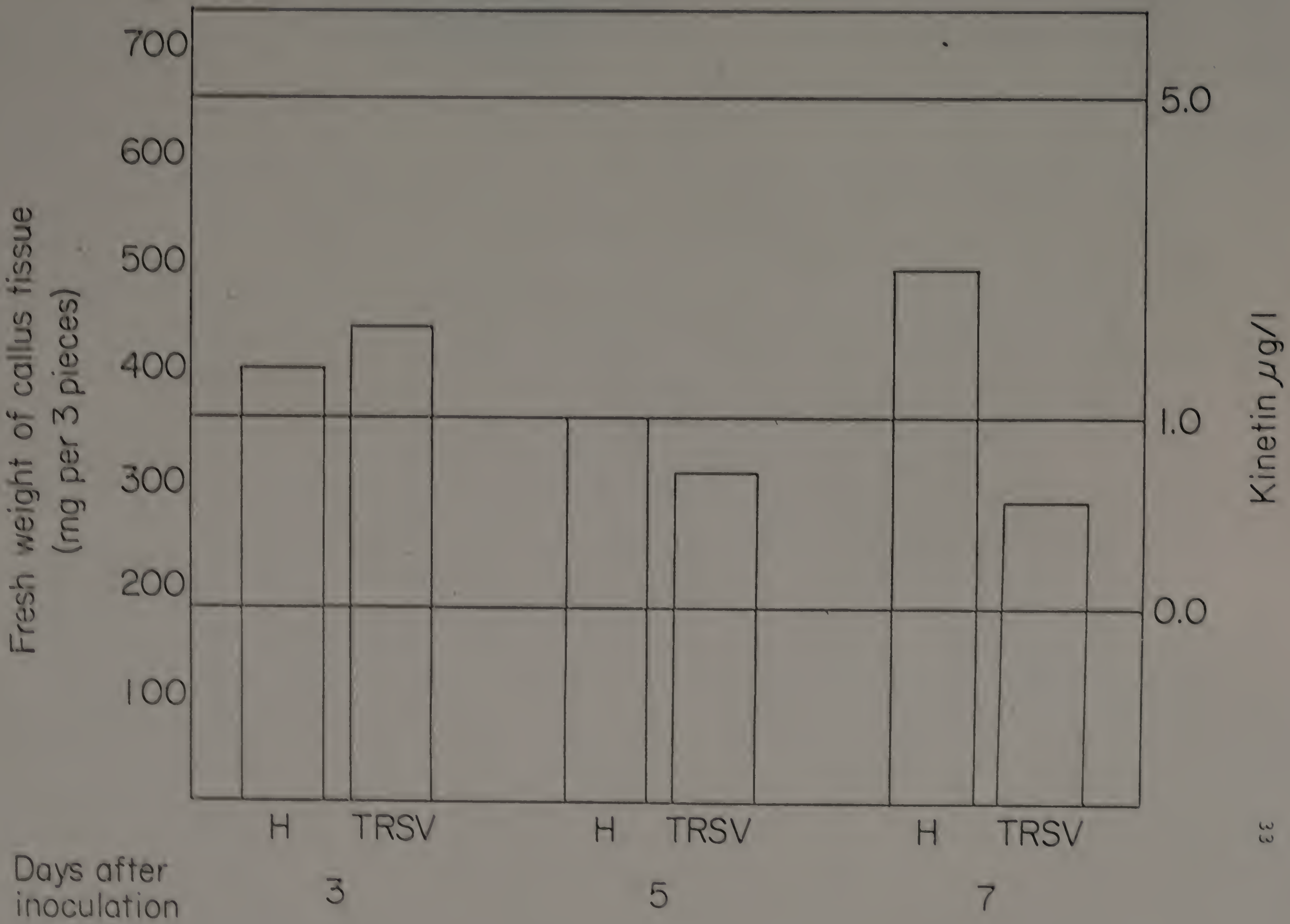
Two peaks of cytokinin activity were observed in bioassays of strips of paper chromatograms obtained with

Table 5. Cytokinin levels in crude, centrifuged sap collected from healthy (H) and tobacco ringspot virus (TRSV)-infected cowpea plants determined with the tobacco stem pith callus bioassay.

Experiment Number	Days after inoculation					
	3		5		7	
	H	TRSV	H	TRSV	H	TRSV
1.	496.2[a]	722.0	402.7	262.0	846.2	379.0
2.	498.0	377.0	459.0	277.3	277.3	279.3
3.	351.6	287.5	257.0	332.6	402.0	235.0
4.	253.5	331.0	304.0	264.0	411.0	207.0
Average	399.8	429.3	355.6	301.3	484.1	275.0
Kinetin standards ug/l	0.0		1.0	5.0	10.0	25.0
	176.0		365.0	648.5	926.6	1,400.0

[a]Each number represents the average weight in milligrams of 3 replications of callus tissue growing on 10 ml of Linsmaeir and Skoog medium.

Figure 1. Histogram of tobacco stem pith callus bioassay of 2 ml crude, centrifuged sap from healthy (H) and tobacco ringspot virus (TRSV) infected cowpea plants collected 3, 5, and 7 days after inoculation.


Fresh weight of callus tissue
(mg per 3 pieces)
700
600
500
400
300
200
100
5.0
1.0
0.0
Kinetin μg/l
H
TRSV
H
TRSV
H
TRSV
Days after
inoculation
3
5
7

purified extracts from healthy and TRSV-infected cowpea plant roots (Table 6, Fig. 2). In extracts from healthy plants the sections with an Rf value of 0.5 produced a small peak of activity corresponding to approximately 1µg/l kinetin. The cytokinin activity of this peak was 27.5% greater than the activity of the corresponding peak produced by extracts from TRSV-infected plant roots. The difference between the two activities, however, was not statistically significant. The second, major peak of cytokinin activity in healthy extracts was at Rf 0.8 and it produced slightly greater callus tissue growth than that produced by the kinetin standard of 5.0 µg/l. The activity from healthy extracts was 31.5% greater than that produced by extracts from TRSV-infected roots. The difference in cytokinin activity between healthy and TRSV-infected root tissue extracts at the 0.8 Rf value was statistically significant at the 0.05 probability level. On the chromatogram, this peak corresponded to the migration of zeatin riboside and zeatin in a 12:3:5 solvent of n-butanol/acetic acid/water.

Kinetin Effects on TRSV Multiplication in Inoculated Primary Leaves

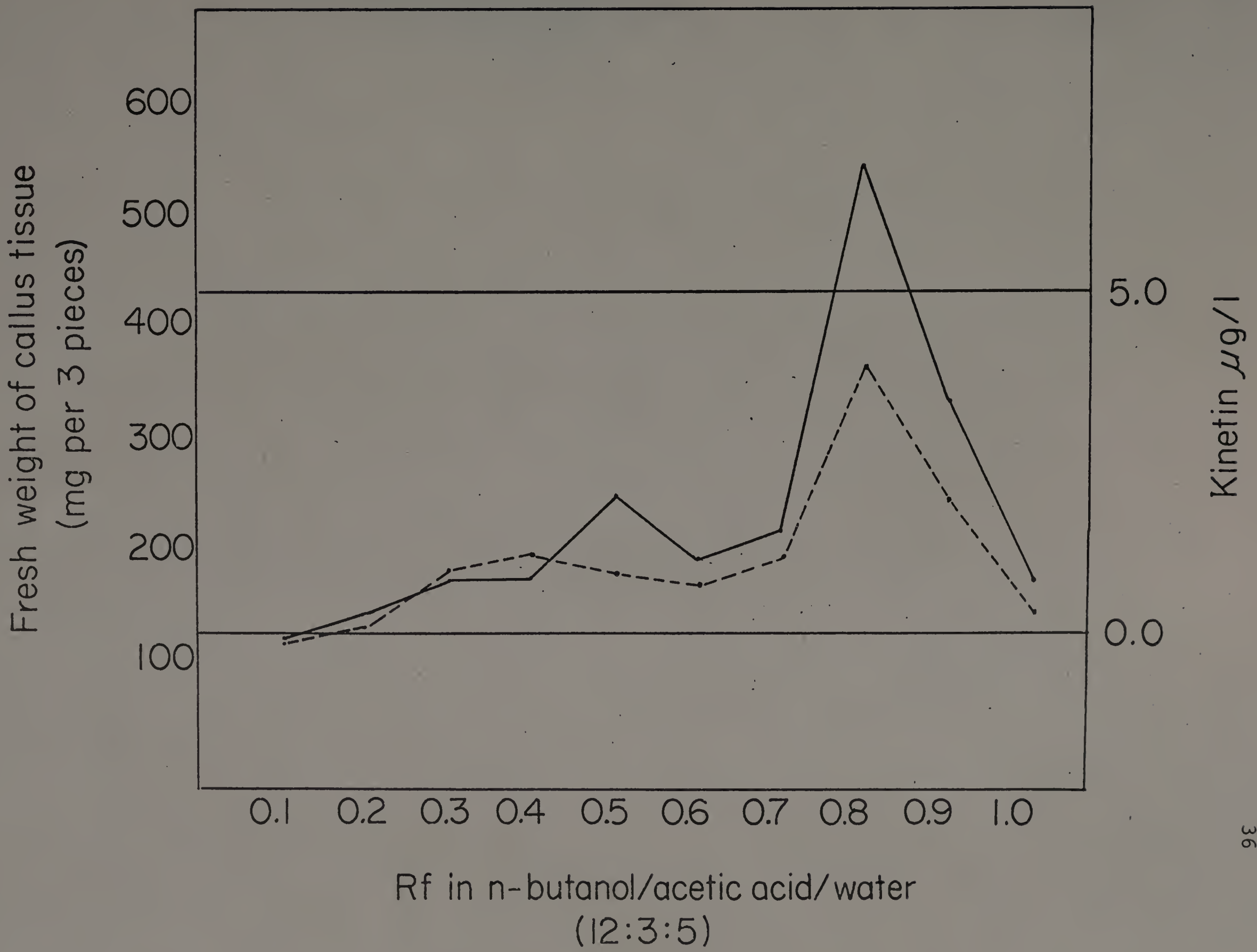
Treatment of cowpea primary leaves with 10^{-4}M kinetin for 4 days before inoculation caused a reduction in the

Table 6. Cytokinin levels in extracts from healthy and tobacco ringspot virus (TRSV)-infected cowpea plant roots determined with the tobacco stem pith callus tissue bioassay.

	Experiment Number	Rf									
		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Healthy	1.	108[a]	166	156	147	362	183	286	659	351	169
	2.	227	191	337	313	336	299	305	577	295	164
	3.	67	92	77	113	117	184	177	487	551	286
	4.	147	163	183	191	262	155	172	495	213	158
	Average	137	153	188	191	269	205	235	554	352	194
TRSV-infected	1.	109	134	176	177	115	137	125	220	237	108
	2.	208	214	300	283	253	232	289	475	254	231
	3.	92	85	168	230	187	195	252	464	384	213
	4.	135	150	134	150	227	181	187	360	191	107
	Average	136	145	194	210	195	186	213	379	266	164
Kinetin standards µg/l		0.0	1.0	5.0	10.0	25.0					
		138	284	448	876	3,055					

[a]Each number represents the average weight of callus tissue in 3 jars on Linsmaeir and Skoog medium containing the eluant from individual Rf strips of paper chromatograms developed in n-butanol/acetic acid/water 12:3:5.

Figure 2. Tobacco stem pith callus tissue bioassay of purified extracts of healthy (—) and tobacco ringspot virus (--) infected cowpea plant roots after paper chromatography.


Fresh weight of callus tissue
(mg per 3 pieces)
600
500
400
300
200
100
5.0
0.0
Kinetin µg/l
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf in n-butanol/acetic acid/water
(12:3:5)

numbers of local lesions formed on the primary leaves after inoculation with TRSV compared to the numbers of local lesions produced on inoculated leaves that were not treated with kinetin (Table 7). The average reduction in local lesion numbers in 3 experiments was 75.7% on the treated leaves. The reduction in local lesions was statistically significant in each experiment. Local lesions on treated primary leaves appeared at the same time as on untreated leaves and showed more variability in size and shape in the 3 experiments compared to lesions on untreated leaves.

Fresh Weight Determinations

Kinetin treatment of cowpea primary leaves did not affect the fresh weight of shoots that were not inoculated with TRSV. Cowpea plants that were not treated with kinetin and were inoculated with TRSV showed a reduction in shoot growth beginning 3 days after inoculation and the reduction became more pronounced after the fifth day. Kinetin treatment and TRSV inoculation caused a similar reduction in shoot growth from non-inoculated controls beginning 6 days after inoculation (Table 8, Fig. 3). However, the difference in weight between plants treated with kinetin and inoculated with TRSV and those not treated with kinetin but inoculated with TRSV was not statistically significant at any time during the course of the experiment.

Table 7. Local lesions produced on cowpea primary leaves treated with 10^{-4}M kinetin for 4 days before and daily after inoculation with tobacco ringspot virus.

Experiment Number	Treatment	
	+ kinetin	- kinetin
1.	44.1[a]	136.3
2.	131.0	547.0
3.	1.2	42.4
Average	58.7	241.9

[a]Each number represents the average number of local lesions on each of 53 to 60 primary leaves counted 4 days after inoculation.

There was a statistically significant difference at the 0.01 probability level between the shoot growth of untreated, uninoculated plants and that of both treated and untreated, inoculated plants beginning 6 days after inoculation and continuing through the remaining 4 days of the experiment. There were also statistically significant differences between treated, uninoculated cowpeas and untreated, inoculated plants from 6 days after inoculation till the end of the experiment. The differences between treated, uninoculated plants and treated, inoculated plants were statistically significant on day 6 after inoculation and again on day 8 and the remainder of the experiment.

Root tissue fresh weight from both kinetin treated and untreated plants inoculated with TRSV began to show a decrease 6 or 7 days after inoculation compared to the root weight of untreated, uninoculated plants (Table 9, Fig. 4).

Eight days after inoculation the root weight of both treated and untreated, uninoculated plants was significantly greater than that of treated and untreated, inoculated plants. The fresh weights of roots from treated, uninoculated plants was significantly lower than that of untreated, uninoculated plants 10 days after inoculation of the plants.

There was no significant difference in root weight between treated, inoculated plants and untreated, inoculated plants at any time during the experiment.

Kinetin Effects on TRSV Multiplication in Root Tissue

Multiplication of TRSV in roots was affected by treatment of primary leaves with kinetin. The time of the initial increase in virus concentration in root tissue was delayed in plants that were treated with kinetin compared to the time of the initial increase in plants that were not treated and inoculated with TRSV. In plants that were not treated with kinetin, TRSV could be detected in the roots 3 days after inoculation of the primary leaves and the initial increase in virus concentration occurred 5 or 6 days after inoculation. After the initial increase in virus multiplication, the concentration of TRSV was reduced to less than half. This decrease was followed by a relatively constant level of virus in the roots for 3 days, after which there was a decline in virus concentration in the root tissue sampled. In plants that were treated with kinetin, the initial increase in TRSV concentration in the roots occurred 7 days after inoculation in experiments 1 and 2 and did not appear at any time during experiment 3 (Table 10). Only plants that showed local lesions on primary leaves were used for assays of TRSV multiplication in roots in experiment 3.

Table 8. Fresh weight of shoots from cowpea plants treated with 10^{-4}M kinetin for 4 days before and daily after inoculation with tobacco ringspot virus.

Treatment	Days after inoculation							
	3	4	5	6	7	8	9	10
- kinetin - TRSV	3.4[a]	4.0	3.7	4.7[b]	5.6[b]	6.0[b]	5.5[b]	6.3[b]
+ kinetin - TRSV	3.3	3.9	3.9	5.9	4.5	5.3	5.7	5.5
- kinetin + TRSV	2.8	3.2	3.5	3.1	2.8	2.5	2.3	2.1
+ kinetin + TRSV	3.2	3.6	3.8	3.5	3.4	3.4	2.7	2.2

[a]Each number represents the average weight in grams of 9 or 10 shoots from 3 experiments.

[b]Each of these numbers represents the average fresh weight in grams of 6 or 7 shoots from 2 experiments

Figure 3. Fresh weight of cowpea shoots treated with 10^{-4}M kinetin 4 days before and daily after inoculation with tobacco ringspot virus.

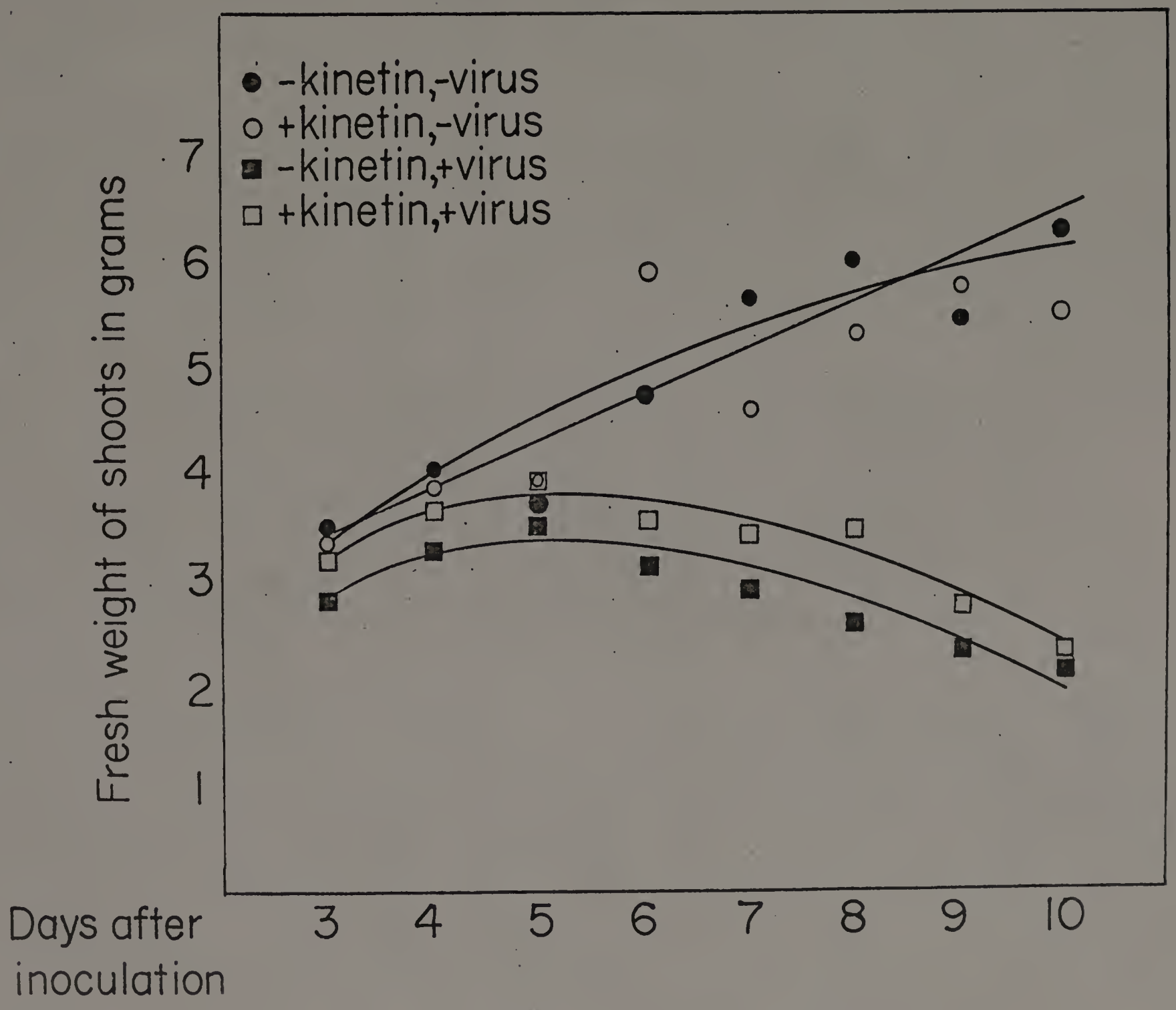

-kinetin,-virus
+kinetin,-virus
-kinetin,+virus
+kinetin,+virus
Fresh weight of shoots in grams
1
2
3
4
5
6
7
Days after inoculation
3
4
5
6
7
8
9
10

Table 9. Fresh weight of roots from cowpea plants treated with 10^{-4}M kinetin for 4 days before and daily after inoculation with tobacco ringspot virus.

Treatment	Days after inoculation							
	3	4	5	6	7	8	9	10
- kinetin - TRSV	2.9[a]	1.8	1.9	2.7[b]	2.8[b]	3.7[b]	3.5[b]	4.8[b]
+ kinetin - TRSV	2.0	2.1	2.0	2.2	2.8	3.0	2.6	2.5
- kinetin + TRSV	1.6	1.7	1.9	2.0	2.0	1.7	2.2	1.1
+ kinetin + TRSV	1.8	1.8	2.0	1.6	2.2	2.1	2.4	2.3

[a]Each number represents the average weight in grams of roots of 9 or 10 plants from 3 experiments.

[b]Each of these numbers represents the average weight in grams of 6 or 7 root systems from 2 experiments.

Figure 4. Fresh weight of cowpea roots treated with 10^{-4}M kinetin 4 days before and daily after inoculation with tobacco ringspot virus.

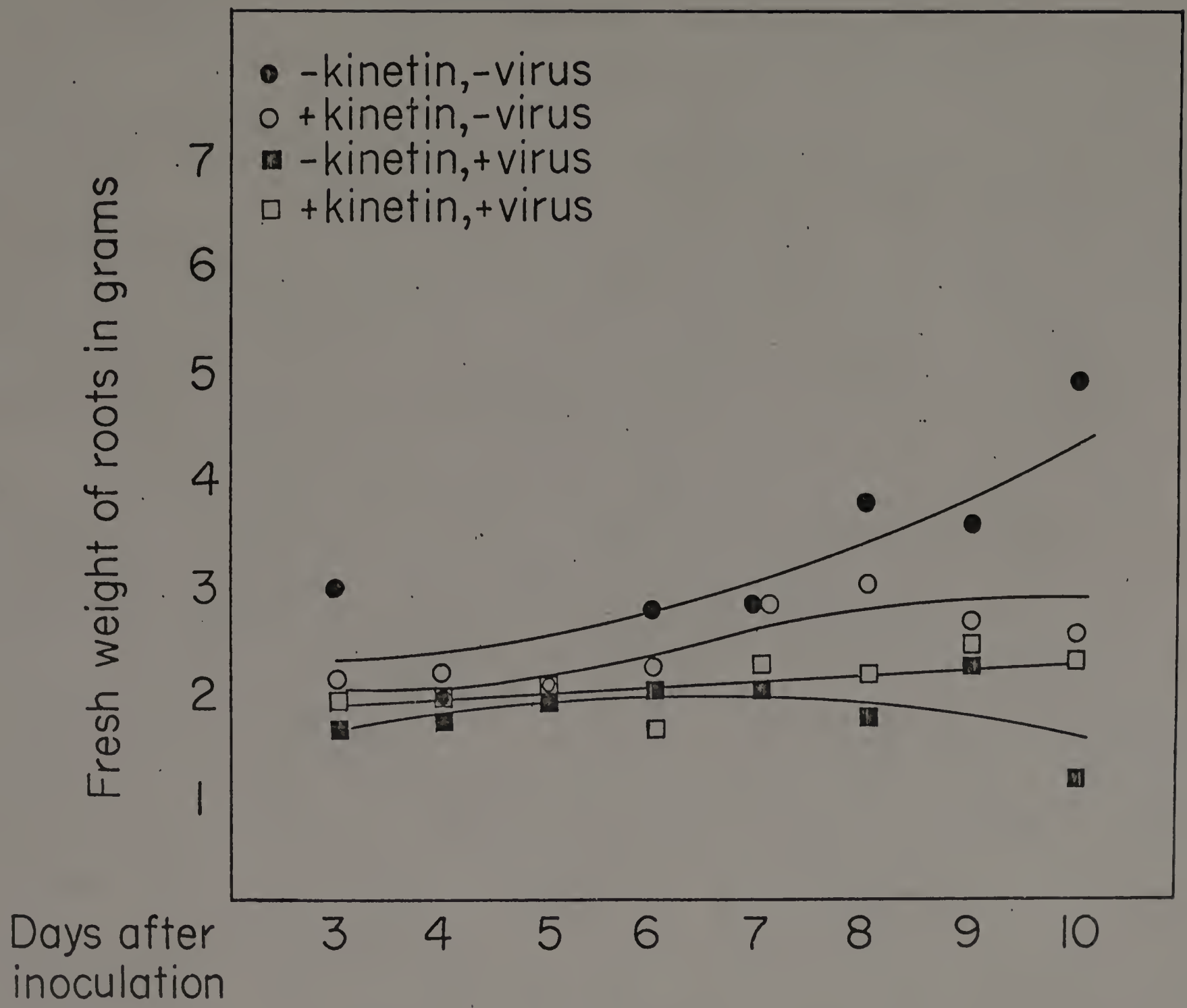

-kinetin,-virus
+kinetin,-virus
-kinetin,+virus
+kinetin,+virus
Fresh weight of roots in grams
1
2
3
4
5
6
7
Days after inoculation
3
4
5
6
7
8
9
10

Table 10. Number of local lesions produced on cowpea primary leaves inoculated with root sap from plants treated with 10^{-4}M kinetin 4 days before and daily after inoculation with tobacco ringspot virus.

Treatment	Experiment Number	Days after inoculation							
		3	4	5	6	7	8	9	10
- kinetin + TRSV	1.	1.7[a]	4.2	116.1	28.0	32.6	42.3	5.5	5.5
	2.	4.8	0.1	12.8	2.0	8.5	16.3	1.2	0.0
	3.	0.0	0.0	2.1	86.1	31.6	31.1	56.5	1.0
	Average	2.1	1.4	43.6	38.7	24.2	29.9	21.0	2.1
+ kinetin + TRSV	1.	0.0	1.3	3.7	0.3	46.1	89.8	26.0	6.8
	2.	1.0	5.8	5.6	3.5	38.2	29.5	1.5	0.2
	3.	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.6
	Average	0.3	2.3	3.3	1.2	28.1	39.7	9.1	2.5

[a]Each number represents the average number of local lesions produced on each of 8 half-leaves from 1 gram of root tissue ground in 3 ml of 0.1 M phosphate buffer pH 7.0.

Symptom Development

Plants that were treated with kinetin and inoculated with TRSV showed a delay in symptom appearance and development compared to inoculated plants that were not treated with kinetin. Symptoms that differed most consistently between kinetin-treated and untreated plants included the time of yellowing of primary leaves and the abscision of yellowed primary leaves. Yellowing of the primary leaves began to appear 5 days after inoculation in untreated, inoculated plants and was followed by abscision during the next 2 days. In kinetin-treated, inoculated plants yellowing began to occur 7 to 8 days after inoculation and the amount of abscision in these plants during the next 2 days was much less than in untreated, inoculated plants. Yellowing proceeded faster in primary leaves that were inoculated but not treated with kinetin. At the end of each experiment (10 days after inoculation) fewer of the remaining plants that were treated with kinetin and inoculated showed symptoms of complete yellowing and leaf abscision than inoculated plants not treated with kinetin.

Figure 5. Cowpea plants showing the effect of kinetin treatment on symptoms caused by tobacco ringspot virus 8 days after inoculation. Kinetin was applied on the primary leaves 4 days before inoculation and daily after inoculation. From left to right: Untreated, uninoculated control; treated, inoculated; untreated, inoculated.

DISCUSSION

Tobacco ringspot virus was detected in the distal millimeter sections of cowpea roots 3 days after leaf inoculation and the greatest concentration of virus was reached 5 days after inoculation (Table 1). Virus multiplication in root tips steadily declined after day 5. The ability of TRSV to infect cowpea root meristem tissue agrees with results obtained by Crowley et al., (1969) and Atchison and Francki (1972) with French bean root tips. In their studies, following initial invasion and multiplication by the virus in the terminal root tip millimeter, the virus concentration remained constant thereafter. Marked root stunting also occurred but bean plants were not killed during the course of their experiments. In the present study, shoot growth was more dramatically affected than root growth, as shown by fresh weight data (Table 2). The reduction in root growth of cowpeas did not appear to be as severe as in the above studies with French beans. However, Crowley et al., and Atchison and Francki compared the length of healthy and TRSV-infected roots while in the present study the comparison was between the fresh weights of healthy and TRSV-infected roots.

The use of cowpea seedlings in this study allowed TRSV to become systemic and to kill cowpea plants in 10 days. Sap amounts collected from TRSV-infected plants 7 days after inoculation were smaller than those of healthy plants, indicating a reduction in the amount of water absorbed by the roots and translocated to the stem in the TRSV-infected plants (Table 3). This reduction in water absorption and/or translocation was very pronounced in trial collections made later than 7 days after inoculation. This indicates that infection by TRSV affects the ability of the roots to absorb and/or translocate water to the stem of the cowpea plant. The effects of TRSV on stem and leaf tissues, including the vascular tissues, may also be involved in the decline of water movement from the root to the stem in cowpea plants, but no study of any such effects was attempted here.

Cytokinin activity in sap collected from either healthy or TRSV-infected plants was low (Table 5). Cytokinin flux in plant sap has been shown to increase with flower induction in Perilla frutescens and in Helianthus annus L. (Beever and Woolhouse, 1973; Sitton et al., 1967). In Perilla frutescens, after 18 inductive cycles of short days, when flower buds were visible, cytokinin concentration was about twice that found in

vegetative plants. With 35 inductive cycles, when the embryos of fruit had reached their final size, the cytokinin content was 5 times that in vegetative controls. Vegetative plants, therefore, appear to contain less cytokinins in sap than do plants in the process of flowering and fruiting. The results of the present study, in which sap was collected when the plants were 13 to 21 days old and too young to begin flowering, indicate that low concentrations of cytokinins are present in the sap of young cowpea plants still in their vegetative stage of growth.

Purified extracts from cowpea plant root tissue also showed low cytokinin activity in the tobacco stem pith callus tissue bioassay (Table 6). Short and Torrey (1972), using 2000 root tips from 2-day-old pea seedlings, found zeatin and zeatin riboside at Rf 0.8 on paper chromatograms developed in n-butanol/acetic acid/water 12:3:5. The total concentration of these two cytokinins combined was equivalent to about 50 µg/l of kinetin. The cytokinin factor or factors observed at Rf 0.8 on paper chromatograms developed in the same solvent system in the present study were present at a concentration equivalent to 5 µg/l kinetin. Letham (1974) determined that, when ethyl acetate was employed as a solvent for cytokinin extraction, 14.4% of the total amount of zeatin and 11.1% of the total

amount of zeatin riboside present originally were lost into the ethyl acetate phase after shaking. If the activity at Rf 0.8 in the present study represents the presence of zeatin and/or zeatin riboside, the losses of these compounds into the ethyl acetate phases would not account for the 10-fold difference in cytokinin activity observed in this study as compared to the results of Short and Torrey. The age of the plant roots used may have been a factor in the observed low level of cytokinin activity. Radin and Loomis (1971), investigating cytokinin activity in maturing radish roots, found no activity in extracts from 9-day-old radish roots but found increasing cytokinin activity in extracts as roots matured to 38 days after planting. Zeatin, zeatin nucleotide and zeatin riboside were found to be present in their study. Gordon et al., (1974), found that, 5 hours after application of exogenous zeatin to intact radish seedling roots, the principal metabolite formed in the roots was 7-glucosylzeatin, termed raphanatin. Raphanatin is a stable metabolite of zeatin. Gordon et al., called raphanatin a storage form of zeatin which may be weakly active in bioassays, and suggested that this form of zeatin may have been present in extracts used in Radin and Loomis' study and may have contributed to lack of activity.

Cytokinin levels were lower in plant sap and root tissue from TRSV-infected cowpea plants compared to those of healthy plants (Tables 5 and 6). The reduction of cytokinins in sap became evident 7 days after inoculation, at which time the cytokinin levels in sap from TRSV-infected cowpea plants produced the lowest fresh weight increase in the tobacco stem pith callus tissue bioassay of all the determinations made. The results indicate that when the amount of water translocated from roots to the stem begins to decrease there is also a decrease in cytokinin concentration in the translocated sap. The reduction in cytokinin concentration in a volume of sap and the decrease in the amount of sap moving through the TRSV-infected cowpea plants may represent a greater decrease in cytokinins in cowpea plant sap than were observed from bioassays of equal volumes of sap from healthy and TRSV-infected plants. Extraction from root tissue 7 days after inoculation also showed that cytokinin activity was lower in TRSV-infected than in healthy root tissue. The major cytokinin activity in both healthy and TRSV-infected tissue appeared at Rf 0.8 and corresponded to the migration of zeatin and zeatin riboside in n-butanol/acetic acid/ water 12:3:5. The presence of zeatin and zeatin riboside in root tissue has been frequently reported, as indicated above. Two other

naturally occurring cytokinins, isopentenyladenosine (IPA) and 6-(3-Methyl-2-butenylamino) purine (2iP), also migrate to Rf 0.8 to 0.9 in n-butanol/acetic acid/water 12:3:5. However, IPA is commonly found in extracts from tRNA of plant tissues and is not prevalent in a free form in these tissues (Dyson and Hall 1972; Skoog and Armstrong 1970; Short and Torrey, 1972). Letham (1974) states that 2iP contributes little to cytokinin activity of many higher plant extracts but has been found to occur in a strain of tobacco pith callus tissue that did not require added cytokinins for growth on a defined medium. Therefore, based on the identical migration of known zeatin and zeatin riboside and of the cytokinin factors in the cowpea extracts it may be reasonably concluded that zeatin and zeatin riboside were present in this study. Adenine and adenosine are known to migrate to Rf values around 0.5 in n-butanol/acetic acid/water 12:3:5. However, there is no literature on the chemical characterizations of naturally occurring cytokinins that migrate to Rf 0.5 in this solvent system.

The reduction in cytokinin levels in TRSV-infected plants was preceded by a high rate of virus synthesis in cowpea root tips 5 days after primary leaf inoculation. Atchison (1973) found that, after TRSV invaded the root

tip of French beans 3 days after inoculation, there was a marked reduction in the mitotic index which reached a minimum 5 to 6 days after inoculation and then returned to normal. Atchison states that the observed decrease was a reflection of the failure of cells to enter mitosis. He also found that root stunting was always preceded by infection of the root meristem. Since cytokinins are known to be produced in the root meristem of young plants (Short and Torrey, 1972; Weiss and Vaadia, 1965), the reduction of cytokinin levels in TRSV-infected cowpeas may occur as a result of virus synthesis in these same meristematic cells. Tobacco ringspot virus is obviously a stress factor in cowpea plants and in the apical meristem of roots as evidenced by the concentration of virus in root tips 5 days after inoculation. The products and nutrients produced in or translocated from the roots to the aerial part of the plant can be affected by various stress conditions. Flooding, drought, fungal infections of roots, and other stress conditions are known to decrease the cytokinin levels in roots, root exudate and extracts from stem and leaf tissue (Burrows and Carr, 1969; Misaghi et al., 1972). Our results indicate that TRSV is capable of invading and multiplying in the apical meristem of cowpea roots, after which there is a reduction in the amount of cytokinins present in sap and root tissue.

Treatment of cowpea primary leaves with kinetin for 4 days before and daily after inoculation did not affect the growth of shoots but it did reduce the growth of root tissue in plants that were not inoculated (Tables 8, 9, Figures 3, 4). There were no differences in shoot or root weight between kinetin treated or untreated, inoculated plants, although symptoms on leaves differed.

Kinetin treatment of primary leaves of cowpea plants for 4 days before inoculation caused a drastic reduction in the numbers of local lesions formed on these leaves following inoculation (Table 7). The results suggest that TRSV multiplication, stability or infectivity is affected by kinetin treatment of the leaves before inoculation. The nature of cytokinin-plant virus interactions are not clear at this time. Recent evidence that cytokinin-binding sites, protein in nature, exist on plant ribosomes and bind cytokinins apart from the ribosome may help to explain the mode of action of cytokinins and the effect they may have on virus synthesis (Erion and Fox, 1975).

Kinetin treatment of primary leaves before and after inoculation delayed and reduced the concentration of infective virus in the roots (Table 10). In kinetin treated plants, the initial appearance of increased virus concentration did not take place until 7 days after inoculation, while in untreated plants virus concentration

began to increase within 5 days after inoculation. This delay probably occurred partly because of the reduced virus in the leaf which resulted in less virus transported to the roots, but also because the kinetin treatment of the leaves may have affected the rate of virus movement through the plant and the physiology of the roots.

Symptom development also differed in the kinetin treated and untreated, inoculated plants. In most cases (Table 7), virus was present at high concentrations in inoculated primary leaves and, 5 days after inoculation, these leaves showed yellowing which was followed by abscision during the next 2 days. Treated, inoculated leaves did not begin to turn yellow until 7 or 8 days after inoculation and the symptoms were less frequent than among the untreated plants. The number of local lesions produced on treated leaves in experiments 1 and 2 (Table 7), were certainly enough to induce yellowing and abscision of leaves before the 7th or 8th day.

The ability of cytokinins to reduce and even reverse chlorophyll destruction in senescing leaves, and the induction of premature leaf yellowing and senescence by stresses on roots are well documented and are known to be related to the availability of cytokinins to the senescing tissues (Bagi and Farkas, 1968; Richmond and

Lang, 1957; Itai and Vaadia, 1965; Burrows and Carr, 1969). Also, stresses applied to leaf tissue only are known to cause reductions in cytokinins in root exudate and in leaves under stress (Itai and Vaadia, 1971). Therefore the evidence from this study suggests that TRSV-infection in cowpea causes a reduction in the amount of cytokinins produced in root tissue and translocated to the shoot. The reduction in cytokinin becomes apparent 7 days after inoculation and the symptoms of senescence of primary leaves, which begin to appear 5 days after inoculation, can be delayed by application of kinetin to the leaves. Since cytokinins already present in the leaves at the time of inoculation may be inactiviated or reduced while virus multiplication takes place, the difference in the time of primary leaf yellowing and the reduction of cytokinins in roots and in sap may still be related. It appears that, although other factors, including other growth regulators, may be involved in symptom development in TRSV-infected cowpeas, cytokinins play a role in the premature yellowing of leaves. It is possible, for example, that wilting, necrosis and abscision of leaves indicate that changes in abscisic acid and ethylene may be involved in symptom expression in TRSV-infected cowpeas (Pratt and Goesch, 1969; Wright and Hiron, 1972).

SUMMARY

Tobacco ringspot virus (TRSV) reached maximum concentration in cowpea plant root tips 5 days after inoculation. Subsequently, virus concentration in root tips declined steadily as the inoculated plants developed systemic symptoms, wilted and died.

Seven days after inoculation the fresh weight of shoots of TRSV-infected cowpea plants was reduced by 50 percent and the fresh weight of roots of the same plants was reduced by 29 percent. Amounts of sap collected from TRSV-infected cowpea plants 7 or more days after inoculation were lower than those collected from healthy plants. This indicates that less water moves from the roots to the shoots as the disease progresses. Cytokinin content was also reduced in sap collected from TRSV-infected cowpea plants 7 days after inoculation. The difference was statistically significant at the 0.1 probability level.

Extracts from healthy and TRSV-infected root material collected 7 days after inoculation showed two peaks of cytokinin activity in the tobacco stem pith callus tissue bioassay. The major peak of activity from TRSV-infected root tissue was lower than in healthy extracts and the difference was statistically significant at the 0.05 probability level. This major peak of activity corresponded to the migration of zeatin and zeatin riboside in the same solvent system.

Kinetin treatment of primary leaves of cowpea plants did not affect the shoot growth of plants but reduced the growth of roots. Kinetin treatment of plants that were inoculated with TRSV 4 days after the start of kinetin applications did not affect shoot or root growth in these plants compared to inoculated plants that did not receive kinetin treatment.

Numbers of local lesions produced on cowpea primary leaves treated with kinetin 4 days before inoculation and daily after inoculation were significantly lower than those of control plants. Virus production in roots of kinetin-treated plants was lower and slower than in plants not treated with kinetin.

Premature yellowing and abscision of leaves was also delayed in inoculated, kinetin-treated leaves and the rate of yellowing of individual leaves was reduced.

LITERATURE CITED

Aldwinckle, H. S. and I. W. Selman. 1967. Some effects of supplying benzyladenine to leaves and plants inoculated with viruses. Ann. Appl. Biol. 60:49-58.

Atchison, B.A. 1973. Division, expansion and DNA synthesis in meristematic cells of French bean (Phaseolus vulgaris L.) root tips invaded by tobacco ringspot virus. Physiol. Plant Pathol. 3:1-8.

Atchison, B. A. and R. I. B. Franchi. 1972. The source of tobacco ringspot virus in root tip tissue of bean plants. Physiol. Plant Pathol. 2:105-111.

Bagi, G. and G. L. Farkas. 1968. A new aspect of the anti-stress effect of kinetin. Experimenta 24:397-398.

Beever, J. E. and H. W. Woolhouse. 1973. Increased cytokinin from root system of Perilla frutescens and flower and fruit development. Nature New Biology 246:31-32.

Burrows, W. J. and D. J. Carr. 1969. Effects of flooding the root system of sunflower plants and the cytokinin content in the xylem sap. Physiol. Plant. 22: 1105-1112.

Crowley, N. C., E. M. Davison, R. I. B. Francki and G. K. Owusu. 1969. Infection of bean root-meristems by tobacco ringspot virus. Virology 39:322-330.

Crowley, N. C. and J. B. Hanson. 1960. The infection of apical meristems of tomato roots with tobacco mosaic virus after treatment with ethylene-diaminetetraacetic acid. Virology 12: 603-606.

Daft, M. J. 1963. Some effects of pancreatic ribonuclease and kinetin on tomato aucuba mosaic virus. Ann. Appl. Biol. 52:393-398.

Daft, M. J. 1965. Some interactions of kinetin and temperature on tobacco leaves infected with tomato aucuba mosaic virus. Ann. Appl. Biol. 55:51-56.

Dyson, W. H. and R. H. Hall. 1972. N^6-(Δ^2-Isopentenyl) adenosine: Its occurrence as a free nucleoside in an autonomous strain of tobacco tissue. Plant Physiol. 50:616-621.

Erion, J. and J. E. Fox. 1975. A cytokinin binding protein from higher plant ribosomes. Plant Physiol. 56:28.

Gordon, M. E., D. S. Letham and C. W. Parker. 1974. The metabolism and translocation of zeatin in intact radish seedlings. Annals of Botany 38:809-825.

Hemberg, T. and P. E. Westlin. 1973. The quantitative yield in purification of cytokinins. Model experiments with kinetin, 6-furfurylamino purine. Physiol. Plant. 28:228-231.

Itai, C. and Y. Vaadia. 1965. Kinetin-like activity in root exudate of water stressed sunflower plants Physiol. Plant. 18:941-944.

Itai, C. and Y. Vaadia. 1971. Cytokinin activity in water-stressed shoots. Plant Physiol. 47:87-90.

Kende, H. 1965. Kinetin-like factor in the root exudate of sunflower. Proc. Nat. Acad. Sci. 53: 1302-1307.

Kiraly, Z. and J. Szirmai. 1964. The influence of kinetin of tobacco mosaic virus production in Nicotiana glutinosa leaf disks. Virology 23:286-288.

Letham, D. S. 1967. Chemistry and physiology of kinetin-like compounds. Annu. Rev. Plant Physiol. 18:349-364.

Letham, D. S. 1974. Regulators of cell division in plant tissues. XXI. Distribution coefficients for cytokinins. Planta 118:361-364.

Linsmaier, E. M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.

Miller, C. O. 1963. Kinetin and kinetin-like compounds. In: "Modern methods of plant analysis." H. F. Linsker and M. V. Tracey, (Eds.), Vol. 6:194-202. Berlin-Gottingen-Heidelberg: Springen.

Misaghi, I., J. E. Dewey and T. Kosuqe. 1972. Changes in cytokinin activity associated with the development of Verticillium wilt and water stress in cotton plants. Physiol. Plant Pathol. 2:187-196.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue. Physiol. Plant. 15:473-497.

Pratt, H. K. and J. D. Goesch. 1969. Physiological roles of ethylene in plants. Annu. Rev. Plant Physiol. 20:541-584.

Radin, J. W. and R. S. Loomis, 1971. Changes in the cytokinins of radish roots during maturation. Physiol. Plant. 25:240-244.

Richmond, A. E. and A. Lang. 1957. Effect of kinetin or protein content and survival of detached Xanthium leaves. Science 125:650-651.

Selman, I. W. 1964. The effect of kinetin on infection of petunia leaves and tomato leaves with tomato spotted wilt virus. Ann. Appl. Biol. 53:67-76.

Short, K. C. and J. G. Torrey. 1972. Cytokinins in seedling roots of pea. Plant Physiol. 49:155-160.

Skoog, F. and D. J. Armstrong. 1970. Cytokinins, Annu. Rev. Plant Physiol. 21:359-384.

Sitton, D., C. Itai and H. Kende. 1967. Decreased cytokinin production in the roots as a factor in shoot senescence. Planta 73:296-300.

Smith, S. H. and D. E. Schlegel. 1964. The distribution of clover yellow mosaic virus in Vicia faba root tips. Phytopathology 54:1273-1274.

Steel, R. G. D. and J. H. Torrie. Principles and Procedures of Statistics. McGraw-Hill New York 1960. p. 73-76.

Weiss, C. and Y. Vaadia. 1965. Kinetin-like activity in root apices of sunflower plants. Life Science 4:1323-1326.

White, P. R. 1943. A handbook of plant tissue culture. Jacques Cattell Press, Lancaster Pennsylvania.

Wright, S. T. C. and R. W. F. Hiron. 1972. The accumulation of abscisic acid in plants during wilting and under other stress and conditions. Plant growth substances 1970. Springer-Verlag, Berlin. p. 291-298.

Yoshida, R. and T. Oritani. 1971. Kinetin-like factors in the root exudate of rice plants. Plant and Cell Physiol. 12:89-94.