Cytokinin Levels and Kinetin-Virus Interactions in Tobacco Ringspot Virus-Infected Cowpea Plants

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ABSTRACT

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Tobacco ringspot virus was detected in cowpea root tips 3 days after leaf inoculation and reached its highest concentration 2 days later. Cytokinin levels in root exudate and root tissue extracts were lower in TRSV-infected cowpea plants 7 days after inoculation than in healthy plants. Purified extracts from healthy and TRSV-infected root tissue collected at that time and separated by paper chromatography showed a minor peak of cytokinin activity at R_f 0.5 and a major peak at R_f 0.8 in the tobacco stem pith callus tissue bioassay. The peak at R_f 0.8 from TRSV-infected root tissue was 31% less than that produced by healthy extracts. Daily treatment of primary leaves of cowpea plants with 10^{-4} M kinetin had no effect on shoot growth but reduced the growth of roots. Kinetin treatment of cowpea plants inoculated with TRSV had no effect on either shoot or root growth. Cowpea primary leaves treated with kinetin and inoculated with TRSV produced 75% fewer local lesions than untreated, inoculated leaves. Virus production in roots of kinetin-treated plants was lower and delayed compared to untreated plants. Yellowing and abscission of inoculated, kinetin-treated leaves was also delayed and the rate of yellowing of individual leaves was slower than in inoculated, untreated leaves.

Plant virus multiplication in systemically infected plants, leaves, and leaf-disk tissue can be altered by exogenously applied cytokinins prior to or after inoculation (1, 11, 17). The effect of plant virus multiplication on endogenous cytokinins has not been investigated.

In seedling plants, cytokinins are produced in root apical meristem tissue (18, 19). Tobacco ringspot virus (TRSV) has been shown to have the unusual ability of invading and accumulating in high concentrations in bean root tissue meristems (3, 7). In these studies root stunting was shown to be preceded by virus invasion of the root meristems. Invasion of bean root meristems by TRSV also produced a temporary decrease in DNA synthesis and in the mitotic index of the invaded meristematic cells (2).

The present study was undertaken to determine the length of time between TRSV inoculation of cowpea primary leaves and the subsequent infection of root tips; the relative concentration of virus, as indicated by infectivity assay, in cowpea root meristems; the relationship between infection of root meristems and cytokinin levels in the roots and in root exudates; and the effects of daily kinetin treatment on virus multiplication in leaves and roots and on symptom development in TRSV-infected cowpea plants.

MATERIALS AND METHODS

Tobacco ringspot virus strain ATCC 157 was maintained on plants of the tobacco cultivar Samsun NN. Cowpea plants of the cultivar Early Ramshorn were inoculated with sap obtained from tobacco leaves showing systemic symptoms and ground in 10 parts of 0.1 M phosphate buffer, pH 7.0, to which 1.5% Celite had been added.

Detection of tobacco ringspot virus.—Terminal 1-mm sections of cowpea root tips were assayed daily beginning 3 days after inoculation of the primary leaves of cowpea plants grown in water culture at 27 C and a 14-hr light period. Four terminal millimeter root tips were excised and ground between a ground glass and a porcelain homogenizer in two drops of phosphate buffer 0.01 M, pH 7.2, and a small amount of Celite. This extract was then rubbed onto two cowpea half-leaves used as assay plants. Local lesions were counted 4 days after inoculation. Eight half-leaves were inoculated on each day of the experiment. Symptom development was recorded daily.

Collection of sap.—Cowpea plants were grown in a mixture of soil, sand, and peat moss (1:1:1, v/v) in 15-cm diameter pots in a greenhouse. A group of 300-350 TRSV-infected and an equal number of healthy cowpea plants were used for sap collection 3, 5, and 7 days after inoculation. The pots in which the plants were growing were saturated with water 12 hr and 2 hr before the tops

were cut off 2.5 cm above the soil line. Sap droplets were collected from each cut stem with a Pasteur pipet over a 5-hr period by alternately collecting from all the TRSV-infected and then from the healthy cowpea plants. An equal number of collections was made. The sap was kept on ice during the collection and then frozen at −20 C until it was bioassayed. This part of the experiment and the subsequent cytokinin extraction and assay were repeated four times.

Cytokinin extraction.—Cowpea plants used for extraction of cytokinins from root tissue were grown in a steam-sterilized mixture of soil and sand (1:1, v/v) in the greenhouse. Seven days after inoculation, the roots of an equal number of healthy and TRSV-infected cowpea plants were harvested by shaking the soil from the roots and then rinsing them twice in tap water. The roots were separated from the stems, both were blotter dried and the fresh weights of stems and roots were measured. Eighty grams (fresh weight) each of healthy and TRSV-infected root tissue was ground in a Waring Blendor in 200 ml of cold 80% ethanol, washed with an additional 100 ml of cold 80% ethanol, and set in a refrigerator at 5 C for 6-12 hr. The extracts were filtered through a Büchner funnel and a coarse Millipore funnel and the residue was washed with 100 ml of 80% ethanol. The filtered extracts and washings were combined and the ethanol evaporated in vacuo at 50 C. The aqueous residue was brought to 100 ml with distilled water, and the pH was adjusted to 9.0 with 1.0 N NaOH. The extract was then washed three times with petroleum ether. The aqueous fractions were acidified to pH 2.5 with 1.0 N HCl and washed three times with ethyl acetate. The pH of the aqueous fractions was adjusted to 2.3 and 10 ml of cold 50% saturated $AgNO_3$ was added to each aqueous fraction followed by stirring in a coldroom for 12 hr. The precipitate that formed was collected by centrifugation at 7,000 *g* for 15 min. The supernatant liquid was removed and the pellets were mixed with 40 ml of 0.2 N HCl and stirred at 50 C for 30 min. The reaction precipitates were collected by centrifugation at 7,000 *g* for 15 min and the supernatant liquid was saved. This procedure was repeated and the two 0.2 N HCl fractions were combined. The pH of these fractions was adjusted to pH 8.0 with 1.0 N NaOH and shaken four times with an equal volume of *n*-butanol. The combined *n*-butanol phases were evaporated in vacuo at 50 C to dryness. The dried residue was washed with 10 ml of 80% ethanol, transferred to a small evaporatory flask, and evaporated to dryness again. The residues were washed with 2 ml of 80% ethanol and streaked onto Whatman 3 MM chromatography paper. The chromatograms were run in descending fashion in *n*-butanol:acetic acid:water (12:3:5, v/v) overnight (30 cm), air-dried, and prepared for bioassay.

Bioassay procedure.—Increase in the growth of tobacco stem pith callus tissue was used for bioassays of sap and purified root extracts. Callus tissue initially obtained from pith tissue of tobacco cultivar Samsun NN was maintained on Linsmaier and Skoog medium (13). Sap from healthy and TRSV-infected cowpea plants was bioassayed by incorporating 2-ml aliquots of thawed, centrifuged crude sap into Linsmaier and Skoog medium which lacked cytokinin, but contained gibberellic acid at 1 mg/liter before autoclaving. To bioassay purified root extracts each dried chromatogram was divided into three sections, each corresponding to 26.6 g fresh weight of tissue. Individual R_f sections from each strip were cut and eluted with water in 110 ml wide-mouthed jars before the addition of 20 ml of Linsmaier and Skoog medium and autoclaving. After the jars had cooled, three pieces of tobacco stem pith callus tissue, each about 10 mg, were placed in each jar. The jars then, along with jars containing callus tissue on kinetin standards, were placed in an incubator at 27 C for 28 days.

Kinetin treatment.—Cowpea plants were grown in a steam-sterilized mixture of equal parts soil and sand in the greenhouse. Twelve 10-cm pots containing three plants per pot were used for each treatment. Treatments included plants treated with kinetin and either inoculated or not inoculated with TRSV and plants not treated with kinetin and either inoculated or not inoculated with TRSV. Primary leaves of cowpea plants were treated by rubbing with a wad of cheesecloth that had been soaked in distilled water or in a 10^{-4} M solution of kinetin. Treatment began 6 or 7 days after planting and was applied daily for 4 days before inoculation and daily after inoculation. Symptom development was recorded and the fresh weight of shoots and roots of a group of plants from each treatment was determined daily.

Kinetin treatment and tobacco ringspot virus multiplication.—Local lesions produced on inoculated kinetin

TABLE 1. Detection and measurement of tobacco ringspot virus (TRSV) in the terminal millimeter of roots from leaf-inoculated cowpea plants

Days after leaf inoculation with TRSV								
2	3	4	5	6	7	8	9	10
0.0[a]	1	4	189	109	77	52	1	0

[a]Each number represents the mean calculated from two replications of the number of local lesions produced on each of eight half-leaves inoculated with sap from the terminal millimeter of roots of leaf-inoculated cowpea plants.

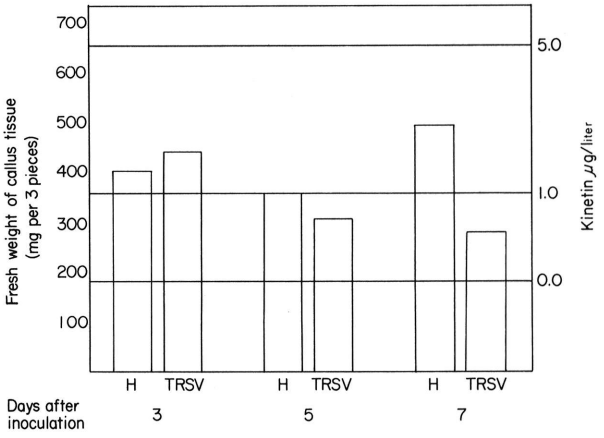

Fig. 1. Histogram showing the increase in growth of tobacco stem pith callus tissue in the presence of 2 ml of crude, centrifuged sap from healthy (H) and tobacco ringspot virus (TRSV)-infected cowpea plants collected 3, 5, and 7 days after inoculation.

treated and untreated primary leaves were counted 3 or 4 days after inoculation. Beginning 3 days after inoculation, 1 g of washed and blotter-dried root tissue was obtained each day from kinetin-treated, inoculated plants and from untreated, inoculated plants. The tissue was ground in 3 ml phosphate buffer, pH 7.0, to which 1.5% Celite was added and assayed for TRSV concentration on eight cowpea half-leaves. Local lesions were counted 4 days later.

RESULTS

Tobacco ringspot virus was first detected in the terminal 1-mm of root tips of cowpea plants 3 days after inoculation. Not all root tips sampled 3 and 4 days after inoculation contained detectable virus concentrations. At that time local lesions were present on the inoculated leaves. Five days after inoculation, when symptoms consisted of necrotic local lesions, the beginning of yellowing of primary leaves and petiole necrosis, all root tips sampled contained detectable virus, and concentrations of TRSV reached their highest levels of all the daily samplings (Table 1). After day 5, virus concentrations steadily declined. Seven days after inoculation, shoot symptoms consisted of yellowed and abscising primary leaves, petiole and stem necrosis, and the majority of inoculated plants began to wilt; roots showed only a lack of new root growth compared to healthy noninoculated plants. Infectivity in root tips assayed 7 days after inoculation was 41% that of root tips assayed 2 days earlier. The fresh weight of TRSV-infected roots was 27% lower and the fresh weight of TRSV-infected shoots was 50% lower than that of healthy plants. Nine days after inoculation, when the shoots of plants were totally necrotic, the virus concentration in assayed root tips was less than 1% of its maximum concentration reached on day 5.

Equal amounts of root exudate were collected from healthy and TRSV-infected plants 3 and 5 days after inoculation. Seven days after inoculation the amount of sap collected from TRSV-infected plants was consistently smaller than that collected from healthy plants in each of four replications. Further reductions in amounts of exudate were observed in trial collections made later than 7 days after inoculation.

Cytokinin activity of crude, centrifuged plant exudate from cut stems of healthy and TRSV-infected cowpea plants collected 3 and 5 days after inoculation produced similar increases in growth in the tobacco stem pith callus tissue bioassay (Fig. 1). Exudate from TRSV-infected plants collected 7 days after inoculation showed less cytokinin activity than sap from healthy plants. The difference was statistically significant, $P = 0.1$. Cytokinin activity of plant exudate collected at various periods after inoculation was generally low. Thus, cytokinin activity in 2-ml aliquots of exudate collected 3 and 5 days after inoculation was equivalent to kinetin standards of about 2.0 μg/liter and 1.0 μg/liter, respectively. Seven days after inoculation, exudate from healthy plants showed cytokinin activity that was equivalent to the activity of 2.5 μg/liter kinetin whereas the activity of exudate from TRSV-infected plants was equivalent to about 0.6 μg/liter kinetin.

Purified ethanolic extracts of cowpea plant roots that were further separated by paper chromatography and then were included in the tobacco stem pith callus bioassay, showed two peaks of cytokinin activity (Fig. 2). In extracts from healthy plants the eluant from sections of R_f value 0.5 produced a small peak of activity corresponding to about 1.0 μg/liter kinetin. The peak from healthy plant root extracts was 28% greater than that produced by extracts from TRSV-infected plant roots, but owing to the large standard deviation of the readings at R_f 0.5 in each of the four experiments, the difference in cytokinin levels between healthy and TRSV-infected sap at R_f 0.5 was not statistically significant. The second, major peak of cytokinin activity in healthy extracts occurred at R_f 0.8 and was 32% greater than the peak produced by extracts from TRSV-infected plant roots. This activity was equivalent to that shown by more than 5.0 μg/liter kinetin. The difference between healthy and TRSV-infected plant root extracts appeared consistently in all four experiments and was statistically significant at the $P = 0.05$ probability level. This peak corresponded to the migration of zeatin riboside and zeatin in *n*-butanol:acetic acid:water (12:3:5, v/v).

Treatment of cowpea primary leaves with 10^{-4} M kinetin for 4 days before and daily after inoculation caused a reduction in the numbers of local lesions formed on the primary leaves by TRSV compared to the numbers of local lesions produced by TRSV on leaves that were not treated with kinetin (Table 2). The average in local

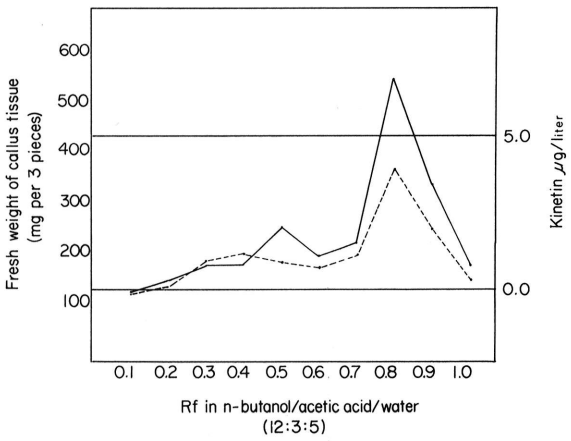

Fig. 2. Tobacco stem pith callus tissue bioassay of purified extracts of healthy (——) and tobacco ringspot virus (- - - -) infected cowpea plant roots after paper chromatography. Each point is the average of four replications.

TABLE 2. 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

Treatment	Local lesions (no.)
− kinetin	242[a]
+ kinetin	59

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

lesion numbers in three experiments was reduced by 75% on the treated leaves. The difference was statistically significant at the $P = 0.01$ level. Local lesions on treated leaves appeared at the same time as on untreated ones but showed more variation in size and shape compared to lesions formed on untreated leaves.

Kinetin treatment of cowpea primary leaves did not affect the fresh weight of shoots that were not inoculated with TRSV (Table 3). Roots of kinetin-treated, uninoculated plants showed an almost 50% reduction in fresh weight 14 days after kinetin treatments began compared to untreated uninoculated plants. There was a significant reduction in the weight of treated and untreated, inoculated shoots compared to treated and untreated, uninoculated shoots. The difference became significant 6 days after inoculation and continued throughout the remainder of the experiment. Root tissue fresh weight from treated and untreated, inoculated plants was significantly lower than the root fresh weight of uninoculated plants after the 8th day from inoculation. The fresh weights of shoots or roots from treated and untreated, inoculated plants were not significantly different at any time during the experiment.

Yellowing of primary leaves of untreated, inoculated plants began to appear 5 days after inoculation. Complete yellowing and abscission of the primary leaves occurred during the next 2 days. In kinetin-treated, inoculated primary leaves, yellowing began to occur later, usually about 7-8 days after inoculation, and the amount of yellowing and abscission in these plants during the remainder of the experiment was less than in untreated, inoculated plants. Yellowing proceeded slower in kinetin treated, inoculated plants than in untreated, inoculated plants.

The concentration of TRSV in roots was affected by treatment of the primary leaves with kinetin. Plants that were not treated with kinetin contained detectable TRSV concentrations 3 days after inoculation of the primary leaves and virus concentration increased through the 5th or 6th day after inoculation (Table 4). After the 5th or 6th day, the virus concentration in root tissue was reduced to less than half. It remained at that level for 3 days and then declined by the 10th day after inoculation. In kinetin-treated, inoculated plants the increase in virus concentration occurred 7 or 8 days after inoculation and then declined rapidly by the 10th day after inoculation.

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

Treatment		Fresh weight (g) following inoculation after: 3 days	4 days	5 days	6 days	7 days	8 days	9 days	10 days
− kinetin − TRSV	Shoots	3.4[a]	4.0	3.7	4.7[b]	5.6[b]	6.0[b]	5.5[b]	6.3[b]
	Roots	2.9[a]	1.8	1.9	2.7[b]	2.8[b]	3.7[b]	3.5[b]	4.8[b]
+ Kinetin − TRSV	Shoots	3.3	3.9	3.9	5.9	4.5	5.3	5.7	5.5
	Roots	2.0	2.1	2.0	2.2	2.8	3.0	2.6	2.5
− kinetin + TRSV	Shoots	2.8	3.2	3.5	3.1	2.8	2.5	2.3	2.1
	Roots	1.6	1.7	1.9	2.0	2.0	1.7	2.2	1.1
+ kinetin + TRSV	Shoots	3.2	3.6	3.8	3.5	3.4	3.4	2.7	2.2
	Roots	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 nine or ten shoots or roots from three experiments.
[b]Each of these numbers represents the average fresh weight in grams of six or seven shoots or roots from two experiments.

TABLE 4. Number of local lesions produced on cowpea primary leaves inoculated with root sap from 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[a]	1	44	39	24	30	21	2
+ kinetin + TRSV	0	2	3	1	28	40	9	3

[a]Each number represents the mean of three replications and shows the average number of local lesions produced on each of eight half-leaves from 1 g of root tissue ground in 3 ml of 0.1 M phosphate buffer, pH 7.0.

DISCUSSION

The ability of tobacco ringspot virus to infect cowpea root meristem tissue agrees with results obtained by Crowley et al. (7), and Atchison and Francki (3) with French bean root tips. In their studies, following initial invasion and multiplication by the virus in the terminal root tip, the virus concentration remained constant thereafter. We detected TRSV in the distal 1-mm sections of cowpea roots 3 days after leaf inoculation; its greatest concentration was reached 5 days after inoculation, and then the virus concentration in root tips declined steadily (Table 1).

The strain of TRSV used became systemic in the cowpea seedlings and killed them in 10 days. The smaller amounts of sap collected from TRSV-infected plants 7 days after inoculation, compared to those of healthy plants, indicate a reduction in the amount of water absorbed by the roots and translocated to the stem in the TRSV-infected plants. This reduction in water absorption and/or translocation was very pronounced in trial collections made later than 7 days after inoculation. This may suggest that TRSV infection affects the ability of the roots to absorb and/or translocate water to the stem of the cowpea plant, but it is also possible that these effects are the result of the damage caused by TRSV on the stem and leaf tissues, including the vascular tissues.

Cytokinin activity was low in sap or root tissues collected from either healthy or TRSV-infected plants. These, of course, were young, vegetative plants 13-21 days old. It has been shown that cytokinin activity in plant sap or root tissues of certain plants remains low until flower induction, at which time it increases (5, 10, 15). The purified compounds that exhibited major cytokinin activity had an R_f value of 0.7 – 0.9, which corresponded to the migration of zeatin and zeatin riboside in the same *n*-butanol:acetic acid:water (12:3:5, v/v) descending system. The presence of these compounds in root tissue has been previously reported (15, 18). If these were indeed the compounds present, losses of zeatin during the purification process were probably slight (12); however, large losses of zeatin riboside may have occurred during precipitation with silver nitrate (14).

The reduction in cytokinin levels in plant sap and root tissue of TRSV-infected plants was preceded by a high rate of virus synthesis in cowpea root tips 5 days after primary leaf inoculation. Atchison (2) found that, following TRSV invasion of root tips of French beans 3 days after inoculation, the mitotic index of root tip cells dropped markedly at 3 days, reached a minimum at 5-6 days, and then returned to normal. Atchison suggests 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 (18, 19), the reduction in cytokinin levels in TRSV-infected cowpea roots, 2 days after virus synthesis reaches its maximum, may occur as a result of virus synthesis in these same meristematic cells.

Treatment of cowpea primary leaves with kinetin for 14 days did not affect the growth of shoots but, for some unknown reason, it did reduce the growth of roots that were not inoculated (Table 3). When the plants were inoculated with TRSV on the 5th day from the start of kinetin treatments, by the 14th day there was no difference either in shoot or in root weight between kinetin-treated or untreated plants, although symptoms on leaves differed. In the latter case, both shoot and root weight seemed to be reduced proportionately by the TRSV infection, and the reduction apparently could not be counteracted by the kinetin treatment. However, 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 2). The results suggest that kinetin treatment of the leaves before inoculation may affect TRSV multiplication, stability, or infectivity.

Kinetin treatment of primary leaves before and after inoculation delayed virus synthesis and reduced the concentration of virus in the roots (Table 4). In kinetin-treated plants, virus concentration increased drastically the 7th day and reached a maximum the 8th day after inoculation, whereas in untreated plants virus concentration reached the maximum 5 days after inoculation. This delay in the kinetin-treated plants was probably the result of reduced virus in the leaf which in turn resulted in less virus transported to the roots. It is also possible, however, that the kinetin treatment of the leaves may have affected the rate of virus movement through the plant and the physiology of the roots.

The difference in symptom development in the kinetin-treated and untreated inoculated plants was in accordance with the known effects of cytokinins. Leaves in which the virus was present at high concentrations showed yellowing and this was followed by abscission during the next 2 days. Kinetin-treated inoculated leaves in which virus synthesis was apparently slower began to turn yellow later and the symptoms among treated leaves were less frequent than among leaves of untreated plants.

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 (4, 6, 8, 16). Also, stresses applied to leaf tissue are known to cause reductions in cytokinins in root exudate as well as in leaves under stress (9). Therefore, the evidence from this study suggests that TRSV infection in cowpea plants causes a reduction in the amount of cytokinins produced in root tissue and translocated to the shoot. The reduction in cytokinin becomes measurable 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 inactivated 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.

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