Enhanced synthesis of messenger RNA in relation to resistance-expression in cowpea *(Vigna unguiculata)* infected with the root-knot nematode, *Meloidogyne incognita*

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SUMMARY

Evidence was sought, for enhanced synthesis of messenger RNA in two cultivars of cowpea, C-152 and Pusa Barsati, resistant and susceptible to the root-knot nematode, *Meloidogyne incognita*, with an objective to elucidate molecular mechanisms of plant-nematode interactions.[^{3}H] uridine labelled RNA isolated from C-152 and Pusa Barsati inoculated with *M. incognita* was fractioned and analyzed employing poly (U)-Sepharose 4B affinity chromatography. Poly (A)-containing mRNA and MAK column bound RNA constituted less than one per cent of the total labelled RNA; the remaining radioactivity was distributed among the bulk RNAs consisting of sRNA and rRNA. [^{3}H] labelled uridine incorporations into RNA fractions indicated that the resistant reacting cowpea cultivar C-152 synthesized poly (A)-containing mRNA about six times more rapidly than the uninoculated plant within 96 hours of inoculation. A similar trend was also observed in the case of the susceptible cultivar Pusa Barsati, although, the degree of enhanced synthesis of mRNA, compared to the resistant cultivar was lesser. A comparison of data of bulk RNA and mRNA clearly indicates activated synthesis of mRNA in the resistant cultivar during the early stage of infection. Treatment of infected roots with actinomycin D (ACTD) reduced enhanced synthesis of mRNA indicating that accumulation of RNAs observed during the post-infection period arose due to *de novo* synthesis. Time course of peroxidase activity, lignin and polyphenols in both the cultivars synchronized with activated synthesis of mRNA, especially in the resistant-reacting cultivar. Application of ACTD (10 µg/ml) prior to the establishment of visible symptoms not only checked further synthesis of mRNA, but also resulted in cessation of further increase in activity or concentration of the macromolecules, lignin and chlorogenic acid. The direct contribution of the nematode towards increased synthesis of mRNA was also examined. It appears from the data thar the invading parasite triggered the host to synthetise mRNA as a feed back defence machanism to counter the animal. The role of newly synthesized mRNA in the resistant and susceptible cultivar, and the role of peroxidase, lignin like polymers and chlorogenic acid are discussed in terms of plant-nematode interaction. On the basis of the results the conclusions are : (1) Synthesis of one or more additional RNA species of the enhanced synthesis of poly (A)-containing mRNA is a specific phenomenon elicited by plant-nematode interaction. (2) Plant-nematode interactions result in gene activation at transcriptional level, eventually leading to synthesis of macromolecules which influence the fate of the infected host. (3) The phenomenon of resistance in cowpea cultivar C-152 is an active one, and both the susceptible and resistant hosts have the tendency to resist the entry of alien entity like nematodes. (4) The ability of the resistant host to counter the nematode is one due to its ability to invoke an early biochemical defence mechanism which is lacking in the susceptible host.

RÉSUMÉ

Accroissement de la synthèse du RNA messager en relation avec l'expression de la résistance chez le pois d'Angole (Vigna unguiculata) *infesté par le nématode galligène* Meloidogyne incognita

Dans le but d'élucider les mécanismes moléculaires des relations entre plantes et nématodes, il a été cherché à mettre en évidence un accroissement de la synthèse du RNA messager chez les cultivars de pois d'Angole C-152 et Pusa Barsati, respectivement résistant et sensible à *Meloidogyne incognita*. Du RNA, marqué sur l'uridine [^{3}H] et isolé des cultivars C-152 et Pusa Barsati inoculés avec *M. incognita*, a été fractionné et analysé par chromatographie d'affinité sur colonnes de poly (U)-sepharose 4B. Le RNA messager poly (A)+ et le RNA fixé sur la colonne de MAK constituent moins de un pourcent du RNA marqué total; la radioactivité restante est répartie dans la masse de RNA, constituée de sRNA et rRNA. L'incorporation d'uridine [^{3}H] marquée dans les fractions de RNA permet de constater que, 96 heures après l'inoculation, le cultivar résistant C-152 a synthétisé le mRNA poly (A)+ six

fois plus rapidement que la plante non inoculée. Une tendance identique a également été notée chez le cultivar sensible Pusa Barsati quoiqu'ici le taux d'augmentation de la synthèse du mRNA fut moindre que celui observé chez le cultivar résistant. La comparaison des données concernant la masse de RNA et le mRNA indique clairement une activation de la synthèse du mRNA pendant les premiers stades de l'infestation. Le traitement par l'actinomycine D (ACTD) de racines infestées diminue l'accroissement de la synthèse de mRNA : ceci indique que l'accumulation de RNA observée pendant la période suivant l'infestation est due à une synthèse *de novo*. La cinétique de l'activité peroxydasique, de la lignine et des polyphénols est chez les deux cultivars synchronisée avec l'activation de la synthèse du mRNA, plus particulièrement d'ailleurs chez le cultivar résistant. L'application d'ACTD (10 µg/ml) avant l'apparition de symptomes visibles non seulement empêche une synthèse ultérieure du mRNA, mais provoque également un arrêt de l'accroissement d'activité ou de concentration des macromolécules (lignine et acide chlorogénique). Le rôle direct du nématode dans l'accroissement de la synthèse du mRNA a été également pris en considération. D'après les données recueillies, il apparaît que le parasite déclenche chez l'hôte la synthèse du mRNA, mécanisme de réaction de défense destinée à bloquer l'animal. Le rôle du mRNA néo-synthétisé chez les cultivars résistants et sensibles, et le rôle de la peroxidase, des polymères voisins de la lignine et de l'acide chlorogénique sont discutés à la lumière des interactions entre plante et nématode. Sur la base de ces résultats, les conclusions suivantes sont avancées : (1) la synthèse de un ou plusieurs types additionnels de RNA lors de l'augmentation de la synthèse du mRNA poly (A)+ est un phénomène spécifique provoqué par une interaction entre plante et nématode. (2) Les interactions entre plante et nématode provoquent une activation des gènes au niveau transcriptionnel conduisant en fin de compte à la synthèse de macromolécules qui influencent le devenir de l'hôte infesté. (3) Le phénomène de résistance chez le cultivar C-152 de pois d'Angole est un phénomène actif, et les cultivars tant résistants que sensibles montrent une tendance à s'opposer à l'entrée du corps étranger que représente le nématode. (4) La capacité de la plante résistante à stopper le nématode est due à sa possibilité de faire appel à un mécanisme de défense qui manque chez la plante sensible.

A great many publications report about the production of phytoalexins as a defence machanism of hosts infected with plant parasitic nematodes (Rich, Keen & Thomason, 1977; Kaplan & Keen, 1980; Kaplan, Keen & Thomason, 1980; Veech, 1982) and examine relationships between synthesis of these classical antibiotic molecules and hypersensitive reactions of hosts (Thomason, Rich & O'Melio, 1976; Rich, Keen & Thomason, 1977; Kaplan & Keen, 1980; Ganguly & Dasgupta, 1982). The results from our laboratory (Premachandran & Dasgupta, 1983 *a, b, c*) coupled with those reported previously (Giebel, 1974, 1979, 1982) explain in part, biochemical mechanism of plant resistance to the nematodes and hint towards the possibility of existence of a biochemical mechanism of host resistance to these parasites operating at molecular level. On the basis of these results, a useful model was proposed by Premachandran and Dasgupta (1983 *c*). This model predicts that incompatibility reaction in root-knot nematode-infected plants is mediated by *de novo* gene activation at transcriptional level resulting in the synthesis of new RNAs and enzyme proteins required for the production of metabolites possessing antibiotic properties. If this hypothesis is correct, then there should be a positive experimental evidence for synthesis of new species of mRNA required for the production of phytoalexins. Although, alteration in nucleic acid metabolism has been demonstrated by several workers (Okapnyi, 1980; Masood & Saxena, 1980; Premachandran & Dasgupta, 1983 *a*), we are aware of only one publication which reports a selective and enhanced activated synthesis of MAK bound RNA (TB-RNA) which is recognized as mRNA by several authors (Cherry, 1964; Ewing & Cherry, 1967; Kunz *et al.*, 1970), in the resistant cultivar of tomato infected with *M. incognita* (Parthasarthy & Dasgupta, 1984). Beyond this, little is known about the alteration and rate of synthesis of mRNA in nematode infected plants.

In this study, we wish to seek evidence for activated synthesis of poly (A)-containing mRNA in two cultivars of cowpea, susceptible and resistant to *M. incognita* by employing poly (U)-Sepharose column with an objective to elucidate the biochemical mechanism of plant-nematode interaction at molecular level. Furthermore, we propose to show by gel chromatography, actinomycin D and measurements of peroxidase activity, one of the two key enzymes for lignin formation, that activated synthesis of mRNA, is in fact, causally linked with the expression of resistance in cowpea cultivar C-152 and the production of lignin like polymers and enhanced synthesis of the enzyme peroxidase. Also we examined the relationship between synthesis of mRNA and the expression of resistance and the production of lignin like polymers and polyphenols.

Materials and methods

Chemicals

Bovine serum albumin, Cohn fraction V (BSA); bromophenol blue (3′, 3″, 5′, 5″ - tetrabromo - phenol sulfon - phthalein); chlorogenic acid; diatomaceous earth, grade III; diethyl pyrocarbonate (DEP); sodium lauryl sulfate (SLS); methylated albumin; calf thymus deoxyribonucleic acid, ribonucleic acid, type III; *O*-dianisidine; tris (hydroxymethyl) aminomethane and peroxidase enzyme (EC 1.11.1.7), were the products of Sigma Chemicals Company, St. Louis, Missouri, USA; Scintillation grade 1, 4-bis (5-phenyl-2-oxazolyl)- benzene (POPOP) was obtained from the Center for Bio-

chemical, CSIR, New Delhi; 2, 5-diphenyloxazole (PPO) from British Drug House, UK; Triton X-100, from the Chemicals Division, Glaxo Laboratories; naphthalene scintillation grade from Koch Light Co., UK; actinomycin D (ACTD) from Merck, Sharp and Dohme Research Laboratory, Chemical Division of Merck and Co. Enc., Rahway, N.J.; 1, 4-dioxan, scintillation grade and polyacrylamide gel electrophoresis reagents were obtained from Merck, Federal Republic of Germany; phloroglucinol from Loba-Chemie Indo-austranal Co. and streptomycin sulfate from Sarabhai Chemicals, India; Poly (U)-Sepharose 4B was obtained from pharmacia Fine Chemicals, Uppsala, Sweden. [^{32}P] labelled orthophosphate, [^{3}H] labelled uridine, specific radioactivity 5 000 mCi/mmol and [U—^{14}C] - labelled leucine, specific radioactivity 140 mCi/mmol were purchased from the Bhabha Atomic Research Centre, Trombay, India. Ethanol used during the experiments was purified by double distillation. All other reagents used in this investigation were of the highest purity obtainable commercially and used without further purification.

General procedures

Nematode

Stock cultures of the root-knot nematode, *Meloidogyne incognita* obtained originally from a single egg mass progeny, were maintained and multiplied on tomato (*Lycopersicon esculentum* cv. Pusa Ruby), and grown in 35 cm pots containing sterilized sand, and supplemented whith full strength Hoagland nutrient solution. As and when required, the nematode populations were subcultured at 45-60 days interval, either by periodic replacement and with fresh seedlings of tomato in infested sand or by inoculating new transplants with second-stage larval suspension of *M. incognita*, after removal of old plants to ensure a constant supply of sufficient egg masses and infective larvae.

Plants and inoculation

Cowpea *(Vigna unguiculata)* cv. C-152 (resistant to *M. incognita*) and Pusa Barsati (susceptible to *M. incognita*) obtained from the Project Coordinator, Division of Nematology and the Division of Horticulture, Indian Agricultural Research Institute, New Delhi, respectively, were used in these studies. The selection of C-152 to represent a cowpea cultivar resistant to *M. incognita* was based on an earlier report (Darekar & Patil, 1981). A preliminary study was made to confirm incompatible reaction of C-152 to *M. incognita*.

Seeds of the two cultivars were surface sterilized by immersion in 0.1 per cent $HgCl_2$ for 5 mn followed by repeated washing with sterile water blanks and sown in 6 and 10 cm pots containing sterilized coarse river sand. Procedures for collection, storage and examination of second-stage larvae were the same as described previously (Dasgupta & Ganguly, 1975). One week-old seedlings of each cultivar were inoculated with axenized suspension of approximately 5 000 infective larvae of *M. incognita* per seeedling. A set of plants of each cultivar was left uninoculated to serve as controls.

Microscopic observation of roots

The roots of inoculated plants were carefully removed, washed and stained to study nematode penetration and development.

Time course of [^{3}H]-labelled uridine incorporation into RNA fractions

^{3}H feeding and extraction of RNA

Cowpea cultivars Pusa Barsati and C-152 were harvested at 24, 48, 72, 96 and 720 hours intervals after inoculation. Surface sterilized, infected root samples were placed in [^{3}H]-uridine solution (100 µCi/ml) for 4 hours during which the isotope solution was found to be completely taken up by host materials. The method of extraction of RNA from [^{3}H]-uridine fed root samples was essentially the same as given by Yoshikawa, Masago and Keen (1977).

Isolation of poly (A)-containing mRNA

One gram of ^{3}H-labelled uridine root samples galled/infected or healthy were homogenized in 3.5 ml of 100 mM Tris-HCl buffer, pH 9.0 containing 400 mM NaCl, 1 per cent SLS, 1 mM $MgCl_2$ and 100 µl DEP at 4°. The suspension was vigorously stirred after the addition of 3 ml of phenol : chloroform : isoamyl alcohol mixture (50 : 50 : 2 v/v) at room temperature of 25°, chilled at 4° and centrifuged at 5 000 × *g* for 15 mn. The residue after removal of aqueous phase was re-extracted with 3 ml of the same buffer and combined aqueous phases re-extracted with 5 ml of phenol mixture. The radioactivity in a 50 µl aliquot of aqueous phase was determined in 10 ml of Triton X-100 scintillation mixture consisting of two volumes of toluene scintillation mixture (5 g PPO, 0.2 g POPOP/litre of toluene) and one volume of Triton X-100 for mesauring the total uptake. Crude RNA was precipitated from the aqueous phase by leaving them overnight at —12° with 2.5 volumes of distilled ethanol. The RNA precipitate was collected by centrifugation at 3 000 × *g* for 20 mn. The RNA pellets were washed with ethanol containing 0.2 per cent NaCl (2:1) and dissolved in 1 ml of NETS buffer (10 mM Tris-HCl, pH 7.4; 100 mM NaCl; 10 mM EDTA and 0.2 per cent SLS) and centrifuged at 16 000 × *g* to remove insoluble material. The crude RNA thus extracted was checked for the presence of DNA by determining optical density ratios at 260 nm/280 nm and 260 nm/230 nm or by diphenylamine test.

Fractionation of poly (A)-containing mRNA by Poly (U)- Sepharose 4B column

The isolation of poly (A)-containing mRNA from the crude RNA was accomplished through affinity column chromatography on poly (U)-Sepharose 4B column via methods of Eiden and Nicholas (1973) and Yoshikawa, Masago and Keen (1977). Separate columns of equal sizes were used for each root sample. The columns were prepared as per instructions from the manufacturer. The crude RNA solution (800 µl) was mixed with 1.2 ml of NETS buffer and loaded onto 1 × 6 cm micro-columns containing 2 ml of poly (U)- Sepharose 4B slurry prepared by swelling overnight in 100 mM NaCl, pH 7.5 and pre-washed with 50 ml of NETS buffer, pH 7.5. Unbound RNA was eluted with 15 ml of NETS buffer and fractions of 3 ml were collected. The column was then washed with 15 ml Tris-HCl buffer, pH 7.4 containing 100 mM NaCl. The bound RNA was eluted with 15 ml of deionized water at 50°. The flow rate was maintained constant at 18 ml/hour. The radioactivity in each fraction was determined by counting a 50 µl aliquot in Triton X-toluene scintillation mixture at 1 mn interval.

ACTINOMYCIN D TREATMENT AND *DE NOVO* SYNTHESIS OF MRNA AND PROTEIN

Twentyfour hours after inoculation, inoculated and uninoculated plants were depotted, immersed in deionized water and surface sterilized. Each root system was immersed in beakers containing 10 ml of actinomycin D (10 µg and 5 µg/ml) for one hour with intermittent stirring. Another group of plants of each cultivar was treated with distilled water in the same way to serve as controls. Unless otherwise stated, treated and untreated roots were incubated in a solution of 10 ml 50 mM Tris-HCl buffer, pH 8.8 and streptomycin sulfate (20 µg/ml) for one hour. Plants thus treated were grown for one to 30 days in greenhouse conditions.

At various times after inoculation or actinomycin D treatment, the root samples were fed either [^{3}H]-labelled uridine solution (100 µCi/ml) for measurement of poly (A)-containing mRNA or [^{14}C]-labelled leucine solution (100 µCi/ml) for measurement of protein.

De novo *synthesis of poly (A)-containing mRNA*

The rate of poly (A)-containing mRNA synthesis was determined by extracting [^{3}H]-uridine labelled RNA by phenol-chloroform-isoamyl alcohol method (Yoshikawa, Masago & Keen, 1977) and further fractionation of poly (A)-containing mRNA from total RNA through poly (U)-Sepharose 4B affinity column chromatography as described previously.

De novo *synthesis of protein*

Roots treated with [^{14}C]-labelled leucine were prepared for extraction. The protein extract was suspended in 1 ml of ice-cold 5 % (v/v) trichloroacetic acid with 5 mM leucine (unlabelled) and kept overnight at 0° (Yoshikawa, Masago & Keen, 1977). It was subsequently filtered through whatman filter paper No. 1. The filtrate was washed with 2 ml of ice-cold 5 % trichloroacetic acid containing 5 mM unlabelled leucine. The filter paper was dried on an oven at 50° and the radioactivity on the filters was directly counted in 5 ml of Bray's (1960) scintillation fluid. The radioactivity was measured employing a liquid scintillation spectrometer.

ASSAY FOR PEROXIDASE (EC 1.11.1.7) ACTIVITY

For detection of peroxidase activity, one to 1.5 g of roots were homogenized using 50 mM Tris-HCl buffer, pH 7.0 containing 0.5 % (v/v) β-mercaptoethanol. The filtrate was centrifuged at 16 000 × *g* for 30 min at — 4°. In usual routine assay, the enzymatic activity of peroxidase was determined using pyrogallol as substrate. The enzyme solution (1 ml) was added to a reaction mixture consisting of 2.0 ml of 5 % pyrogallol (freshly prepared in water), 1.0 ml 147 mM H_2O_2, 2.0 ml 100 mM phosphate buffer, pH 6.0, and 14.0 ml water. After exactly 45 sec. the reaction was stopped by adding 1.0 ml of NH_2SO_4 and extracted twice with 5.0 ml portions of ether and optical absorbancy was recorded with spectrophotometer model Spektromom 204 at 420 nm using ether as reference. The rate of reaction was found to be linear during the first 45 sec. A unit of enzyme is defined as the amount of enzyme which will result in increase in absorbancy of 1 *A* per 45 sec per g of fresh root weight under given assay conditions.

PROTEIN ESTIMATION

Concentration of protein in crude extracts was determined according to the method of Lowry *et al.* (1951). The quantity of protein in the sample was determined from the standard curve prepared using bovine serum albumin.

Polyacrylamide gel electrophoresis

The isozymes of peroxidase and protein patterns of roots were determined either via the method of Davis (1964) with 7.5 % running gel without the larger pore spacer gel or by employing SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) (Jones, 1980) containing 12 % running gel and 3 % stacking gel. A sample of 50-100 µg protein was loaded under buffer on top of the running gel or stacking gel along with 20 % sucrose. The runs were made from cathode to anode with a current of 3 mA per tube. On the completion of runs, gels were removed and washed in distilled water. Protein bands were detected by either staining with amido Schwartz or Coomasie brilliant blue and then destaining in 7.0 % acetic acid.

The peroxidase isozymes resolved on the gel surface were detected by incubating them for 30 mn in a reaction mixture containing 3.2 ml of 5 % *O*-dianisidine, 9.6 ml of sodium acetate buffer, pH 5.4 and 83.2 ml of H_2O (Shannon, Uritani & Imaseki, 1971). At the end of the incubation period, the gels were treated with 100 mM H_2O_2 till the bands developed and preserved in 7.0 % acetic acid.

Results

Cowpea cv. C-152 was reported to be a resistant host for the root-knot nematode, *Meloidogyne incognita* (Darekar & Patil, 1981). But in view of the reported existence of physiological races in *M. incognita* (Taylor & Sasser, 1978), we decided, at the onset of this work, to have preliminary information concerning penetration and developement of the nematode, protein patterns of both the cultivars and cellular responses of the host during post-infection period. We consistently found that the population of *M. incognita* used in this study penetrated both the cultivars, Pusa Barsati and C-152 (susceptible and resistant, respectively) almost in equal numbers within 24 hours after inoculation (32.5 and 29.0 % of initial inoculum respectively), which reached maximum by the next 72 hours. Hypersensitive reaction, however, was observed only in cv. C-152 soon after penetration of the nematodes. Employing SDS-PAGE gel electrophoresis it was observed that the anionic protein patterns of both the cultivars were strikingly similar with reference to their movement in electrolytic field and number of bands. On the basis of these observations, it was of interest, in the subsequent steps, to study time course of ^3H incorporation into different classes of RNAs.

Time course of induced synthesis of poly (A)-containing mRNA

Initial observations showed that only poly (A)-containing mRNA remained bound to poly (U)-Sepharose 4B columns and the columns did not bind poly (U) or unlabelled crude RNA from the host plants. The possibility of DNA getting bound to the column was also checked and found to be negative. Hereafter, RNA unbound to poly (U)-Sepharose 4B column will be referred to as bulk RNA (sRNA + rRNA) and RNA bound to columns as poly (A)-containing RNA or mRNA, since it has been demonstrated conclusively that RNA containing poly (A) sequences are indeed mRNA (Greenberg, 1975; Tobin & Kelin, 1975). Elution profiles of incorporations of [^{3}H]-labelled uridine into RNA fractions of two cultivars inoculated with nematodes are shown in Figure 1.

In consistency with the earlier observation, the results furnished in Table 1 provide clear evidence for enhanced synthesis of bulk RNA and especially mRNA in the inoculated, susceptible and resistant cultivars indicating alterations of mRNA metabolism at early stages of infection before the establishment of visible compatible and incompatible interactions*. However, the rate of mRNA synthesis in cultivar C-152 showing incompatible reaction, was substantially more than that of the susceptible cultivar within 24 to 96 hours after inoculation. The hypersensitive reaction of infected tissue in the resistant cultivar was observed only after this period. Furthermore, the magnitude of enhanced synthesis of mRNA was considerably higher than that of bulk RNA in both the hosts. The ratio of [^{3}H]-labelled uridine incorporation into different classes of RNAs from inoculated and uninoculated root samples is shown in Figure 2. It was of interest to note that the ratios of [^{3}H]-labelled uridine incorporation (inoculated/uninoculated) into poly (A)-containing mRNA was considerably higher in the resistant reacting cultivar than its susceptible counterpart during the course of 24-96 hours of post-infection period (3.1-6.7 in the resistant cultivar *vs* 1.3-2.1 in the susceptible cultivar). The specific radioactivities of poly (A)-containing mRNA isolated through affinity column chromatography were determined and the results are presented in Figure 3. The results show that the specific radioactivities of mRNA from the root samples at 24, 48, 72 and 96 hours after inoculation was higher than those of mRNA fractions from the corresponding healthy controls. The increase in specific radioactivities reached maximum at 96 hours after inoculation and was then followed by an apparent decline. These results emphasize that the onset of enhanced synthesis of mRNA in the resistant reacting cultivars which initiated 24 hours after inoculation continued till 96 hours of post-infection period. Thus, the results (Figs. 2-3 and Tab. 1) are considered to indicate specific activation of mRNA synthesis in resistant reacting cultivar C-152 at a relatively early stage of infection preceding or almost synchronizing with the appearance of hypersensitive reaction.

The results reported in the preceding sections raise one important and relevant question to be answered : what could be the possible reasons to account for enhanced synthesis of mRNA in the resistant reacting cultivar, in particular? One possibility is that the elevated level of mRNA observed in inoculated plants in general and the resistant cultivar in particular were synthesized *de novo*. The other possibility is that the invading nematodes themselves contributed towards increased synthesis of mRNA. The subsequent experiments were designed to examine these possibilities and the results reported thereafter provide conclusive evidence for *de novo* synthesis of mRNA and enzyme protein and their link with the synthesis of lignin like polymers and polyphenols is also shown.

*^{32}P incorporation indicated enhanced synthesis of MAK column bound RNA in nematode-inoculated plants.

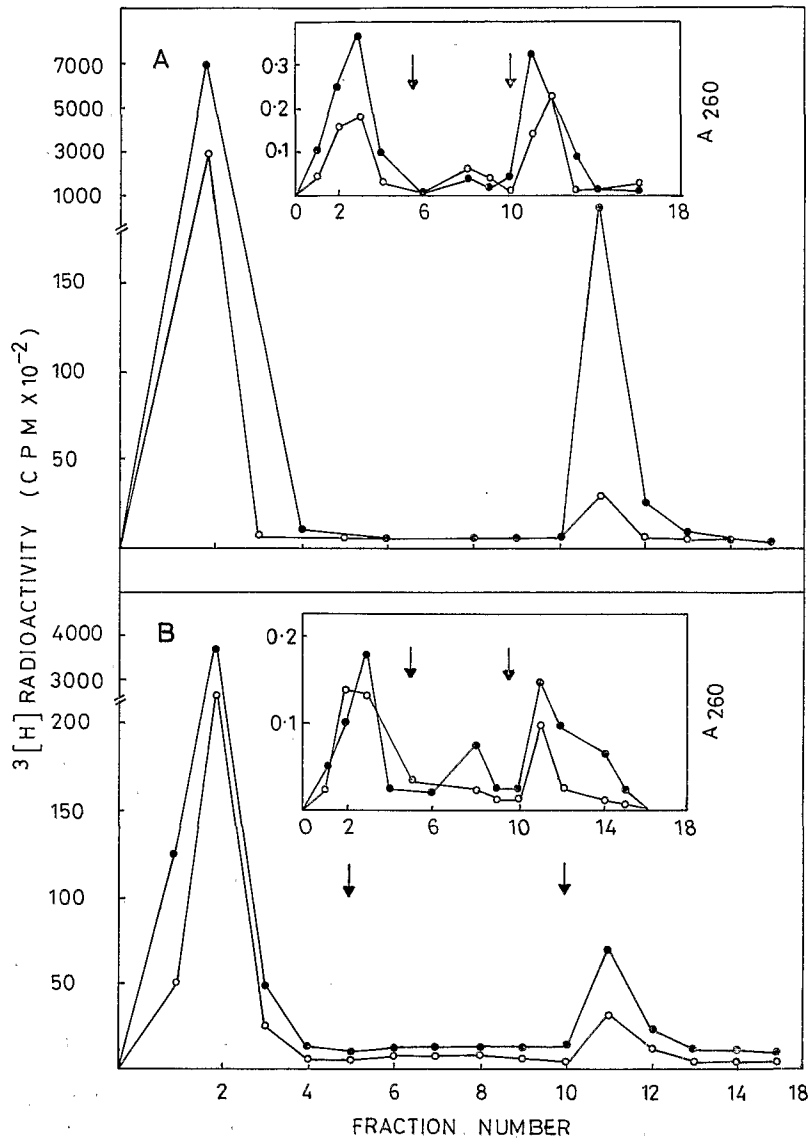

Fig. 1. Affinity chromatography of RNA fractions on poly (U)-Sepharose 4B columns, 96 hours after inoculation. The arrows indicate points of addition of buffer (10 mM Tris-HCl, pH 7.4, 100 mM (NaCl) and deionized water at 50° respectively, in order of increasing fraction No. Data represent three replicates. Inset graphs are absorbancy curves at 260 nm. Uninoculated : open circles; Inoculated : black circles. A : C-152; B : Pusa Barsati.

De novo SYNTHESIS OF mRNA AND PROTEIN

Application of ACTD at 5 µg and 10 µg/ml efficiently inhibited synthesis of mRNA and/or protein in both inoculated and uninoculated plant (Tab. 2). Poly (A)-containing mRNA synthesized in the inoculated resistant cultivar was about six times greater than that of uninoculated plants. The rate of increase of mRNA synthesis in the same cultivar was inhibited by actinomycin D at 5 µg and 10 µg/ml (Fig. 4 A). The trend remained the same for susceptible cultivar although the degree of inhibition by actinomycin D was to a lesser degree. The rate of protein synthesis was also inhibited due to actinomycin D treatment although not to the same extent as that of mRNA (Fig. 4 B).

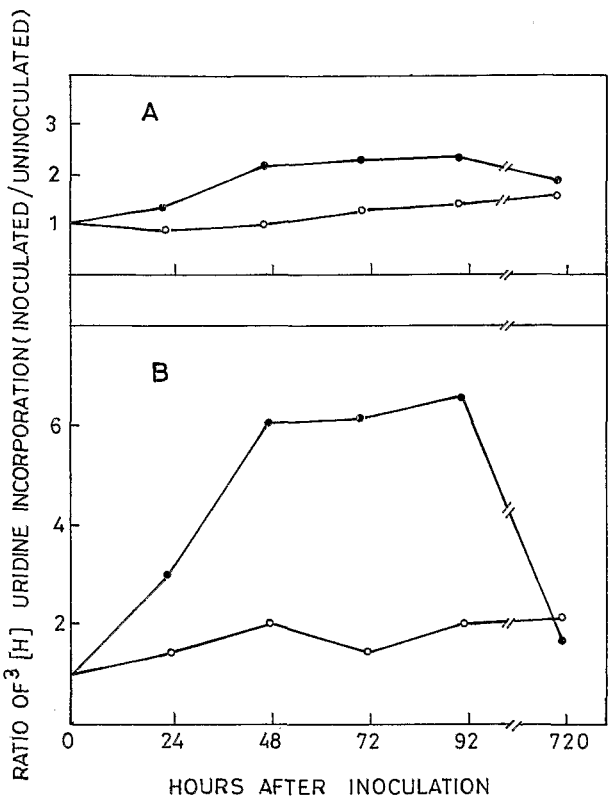

Fig. 2. Synthesis of bulk-RNA (sRNA + rRNA) and poly (A)-containing mRNA in cowpea cultivars C-152 (black circles) and Pusa Barsati (open circles) inoculated with *M. incognita*. A : Bulk RNA unbound to poly (U)-Sepharose 4B column; B : Poly (A)-containing mRNA bound to poly (U)-Sepharose 4B column. Each point indicates average value of three replicates.[^{3} H] uridine incorporation values are correctedvalues.

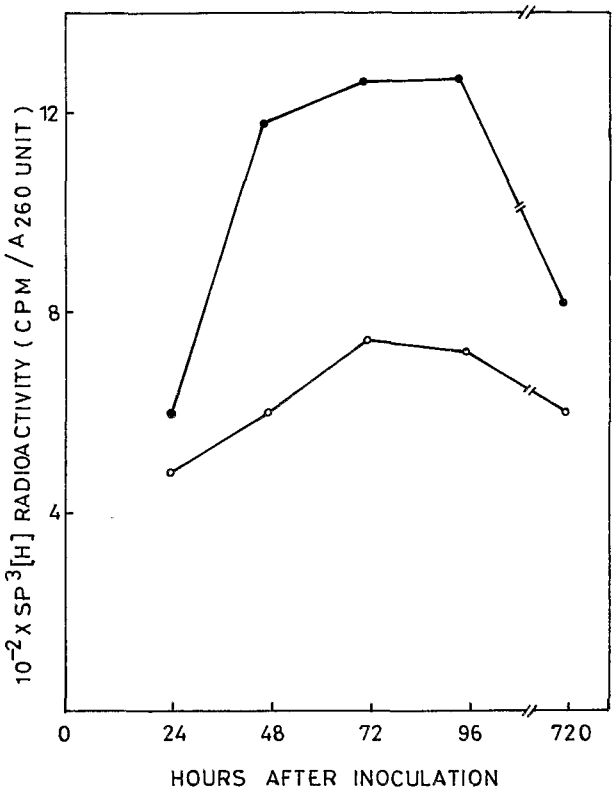

Fig. 3. Effect of inoculation of cowpea cultivar C-152 with *M. incognita* on the specific radioactivity of poly (A)-containing mRNA for uninoculated (open circles) and inoculated (black circles) plants respectively.

Possible contribution of nematode towards enhanced synthesis of mRNA

As stated previously, the metabolism of the invading parasite or contaminant microorganisms could be contributory factors towards enhanced synthesis of mRNA observed during early stages of infection. Results in Table 3 do not provide any evidence to suggest that the observed increased synthesis of poly (A)-containing mRNA was due to *M. incognita*. Likewise, procedures adopted during experimentation ensured complete suppression of microbial growth. Furthermore, axenization of the nematode did not reduce the observed increase in mRNA synthesis in the infected host (Tab. 3).

On the basis of results furnished in Tables 2 and 3 a reasonable explanation for enhanced synthesis of mRNA especially in the resistant-reacting cultivar is that the observed elevated levels of mRNA in incompatible hosts is mainly due to activated *de novo* synthesis of mRNA by host cells. Alternative explanations such as synthesis of mRNA by the parasite within the host or microbial contaminants are ruled out due to lack of experimental evidence.

Because of the observed hypersensitive reaction in the resistant cultivar and because of conclusive evidence of enhanced synthesis of mRNA in the resistant cultivar observed in this study, it was of interest to determine the link, if any, between activated synthesis of peroxidase and the production of lignin like polymers, which have been reported to be associated with the expression of resistance (Mote & Dasgupta, 1979). Therefore, we considered the possibility that the activity of peroxidase, one of the key enzymes of lignin formation could influence the enhanced synthesis of these metabolites in the resistant reacting cultivar. Indeed this appears to be the case as shown in the following experiment.

Time course of peroxidase activity

The data in Table 4 show time course of peroxidase activity in two cultivars inoculated with *M. incognita*. It may be noted that peroxidase activity increased from

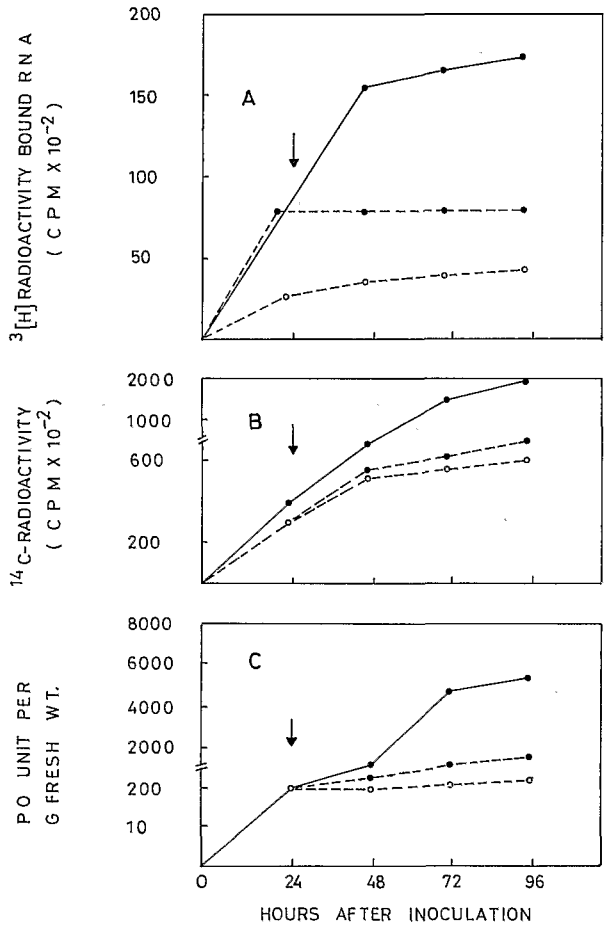

Fig. 4. Effect of ACTD (5 µg/ml) on time course synthesis of poly (A)-containing mRNA (A), protein (B), and peroxidase (C) activity in C-152. Arrows indicate point at which ACTD was applied. Inoculated, untreated (water) : black circles; inoculated, treated (ACTD) : black circles and dotted line; uninoculated, untreated (water) : white circles and dotted line.

120 units in healthy tissue at 0 hours of inoculation to 6 500 units, 96 hours after inoculation in the resistant reacting cultivars. This amounts to nearly 136 fold increase in peroxidase activity during 96 hours of post-inoculation period. Thereafter there was a fall in the enzyme activity. Compared to the phenomenal increase in peroxidase activity of resistant cultivar during the early stage of infection the increase in the activity of enzyme in susceptible cultivar was relatively rather inconspicuous.

Whether or not the remarkable increase in peroxidase activity results from the *de novo* synthesis of the enzyme or from the activation of the existing inactive form was examined through the treatment of infected tissue with actinomycin D and later by analyzing them through gel electrophoresis. It can be seen from the data that there was a complete cessation of further peroxidase synthesis soon after treatment of the host with actinomycin D (Fig. 4 C).

Confidence in the quality of data in Table 4 and Figure 4 C was gained by qualitative determination of peroxidase isozymes in poly acrylamide gel electrophoresis (Figs. 5-6). Application of actinomycin D resulted in almost cessation of synthesis of new peroxidase isozymes indicating the fact that elevated level of enzyme observed in the quantitative studies, by and large, was due to the *de novo* synthesis of peroxidase. It is of interest to note that only the resistant cultivar C-152 showed synthesis of new isozymes which are lacking either in its healthy counterpart or inoculated or uninoculated cultivars of Pusa Barsati (Fig. 5). The number of isozymes in inoculated and uninoculated susceptible cultivars remained the same although the concentration of individual isozymes was more in the inoculated plant as judged on the basis of staining intensity.

Changes in concentration of lignin and polyphenols

The values of the lignin concentration during the course of disease progression are shown in Table 5. Maximum lignin accumulation in resistant cultivar was almost complete at 72 hours after inoculation, whereas in the susceptible cultivar the values for maximum was reached much later. It is noteworthy that 24 hours after inoculation there was far more absorbancy at 245 nm and 358 nm in the inoculated resistant cultivar than the susceptible counterpart. The values for inoculated samples of C-152 were higher than Pusa Barsati.

These results could, perhaps, suggest the greater biosynthesis of phenolic compounds in the resistant rather than susceptible cultivar, which were examined in the following experiments and the results are presented in Table 6. The analysis of chlorogenic acid in the time course studies shows a remarkable increase in the concentration of CGA in the resistant variety during early stage of infection. However, these values were not so conspicuous for its susceptible counterpart.

Discussion

It has been an exciting experience for our laboratory to realize the intrigues and complexities of the biochemical mechanism of plant-nematode interactions which we have been struggling to decipher at the molecular level, linked with the *de novo* synthesis of polypeptides. And, within the conceptual framework of the model proposed by Premachandran and Dasgupta (1983 *c*) important questions are : is there any evidence for activated synthesis of mRNA during early stages of disease development which indicate possible changes in DNA transcription in the infected host, and which may

Table 1

Time course of [^{3}H]-labelled uridine incorporation into poly (A)-containing mRNA of cowpea cultivar C-152 and Pusa Barsati inoculated with *M. incognita* (isolation of poly (A)-containing mRNA was made by poly (U)-Sepharose columns. Separate columns of equal sizes were used for each root sample)

Hours after inoculation	*Cultivar*	*Treatment*	*[^{3}H] radioactivity (cpm × 10^{-2})/g fresh root weight* (1)				
			Total uptake (a)	*Bulk RNA (sRNA + rRNA)* (2) *(b)*	*Percentage of b/a*	*Poly (A)-containing mRNA* (3) *(c)*	*Percentage of c/a*
24	C-152	Control	42010	2838	6.76	26.2	0.06
		Inoculated	42210	3837	9.09	79.0	0.19
	Pusa Barsati	Control	32220	2538	7.88	22.0	0.07
		Inoculated	31050	2638	8.49	30.0	0.10
48	C-152	Control	41352	2996	7.25	30.4	0.07
		Inoculated	41565	6597	15.87	190.6	0.47
	Pusa Barsati	Control	33350	2648	7.94	27.5	0.08
		Inoculated	34251	2735	7.99	55.3	0.16
72	C-152	Control	40343	3125	7.75	32.5	0.81
		Inoculated	40134	6879	17.14	195.2	0.49
	Pusa Barsati	Control	32150	3012	9.37	28.3	0.09
		Inoculated	33756	3617	10.72	45.6	0.14
96	C-152	Control	40343	3101	7.77	30.3	0.08
		Inoculated	40125	7215	17.98	201.3	0.50
	Pusa Barsati	Control	31985	3020	9.44	28.4	0.89
		Inoculated	30965	3823	12.55	60.8	0.19
720	C-152	Control	39126	2998	7.66	30.5	0.08
		Inoculated	38025	6092	16.02	55.2	0.15
	Pusa Barsati	Control	28135	2630	9.35	22.5	0.08
		Inoculated	27350	3723	13.61	45.3	0.17

(1) Data represent a typical experiment, representative of three replicate experiments. Root samples used were either galled/infected (inoculated) or healthy uninoculated.

(2) RNA fractions unbound to poly (U)-Sepharose columns.

(3) RNA fraction bound to poly (U)-Sepharose columns.

occur well ahead of establishment symptom expression? Also, is there any evidence of *de novo* synthesis of mRNA and enzyme mediated biosynthesis of phytoalexin like compounds in nematode infected plant? These questions are addressed to in this report. Keeping these enquiries in mind, the main goal of this investigation as stated earlier, was to provide the experimental evidence for nematode-triggered synthesis of mRNA containing poly (A) sequences during the early stages of infection of the host. Experimental procedures employed in this study fully ensured isolation of poly (A)-containing mRNA and synthesis. It is true that both susceptible and resistant cultivars, exhibited higher rate of mRNA synthesis, soon after the infection, but then, the differences between the resistant and susceptible cultivars in the magnitude of incorporation of [^{3}H]-labelled uridine were more than apparent. Further, it indicates that initiation of symptom expression in nematode-induced-plant diseases is triggered by *de novo* synthesis of new species of RNAs. This observation is in excellent agreement with the reports published earlier (Mote & Dasgupta, 1979). On the basis of these results, we maintain that interaction between the host and nematode during the early stages of infection results in rapid alteration of plant metabolism at gene transcriptional level. The discussion hereafter is primarily aimed to elaborate this point further.

The results presented in this investigation coupled with other published reports (Premachandran & Dasgupta, 1983 *a, b*) suggest that increase in all classes of

Table 2

Effect of actinomycin D (ACTD) on the synthesis of poly (A)-containing mRNA and proteins of cowpea cultivars C-152 and Pusa Barsati uninoculated and inoculated with *M. incognita* (1)

Treatment			Radioactivity/g fresh root weight			
			[^{3}H] uridine incorporation (cpm × 10^{-2})		[^{14}C] leucine incorporation (cpm × 10^{-2})	
			Total uptake	Poly (A)-containing mRNA(2)	Total uptake	Protein (3)
C-152	Uninoculated	Control (H_2O)	40 750	32.3	12 720	1 271
		ACTD (5 µg/ml)	39 394	18.4	12 958	527
		ACTD (10 µg/ml)	37 938	14.5	12 951	504
	Inoculated	Control (H_2O)	40 125	192.5	18 822	1 382
		ACTD (5 µg/ml)	39 683	76.0	11 745	602
		ACTD (10 µg/ml)	39 043	36.2	10 016	575
Pusa Barsati	Uninoculated	Control (H_2O)	30 661	28.3	15 134	1 516
		ACTD (5 µg/ml)	30 851	12.5	14 316	1 021
		ACTD (10 µg/ml)	30 521	13.4	12 314	932
	Inoculated	Control (H_2O)	30 215	59.2	16 803	1 621
		ACTD (5 µg/ml)	31 023	37.4	15 005	1 421
		ACTD (10 µg/ml)	30 521	25.7	14 109	1 041

(1) Results are average of two tests.

(2) The extracted total RNA was separated by poly (U)-Sepharose affinity column chromatography and mRNA was isolated as poly (A)-containing RNA. Separate columns of equal sizes were used for each root sample.

(3) 5 % trichloro acetic acid precipitate fraction.

For details of isotope feeding and ACTD treatment, see text.

RNAs is rather a general feature of host-parasite interactions directing towards either compatible or incompatible reactions. There are several possibilities to explain these observations. The remarkable quantitative enhancement of mRNA synthesis in the resistant-reacting cultivar C-152 at an early stage of infection can be considered as a direct indication of *de novo* synthesis of mRNA and protein. And, once the evidence for *de novo* synthesis of mRNA is established it will be possible to elucidate further whether the *de novo* synthesis of mRNA is causally linked with appearance of new polypeptides and production of antibiotic molecules in the infected plant showing resistance responses. Using ACTD, which is known to prevent transcription of double stranded DNA it has been possible, in this study, to demonstrate *de novo* synthesis of mRNA in the infected plants during the early stage of progression of disease. We propose, on the basis of data obtained that *de novo* synthesis of poly (A)-containing mRNA which initiates as early as 24 hours after inoculation, accounts for the dramatic accumulation of mRNA observed in the infected tissue of the resistant reacting cultivar. The other possibility to account for enhanced synthesis of mRNA during early stages of infection is due to metabolism of parasite or contribution by microbial contaminants. Data recorded in this study, however, do not allow us to accept this possibility. We conclude that accelarated rate of mRNA synthesis in the inoculated resistant cultivar, in particular, is initially due to a parasitically induced activity of the host cells, and a substantial direct contribution by invading parasite or other microorganisms is ruled out. The following lines of arguments are advanced to support this conclusion. First, the dramatic enhancement of mRNA synthesis was observed in the resistant cultivar at 24 hours after inoculation, when the activity of the invading parasite was limited and there was no cytological evidence of the established feeding sites of the nematode. Secondly, mRNA synthesis was much higher at this stage in the resistant reacting cultivar than the susceptible cultivar, although the percentage of nematode penetration in the incompatible host relatively was lower. Thirdly, possible

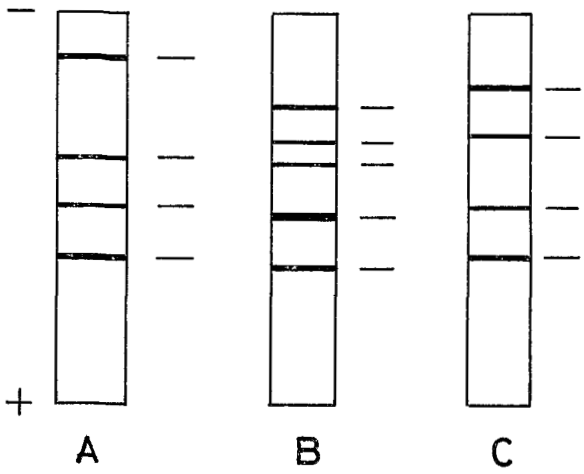

Fig. 5. Effect of ACTD on the synthesis of peroxidase isozymes isolated from C-152 and resolved through polyacrylamide gel electrophoresis. A : healthy, untreated (water only); B : inoculated, untreated (water only) and C : inoculated, treated (ACTD, 10 µg/ml). Fifty µg of enzyme solution was applied onto columns directly under buffer along with 20 % sucrose. For details of time of application of ACTD and inoculation, see text. The arrows indicate number of peroxidase isozymes resolved on gel surface.

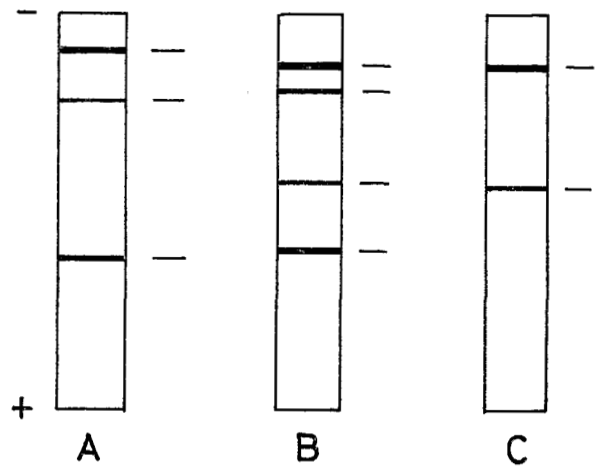

Fig. 6. Effect of ACTD ont he synthesis of peroxidase isozymes isolated from Pusa Barsati and resolved through polyacrylamide gel electrophoresis. A : healthy, untreated (water only); B : inoculated, untreated (water only) and C : inoculated, treated (ACTD, 10 µg/ml). Fifty µg of enzyme solution was applied onto columns directly under buffer along with 20 % sucrose. For details of time of application of ACTD and inoculation, see text. The arrows indicate number of peroxidase isozymes resolved on gel surface.

Table 3

Contribution of host/nematode on the enhanced synthesis of poly (A)-containing mRNA at 96 hours after inoculation of resistant interaction between cowpea cultivar C-152 and *M. incognita*, and susceptible interaction between cowpea cultivar Pusa Barsati and *M. incognita* (mRNA is isolated as poly (A)-containing RNA)

Treatment	*[^{3}H]-radioactivity (cpm × 10^{-2})/g fresh root weight or nematode suspension*				
	Source of RNA extraction	*Total uptake (a)*	*Poly (A)-containing mRNA (b)*	*Percentage of b/a*	*Ratio in % of b/a*
Uninoculated (1)	Nematodes	11 330	6.2	0.054	1.00
Inoculated(3)	C-152	42 232	201.0	0.476	8.81
Uninoculated(2)	C-152	41 230	30.0	0.072	1.33
Uninoculated(2)	Pusa Barsati	31 315	28.3	0.090	1.67
Inoculated(3)	Pusa Barsati	30 675	60.3	0.196	3.62

(1) About 5000 larvae were concentrated in a minimal volume of 3 ml in 5 ml stoppered flask and exposed to [^{3}H] isotopes (100 µCi/ml) for 4 hours. After termination of exposure period, nematode suspensions were filtered and washed repeatedly with sterile water or with acetone and sterile water. Nematodes were then homogenized by ultrasonicator and processed further for radioactivity. Count as detailed in the text under section Materials and methods. Alternatively, for the extraction of nematode-labelled RNA, 1 g of unlabelled healthy root samples was homogenized together with the labelled nematode suspension. The level detected in pellet fraction comprised less than 1 % of the total radioactivity associated with the nematodes.

(2) Uninoculated plant fed with isotope [^{3}H].

(3) Inoculated plants were fed with isotopes, processed and assayed for radioactivity as described under text in " materials and methods ".

Table 4

Time course of peroxidase activity from cowpea cultivars C-152 and Pusa Barsati inoculated with *M. incognita*.

Hours after inoculation	*Enzyme unit/g fresh root weight*[1] *C-152*			*Enzyme unit/g fresh root weight*[1] *Pusa Barsati*		
	Control	*Inoculated*	*Ratio inoculated/ uninoculated*	*Control*	*Inoculated*	*Ratio inoculated/ uninoculated*
0	120	—		112	—	
24	120	632	5.33	112	321	2.87
48	127	802	6.31	135	435	3.22
72	250	1 862	7.44	200	635	3.18
96	240	6 500	27.08	235	1 285	5.47
720	201	1 256	6.24	233	1 298	5.57

(1) For the definition of enzyme unit, see text.
Root material used were galled/infected (inoculated) or uninoculated healthy.

Table 5

Time course of lignin values from the root extracts of cowpea cultivars C-152 and Pusa Barsati inoculated with *M. incognita*

Hours after inoculation	*Lignin* µg *phenol/mg*				*D/mg*[1] *245 nm*				*D/mg*[1] *358 nm*			
	C-152		*PB*		*C-152*		*PB*		*C-152*		*PB*	
	H	*I*	*H*	*I*	*H*	*I*	*H*	*I*	*H*	*I*	*H*	*I*
24	1.6	2.8	1.2	1.3	0.76	0.82	0.25	0.30	0.22	0.34	0.10	0.20
48	1.4	2.0	1.2	1.1	0.67	0.78	0.52	0.50	0.55	0.58	0.21	0.18
72	1.6	3.8	1.3	1.2	0.70	0.92	0.48	0.45	0.58	0.67	0.20	0.35
96	1.3	1.7	1.4	1.8	0.22	0.20	0.52	0.68	0.12	0.10	0.21	0.35
720	4.0	3.8	1.8	2.0	1.20	1.10	1.30	1.50	0.88	0.75	0.70	0.75

(1) Values given as diffference spectrum. The quantitative estimation of lignin-like polymers extracted from methanol insoluble residues was determined according to the method of Stafford (1958). The residue obtained during extraction of polyphenol by methanol was dried and washed with distilled water. The residue was then suspended in 3 ml of 0.5 N NaOH and left overnight at 70°, centrifuged at 4 000 × *g* for 30 mn and the pH of the supernatant solution adjusted to 8.5-9.0. The supernatant was then divided into two aliquots, one adjusted to pH 7.0 with 50 mM phosphate buffer and the other to pH 12.3 with 0.05 N NaOH. The difference spectrum was obtained after substraction of optical density readings taken for the neutral and alkaline solutions at 245, 295 and 358 nm. Qualitative detection of lignin was made by incubating hand sections of root samples with phloroglucinol (Jensen, 1962).

H = Uninoculated control; I = Inoculated; PB = Pusa Barsati.

Table 6
Time course of chlorogenic acid[1]
(µg/g fresh root weight) in two cowpea cultivars C-152
and Pusa Barsati inoculated with *M. incognita*

Hours after inoculation	*C-152*		*Ratio of inoculated/ uninoculated*	*Pusa Barsati*		*Ratio of inoculated/ uninoculated*
	Healthy	*Inoculated*		*Healthy*	*Inoculated*	
0	102			90		
24	102	152	1.49	90	105	1.16
48	104	198	1.90	83	96	1.15
72	112	389	3.47	93	112	1.20
96	114	350	3.07	98	116	1.12
720	105	340	3.23	110	120	1.09

(1) Root samples of various treatments were dried in an oven at 70° and kept in a dessicator over $CaCl_2$. Samples were homogenized in 3 volumes of absolute methanol and centrifuged at 4 000 × *g* for 30 mn. The samples were re-extracted with methanol and processed further as per the method of Zucker and Ahrens (1958). Alcohol extracts (1 ml) were placed on 1 × 10 cm aluminium oxide microcolumn. The column was washed with 5 ml of 80 % ethanol and then with 10 ml of water. Four ml of freshly mixed solutions of equal volumes of 0.5 % $NaNO_3$ and 5.0 % acetic acid. The adsorbed material in the column was washed with 10 ml of water followed by 10 ml of 5 N NaOH to elute polyphenols identified as chlorogenic acid. Measurements were made at 525 nm and the amount of polyphenols present in the sample was calculated from a standard curve prepared from known amounts of chlorogenic acid.

contribution by microorganisms was ruled out because of the constant use of surface sterilants and antibiotics throughout the inoculation. It will be appropriate at this juncture to comment on the significance of activated and *de novo* synthesis of mRNA in the inoculated plants in term of host-parasite interactions. It is conceivable that many major changes are likely to occur during the course of progression of disease development for which new species of RNAs are required. Indeed many adaptive cellular changes like establishment of giant transfer cells in a susceptible host or hypersensitive reaction in the resistant reacting host in root-knot diseases are obviously an enzyme mediated phenomenon. Consistent with this presumption, is the appearance of new peroxidase isozymes observed in the present study in the resistant variety soon after infection. The fact that the lower concentration of ACTD (5 µg/ml) did not inhibit activity of nematode, but it did inhibit *de novo* synthesis of peroxidase isozymes and reduced the production of lignin like polymers in the resistant variety, presumably provides a link between *de novo* synthesis of mRNA and expression of resistance. Furthermore, treatment of plants with actinomycin D later on, 96 hours after inoculation, apparently had no host reaction favorable for the developing parasite, thereby, indicating that DNA dependent mRNA synthesis in incompatible hosts occurred at a very early stage of infection. It will be logical to maintain that the role of RNA transcribed during the early stage of infection must have been different in the susceptible and resistant reacting hosts. We suggest, inoculation of the resistant cultivar with the nematode results in transcription of an mRNA the formation of which is completed within 96 hours of inoculation, and it codes a post-transcriptional synthesis of polypeptides required to initiate incompatible interactions favoring the host to its advantage. Infection of the susceptible cultivar, we feel, induces two mRNAs; the first is the messenger for RNAse which aims to contain the nematode. The second mRNA codes a post-transcriptional repressor of the enzyme synthesis and the accumulation of the repressor blocks the synthesis of the polypeptides completely or partially favoring the parasite and leading to compatible interaction. We feel, it is critical for the resistant reacting host, to provide a biochemical defence mechanism against the invading parasite during the early stage of infection. Apparently such a mechanism is lacking in a susceptible host. Analogous reasoning has been invoked to interpret the significance of *de novo* synthesis of PAL isozymes in the resistant reacting tomato cultivar infected with *M. incognita* during the early stages of infection (Mote & Dasgupta, 1979).

The implication of the above discussion is that the incompatible reaction is under the influence of *de novo* gene activation at transcriptional level thereby resulting in the synthesis of new species of poly (A)-containing mRNA and enzyme protein possibly required for the production of low molecular weight metabolites having antibiotic properties. But then, the final conclusion should be arrived at only after demonstration of increased activity of DNA-dependent RNA polymerase and derepression of a part of the genome.

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