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S.K. Ghosh [a] , Sanchalika Acharyya [a] & J.I. Mir [b]

[a] Plant Virus and Biotechnology Laboratory, Division of Crop Protection, Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, West Bengal, India

[b] Central Institute of Temperate Horticulture, Rangreth, Srinagar, India

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Passage of rice necrosis mosaic virus property induced growth promotion in some plants of commercial importance and its molecular evidence

S.K. Ghosh[a], Sanchalika Acharyya[a] and J.I. Mir[b]*

[a]*Plant Virus and Biotechnology Laboratory, Division of Crop Protection, Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, West Bengal, India;* [b]*Central Institute of Temperate Horticulture, Rangreth, Srinagar, India*

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Rice necrosis mosaic virus (RNMV), upon inoculation, induced higher growth and yield in *Ludwigia perennis* and *Corchorus olitorius*. Crops of commercial importance, including arhar, rice bean, cotton and tomato, were tested for growth promotion and higher productivity upon RNMV inoculation. Plant growth characteristics and biochemical components were measured from control, inoculated and energised plants. To understand the molecular basis behind such phenomenon, tomato plants were selected for subtractive hybridisation and reverse northern analysis due to its known gene sequences. Significant changes in biological properties and biochemical components in all the inoculated test plants over control were observed along with better seed quality. Over-expression of genes falling in different functional categories like photosynthesis, plant growth and development, and membrane transport explained the virus-induced growth promotion phenomenon as well as the temporary passage of this property through seeds of inoculated plants.

Keywords: rice necrosis mosaic virus; growth promotion; cytokinin-like compound; subtractive hybridisation; seed transmissibility

Introduction

Viruses infecting plants often cause severe growth reduction and hampered crop productivity. Rice necrosis mosaic virus (RNMV) stunts the growth of rice plants and causes necrosis and chlorosis in infected leaves (Fujii 1967; Inouye and Fujii 1977; Ghosh 1980). Ghosh (1981, 1982) observed that plants of *Ludwigia perennis* and some fibre crops, artificially inoculated with RNMV, grew faster; leaf size, stem diameter and number of fibre bundles in all the inoculated species examined were increased compared with healthy plants under controlled net-house conditions. Association of RNMV with such growth promotion was confirmed with electron microscopic and serological studies (Ghosh 1982). Furthermore, the property of such virus-induced growth promotion was also found to be evident in plants, especially jute (*Corchorus olitorius* cv. *JRO-524 E*), grown out of the seeds (termed as E for energised seeds) obtained from those of RNMV-inoculated ones (Ghosh 2002) without any change in its existing genetic make-up (Roy et al. 2006). Studies with different hosts indicated that though RNMV retards growth and yield in some monocot plants (Ghosh 1979,

*Corresponding author. Email: javidiqbal1234@gmail.com

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1981), it has growth promoting effect on some plants of dicot type. Earlier work (Ghosh, 1982, 1995) revealed association of enhanced cytokinin-like material and IAA with such growth promotion in inoculated plants. Upon further investigation, this cytokinin-like material obtained from inoculated plants appeared to be a close derivative of zeatin, a naturally occurring cytokinin, having m/z value of 217.1. In addition, different proton–proton coupling and a molecular structure of the highly substituted aliphatic group have been found in this material which possibly aided as one of the factors associated with such higher growth (Dey et al. 2012). Keeping in view the observations on changes in growth pattern properties and effect on cytokinin metabolism in host after RNMV inoculation, this phenomenon was studied further in relation to its possible utilisation for increasing productivity in some crops of commercial importance under natural conditions. Along with this, an attempt was also made to understand the background, at molecular level, of the passage of the property of such virus-induced growth promotion into the next generation by growing one of the test plants like tomato under controlled greenhouse and the results are presented.

Materials and methods

Plant and virus material

Healthy seeds of arhar (*Cajanus cajan* cv. Local) obtained from local sources were surface sterilised and used as test plants. Twenty rows, each row containing 25 plants (0.5 m apart), were grown in field as per recommended practices. The 20 rows for control plants comprising one plot and another plot containing 20 rows for inoculated plants were maintained for the study at the Central Research Institute for Jute and Allied Fibres, West Bengal, India, during 2003. Twenty days after germination, the apical leaf of each of the 500 plants were mechanically inoculated (Ghosh 1982) with the sap obtained from the leaves of rice plants (*Oryza sativa* cv. T(N)1) previously infected with RNMV, kept in the greenhouse. Control plants were also mechanically treated with the sap obtained from the leaves of healthy rice plants kept under identical conditions. All these plants were then allowed to grow till maturity and observations were recorded at maximum growth stage (120 days) and at pod maturity. The recorded observations on growth were then analysed through statistical tools to examine whether the effect of inoculation was significantly different or not. Since during growth period a number of plants both from inoculated and control plots died, a total number of 416 and 365 plants from inoculated and control group respectively constituted as the population for the study. The individual plant height was recorded from the two groups and the difference between mean height of these groups was statistically tested at 5% and 1% level of significance by two sample "*t*" test (assuming equal variance). To compare the uniformity in height between these groups, the co-efficient of variations were estimated by taking the ratio of standard deviation of the mean of the respective group. The overall mean height of all the plants of these populations was calculated and the height of plants was divided into two categories, such as, above the overall mean and below the overall mean. Then, percentage of inoculated and control plants belonging to these categories were calculated with respect to their population sizes.

Further to prove whether RNMV inoculation has any significant effect in increasing the plant height, the recorded observations were tested by *chi-square* test.

One set of surface sterilised healthy seeds of above stated variety of arhar having 20 replications were germinated in sterilised earthen pots containing sterilised soil

(five seedlings/pot), kept in the screened net-house. Out of these, plants of 10 replications were used for inoculation purpose and the rest served as control. All these plants were allowed to grow inside, and characteristic effects of RNMV inoculation were recorded for comparison with those of field plots.

Based on the observations made on arhar plants, healthy seeds of rice bean (*Vigna umbellata* cv. Local), cotton (*Gossypium hirsutum* cv. Ganganagar Agethi) and tomato (*Lycopersicon esculentum* cv. Patharkuchi), all obtained from local source and two more varieties of arhar namely, German White and Hyderabad Local obtained from Tree Oils India Limited, Hyderabad were surface sterilised and were grown for observing the effect of RNMV inoculation on each of them under field conditions at the institute farm during 2004. In all these cases, four rows containing 25 plants in each row for each species were maintained in field separately both for control and inoculation purpose. Twenty days after germination, plants of two rows of each species were mechanically inoculated with RNMV as stated earlier and the plants of rest two rows for each species served as control. The plants were then allowed to grow up to maturity and observations were noted as per requirement. Seeds obtained from above stated inoculated plants including arhar grown during 2004, termed as energised seeds, were grown (for confirmation) in the fashions as were done during 2005 at the institute farm for observations. Additionally, one trial with energised arhar seeds (cv.Local) *vis-a-vis* control seeds was conducted during 2005 at Hyderabad with the help of Tree Oils India Limited, Nallakunda, Hyderabad to assess the growth pattern of such seeds under (for confirmation) different agro-ecological situation and the results were noted. A trial with energised rice bean and cotton seeds was undertaken separately at the farm of Central Institute for Cotton Research, Nagpur, India for observation during this year.

During 2006, three categories of plants (namely, control, RNMV inoculated and RNMV energised) were grown at the institute farm and the observations were noted. Since significant improvement in tomato plants due to RNMV inoculation was noticed, the energised tomato seeds together with control were grown under field conditions separately to assess the significant differences in different plant characteristics between them.

Plant growth characteristics

Plant growth characteristics such as leaf size, plant height, green weight, root weight, fruit weight and 100-seed weight, wherever pertinent were recorded for each of the test plants of each species.

Biochemical parameters

Based on the phenotypic changes noticed in test plants due to RNMV inoculation, studies were also conducted to know the effects, if any, on some biochemical components of these host plants.

Photosynthetic pigments

Apical leaves (third from top with four replications) of all the test plants except tomato (inoculated, energised and control) were collected from field grown plants, thoroughly washed with sterile distilled water and extracted with water–acetone

(80%) for estimation of total chlorophyll content in each of them. Each extract was then centrifuged at 3000 × *g* for 5 min (Welschen and Bergkotte 1994). Each supernatant was then determined spectrophotometrically at 663 nm for chlorophyll *a* and 645 nm for chlorophyll *b* and the contents were calculated according to the method put forth by Lichtenthaler and Wellburn (1983).

Protein content

Apical leaves (third from top with four replications) of tomato plants (energised and control) grown inside net-house were used for the estimation of buffer soluble sap protein following the method of Lowry et al. (1954). After collection, the leaf materials of each type were thoroughly washed with sterile distilled water and homogenised with boron buffer (pH 8.7) in a refrigerated centrifuge at 5000 × *g* for 15 min. The solution absorbance was then determined in presence of Folin reagent at 750 nm wavelength. The protein content was then measured following a standard curve obtained with BSA. Total soluble protein estimation was done to check the effect of RNMV inoculation on total soluble protein expression in the beginning, later on which was confirmed through over-expression of genes through SSH also.

Seed quality

Energised seeds of arhar (cv. Local) were used for quality assessment. Different quality parameters were estimated through National Institute of Nutrition, Hyderabad. Required seed amount of both energised and control seeds were used for the assay.

Other components

Components like total chlorophyll, total free amino acids, phenolics in leaves and lycopene content in fully ripened tomato fruit were estimated as per standard procedures from the energised and control tomato plants grown under net-house conditions.

RNA isolation and generation of subtracted cDNA libraries

Since the impact of RNMV inoculation on tomato plants was observed to be highly significant, an attempt was made to use the crop to observe the molecular change, if any, in the genetic make-up of RNMV inoculated and RNMV energised *vis-a-vis* control tomato plants. For this purpose, sterilised healthy seeds of tomato plants were germinated in sterilised earthen pots containing sterile soils (5 seedlings/pot) with 20 replications in an insect proof screened net-house. Out of these plants, 10 replications were used for inoculation purpose and the rest served as controls. Inoculation was done mechanically on 10-day-old plants as stated earlier. Likewise, 10 replications of energised tomato seeds were also allowed to germinate at the same time and kept under identical conditions for growth and comparison. Apical leaf of 20-day-old plants in each case was used for the study.

The Clontech PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA, USA) was then used to generate a forward cDNA subtraction library (FSL) and a reverse cDNA subtraction library (RSL), respectively, from RNMV-inoculated and control

tomato plants. The cDNA prepared from RNMV-inoculated plants (the one containing specific transcripts) was used as ''tester'' and that from control plants (harbouring the reference cDNAs) as the ''driver'' for the forward subtraction reaction. Reverse subtraction was performed using control plants as ''tester'' and RNMV-inoculated plants as ''driver''. All the RNAs were extracted from a pool of 10–12 plants from control (C) and inoculated (I) tomato plants using TRIzol (Invitrogen). Integrity of RNA was analysed by electrophoresis with 1.2% agarose gel. Quantification and purity were measured spectrophotometrically by relation of absorbance (260/280 nm).

Full-length double-stranded cDNAs, synthesised using the SMART kit (BD Biosciences Clontech), were digested with *Rsa* I to generate short, blunt-ended fragments optimal for adaptor ligation and subtraction. After digestion with RsaI, the experimental tester cDNA preparation for reaction with each direction was divided into two subpopulations, which were ligated to two different adaptors. No adaptor was ligated to ''driver'' cDNA population. The two subpopulations were then hybridised with an excess amount of driver cDNA for each reaction, after which they were combined and hybridised again in the presence of driver cDNA, without denaturing the DNA before the second hybridisation. Following the second hybridisation, two rounds of PCR were performed to enrich and amplify the differentially expressed sequences. The final PCR products were then cloned into the cloning vector pJET 1.2 positive selection vector using CloneJET^{TM} PCR Cloning Kit (Fermentas, USA) and transformed into DH5α' *Escherichia coli* competent cells using a modified heat shock method. Recombinants were picked up from each plate. After confirming through colony PCR and restriction analysis, positive clones were sequenced using automated sequencing services provided by Chromous Biotech Pvt. Ltd., India.

Differential screening

Three hundred colonies of transformed DH5α from both the forward and reverse subtracted libraries were picked and grown overnight in Luria Agar media (Sigma-Aldrich) with Ampicillin. After confirming the presence of inserts through colony PCR and restriction release, 70 clones were subjected to differential screening. cDNA insert of these clones were amplified by colony PCR and blotted on positively charged nylon membrane (provided by BioTrace, Inc.) in duplicate. Digoxigenin-11-dUTP labelled probes were generated from forward- and reverse-subtracted cDNAs following manufacturer's instructions (www.roche-applied-science.com) and hybridised with cDNAs blotted on membranes. The hybridisation signal intensity was analysed through densitometric analysis using AlphaEaseFC (Alpha Innotech) and differentially expressed clones were sequenced using the automated DNA sequencing service of Chromous Biotech Pvt. Ltd., India.

Sequence analysis of putative differentially expressed genes

With the use of a variety of computational tools, sequences were scrutinised to determine their identity. Sequences were subjected to standard nucleotide–nucleotide basic local alignment search tool BLASTn (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al. 1997) to determine their identity using the *E value* obtained as a guide for assigning putative functions based on sequence homology with known genes. The

degree of sequence similarity between the cDNA clone and known sequences was represented by the expect (*E*) value.

Reverse northern analysis

To scrutinise the differential expression pattern of ESTs, plasmids of positive clones were prepared and used as PCR templates. All PCR products were electrophoresed on 1.2% agarose gel to verify the presence of the inserts and were denatured by incubating at boiling temperature for 10 min. Then they were rapidly chilled on ice and dot-blotted in equal volume (5 μl) onto three replicated positively charged nylon membrane sets (provided by Bio Trace, Inc.). The spotted DNAs were crosslinked to the membrane with a UV crosslinker (UVP, Inc.). Double stranded cDNAs were synthesised from total RNAs (isolated from control, RNMV inoculated and energised tomato plants) using manufacture's protocol (www.ambion.com) of RETROscript kit provided by Ambion, Inc. These three cDNAs were then randomly labelled by Digoxigenin-11-dUTP supplied by Roche Diagnostics following manufacture's protocol (www.roche-applied-science.com). These three labelled probes were then hybridised separately to those three membrane sets. Hybridisation was carried out at 42°C for overnight. The hybridised membranes were then subjected to stringency washes and detection was made following manufacture's protocol. After exposing the membrane to suitable substrate, they were further exposed to Kodak photographic X-ray films to detect the chemiluminescence. The X-ray films were then developed following conventional methods. For eliminating the false-positive clones and enhancing the specificity, another reverse Northern analysis was performed using messenger RNAs as probe templates.

Results

Plant growth characteristics in response to RNMV inoculation

No visible virus symptoms of RNMV after inoculation on arhar plants was noticed and the inoculated plants looked better in growth with enhanced juvenility as compared with controls. At 120 days of RNMV inoculation, remarkable variation in plant height (Figure 1), bushiness, branching pattern and total bio-mass were noticed in inoculated arhar plants. In the control plot, most of the plants were below 2.3 m in height with sporadic taller plants. More uniformity with higher plant height (2.3 m and above) was noticed in inoculated arhar plants. Significant variation with respect to mean plant height was observed as revealed by *t*-test. The calculated *t* value was found to be 5.13 which was greater than that of table value of 2.582 at 1% level of significance having 779 as degrees of freedom. Hence, the null hypothesis appeared to be not true indicating that the mean height of the two populations was significantly different.

The overall mean height of all plants of these populations was calculated as 2.27 m. The percentage distribution of inoculated and control plants belonging to above overall mean and below overall mean has been shown in Table 1 and Figure 2 (a)–(c).

The co-efficient of variations with respect to plant height of inoculated and control groups were found to be 10.87% and 12.37%, respectively. The result suggested that there was variation in plant height in both the groups. However, such variation was found to be less within the plants of inoculated group as compared with those of the control ones indicating that there was much reduction in instability

Figure 1. Plant height at 120 days of growth in control (left) and rice necrosis mosaic virus inoculated (right) arhar plants (*Cajanus cajan* cv. Local) under field conditions.

Table 1. Distribution by different size groups of arhar (*Cajanus cajan* cv. Local) plants grown at institute farm.

S. No.	Groups	No. inoculated plants*	No. control plants*
1	Above the average	270 (64.91)	171 (46.85)
2	Below the average	146 (35.09)	194 (53.15)
3	No. observations	416 (100)	365 (100)

*Figures in parenthesis indicate percentage to respective total number of plants. The *Chi-square* statistic for the above data is 25.781 with 1 degree of freedom as compared with table value of 10.8.

of plant height within inoculated group. This also indicated that there was more uniformity in plant height within the inoculated group than the control group. At 150 days of growth, the inoculated arhar plants showed significant improvement in most of the biometrical characteristics (Table 2). Plant basal diameter (6 cm above soil), total branching, main root length, total leaf weight, total plant weight (without leaf) and total plant weight (with leaf) in inoculated plants were found to increase over control by 37.5%, 117%, 0.3%, 233.3%, 125.0% and 150.0%, respectively. At the time of maximum pod formation stage, plant height in control plants was found to be little less than that of inoculated ones but the basal diameter, number of branches and total green weight were found to be much higher (14.5%, 23.8% and 41.3% more) than control ones (Table 3). When the grain yield was recorded, it was found to be 26.4% higher (q/ha basis) in inoculated plants than the control. Total seed weight/plant was also found 24.2% higher in inoculated plant than the control one (Table 4).

In a trial at the institute farm with other varieties of arhar, *viz*. *Cajanus cajan* cv. Hyderabad Local and *C. cajan* cv. German White obtained from Hyderabad, more or less similar effect of RNMV inoculation on them was noticed. Results revealed that due to inoculation in case of the variety German White, the plant height was

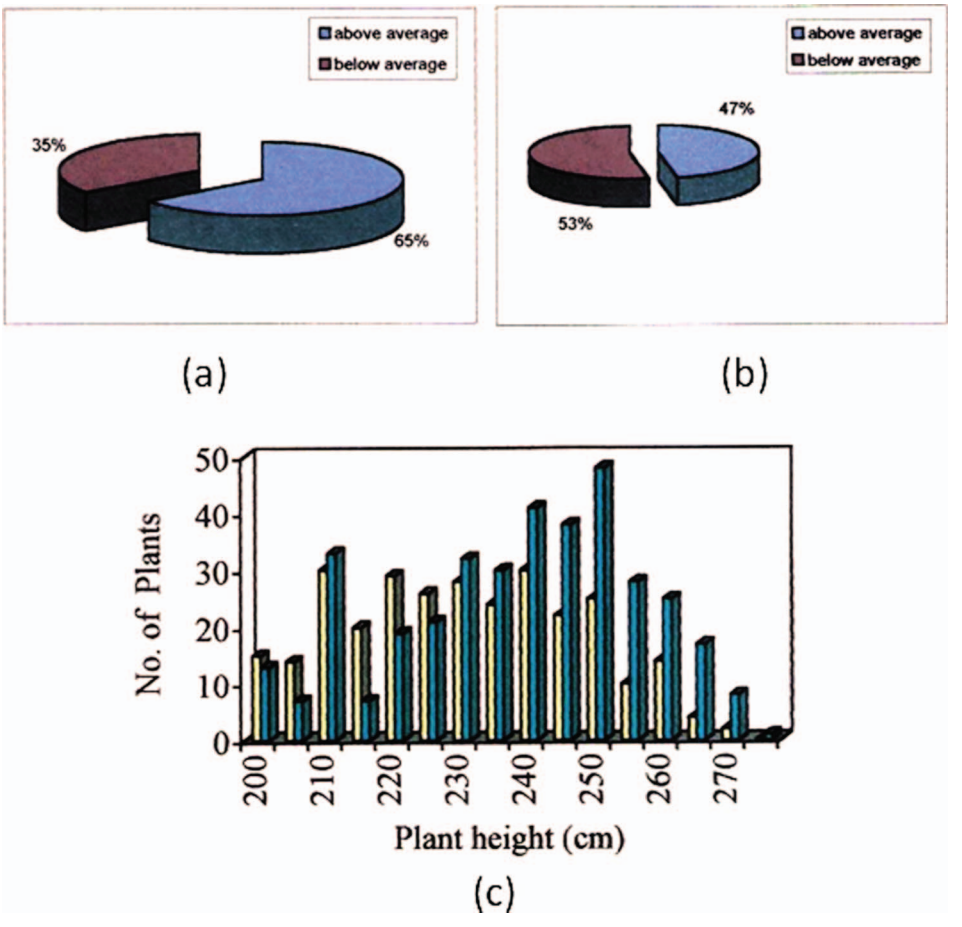

Figure 2. Share of arhar plants within inoculated (a) and control (b) groups with respect to plant height; (c) indicates number of plants in each category of plant height.

Table 2. Effect of rice necrosis mosaic virus inoculation on yield attributing characters in *Cajanus cajan* cv. Local at 150 days of growth at institute farm.

S. No.	Parameters* (per plant basis)	Control	Inoculated	% increase over control
1	Total plant weight (kg) (with leaves)	1.6 ± 1.2	4.0 ± 0.94	150.0
2	Basal diameter (cm)	2.4 ± 0.53	3.3 ± 0.72	37.5
3	Total no. branching	165 ± 15.30	359 ± 20.40	117.6
4	Main root length (cm)	48 ± 0.91	52 ± 0.88	0.3
5	Total leaf weight (g)	300 ± 12.42	1000 ± 18.38	233.3
6	Total plant weight (g) (without leaves)	800 ± 11.00	1800 ± 17.25	125.0

*Mean average of 10 plants ± S.E.

Table 3. Plant characters at maximum pod formation stage in *Cajanus cajan* cv. Local plants grown at institute farm

S. No.	Plant characters*	Control	Inoculated	% increase over control
1	Plant height (m)	3.17 ± 0.42	3.26 ± 0.63	2.8
2	Basal diameter (cm)	4.00 ± 1.03	4.58 ± 0.97	14.5
3	Total green weight (kg)	3.75 ± 0.72	5.30 ± 0.44	41.3
4	No. of branches	462 ± 2.47	572 ± 7.22	23.8

*Mean average of 10 plants ± S.E.

Table 4. Effect of rice necrosis mosaic virus inoculation on grain yield of *Cajanus cajan* cv. Local plants grown at institute farm.

S. No.	Parameters*	Control	Inoculated	% increase over control
1	No. pods/plant	1106 ± 3.64	1492 ± 4.05	34.9
2	Total seed weight/plant (g)	189.5 ± 0.87	235.4 ± 1.05	24.2
3	100-seed weight (g)	7.24 ± 0.16	8.09 ± 0.09	11.7
4	Grain yield (q/ha)**	12.5	15.8	26.4

*Mean average of 10 plants ± S.E. **Calculated on plot size basis.

increased by 15.67% while for the variety Hyderabad Local, the same was 9.31% over respective controls. The green weight/plant in inoculated sample of variety German White was found to increase over control by 75.0% whereas in variety like Hyderabad Local the same was found to decrease by 21.92% in inoculated plants over control (Table 5). After harvest of the pods from the crops (control and inoculated) of all the varieties of arhar, the yield was noted in each case and it was found that yield was greater in inoculated plants in each case. In case of energised plants (i.e. plants grown out of seeds obtained from inoculated plants) of each variety, transmission of growth promotion property was noted. At Hyderabad also trial with energised seeds of arhar cv. Local showed enhanced number of branches and number of pods/plant (Table 6). The plant height at initial stage of growth was significantly higher but the same was found to be slightly lower (Figure 3) in energised plants than control at harvest.

In case of rice bean, cotton and tomato plants also, no visible virus symptoms of RNMV upon inoculation was noticed. In contrast, much improvement in plant growth and yield was noticed in these crops as compared with those of respective controls under field conditions and also under controlled net-house conditions. Furthermore, the property of such growth promotion was also found to be transmitted into the next generation of plants grown out of the seeds obtained from inoculated plants in each case.

The growth characteristics in Arhar plants have been studied in details so that these data may act as the base to add confidence with respect to this unique phenomenon.

When control, inoculated and energised plants (arhar, rice bean, cotton and tomato) were grown in field side by side and under net-house conditions, the germination and initial growth rates for plant in each treatment were similar at first and second weeks after germination. Subsequent growth for plants in treatments was extensively greater than for plants in the control. At 30 days after germination, some

Table 5. Effect of rice necrosis mosaic virus inoculation on different cultivars of *Cajanus cajan* grown under field conditions at institute farm.

S. No.	Parameters	cv. Hyderabad Local*			cv. German White*		
		Control	Inoculated	% decrease(−) or increase over control	Control	Inoculated	% decrease(−) or increase over control
1	Green weight (kg/Plant)	0.73 ± 0.03	0.57 ± 0.02	(−) 21.92	0.36 ± 0.01	0.63 ± 0.02	(+) 75.0
2	Leaf area (sq. cm)	39.0 ± 1.27	57.0 ± 2.41	(+) 46.10	41.0 ± 1.48	57.0 ± 1.94	(+) 39.02
3	Plant height (cm)	185.27 ± 1.68	202.5 ± 1.34	(+) 9.31	152.75 ± 1.37	176.69 ± 1.69	(+) 15.67

*Mean average of 30 plants ± S.E.

Table 6. Main plant characteristics in rice necrosis mosaic virus energised *Cajanus cajan* cv. Local plants grown under field conditions at Hyderabad, India.

S. No.	Parameters*	Control	Energised	% increase over control
1.	Plant height (cm)	245.0 ± 1.10	259.0 ± 0.49	5.71
2.	No. branches/plant	68 ± 2.40	81 ± 3.47	19.11
3.	No. pods/plant	327 ± 1.66	496 ± 1.83	51.68

*Mean average of 30 plants ± S.E.

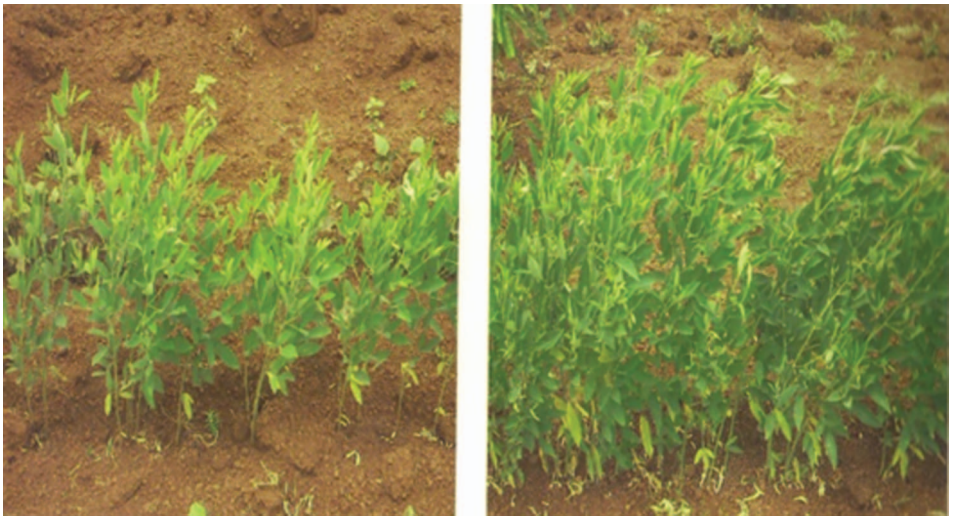

Figure 3. Plant height of arhar plants (*C. cajan* cv. Local) grown at Hyderabad showing plant height at initial stage of growth (60–65 days) under field conditions.

plants in the control were much smaller than plants of the same aged energised plants (Figure 4(a)–(f)). Plants from inoculated set showed growth promotion at 11 days after inoculation. Growth parameters like leaf size, total seed weight for arhar and rice bean, number of fruits/plant and fruit weight for tomato and number of balls/plant in case of cotton were mainly evaluated at the end of the experiment. An evaluation of these growth parameters amongst energised, inoculated and control plants in each species revealed that plants belonging to inoculated and energised group showed significant improvement in them as compared with their respective controls (Table 7 and Figures 5 and 6).

In case of the energised and control tomato plants which were grown side by side in field, remarkable differences in plant characters between the two were noticed (Figure 7). The plant height, leaf length and root length were found to increase over the control by 12.5%, 5.3% and 80%, respectively (Table 8) with enhanced juvenility.

Biochemical parameters

Chlorophyll content

In case of arhar, rice bean and cotton plants, significant increase over control in different constituents of chlorophyll was noticed in each type of test plants upon

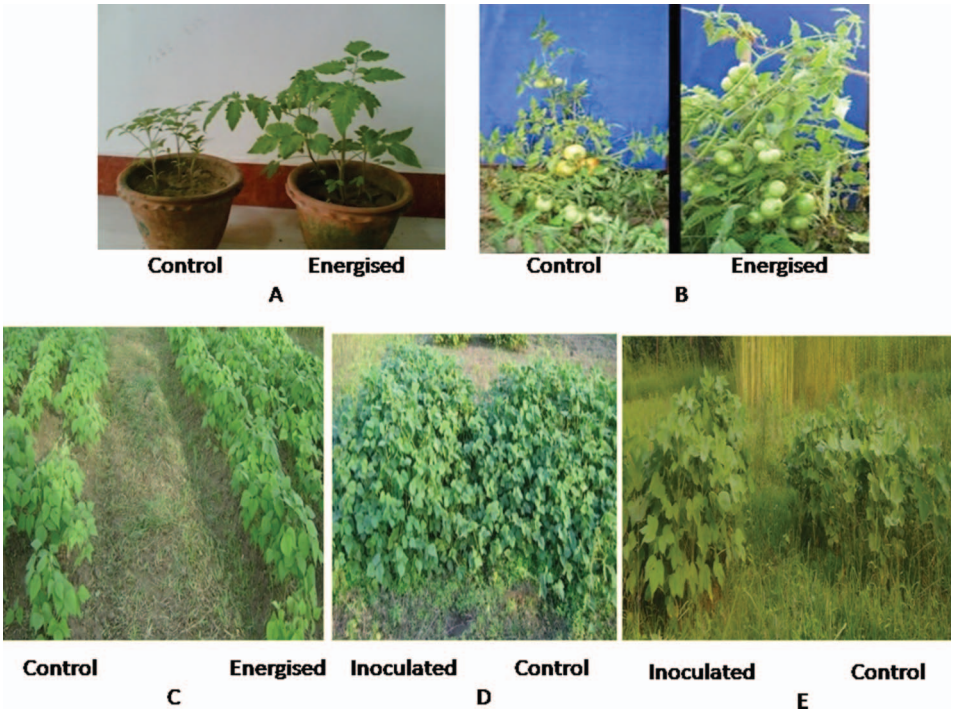

Figure 4. Growth pattern in plants like (a) arhar (cv. Local), (b) tomato under net-house conditions and rice bean (c and d).

Table 7. Comparative analyses on leaf size and yield parameters in different test plants grown under field conditions at institute farm.

S. No.	Plant type	Parameters	Control*	Inoculated*	Energised*
1	*Cajanus cajan* cv. Local	Leaf area (sq. cm)	38.0 ± 3.03	58.3 ± 1.69	52.7 ± 1.05
		Yield (q/ha)**	10.5	13.9	14.0
		100-seed weight (g)	6.9 ± 0.42	7.8 ± 0.53	8.1 ± 0.43
2	*Vigna umbellata* cv. Local	Leaf area (sq. cm)	96.0 ± 1.77	127.0 ± 1.92	146.0 ± 2.01
		Yield (kg/plant)**	1.6	2.1	2.45
		100-seed weight (g)	4.1 ± 0.33	6.1 ± 0.26	6.0 ± 0.37
3	*Gossypium hirsutum* cv. Ganganagar Agethi	Leaf area (sq. cm)	97.0 ± 1.46	144.0 ± 1.11	140.0 ± 0.98
		Boll No./plant	12.0 ± 0.70	16.0 ± 0.55	17.0 ± 0.62
4	*Lycopersicon esculentum* cv. Patharkuchi	Leaf length (cm)	13.9 ± 0.43	14.78 ± 0.22	14.32 ± 0.37
		No. fruits/plant	30.0 ± 0.18	36.0 ± 0.13	39.0 ± 0.37

*Mean average of 30 plants ± S.E. **Calculated on plot size basis.

RNMV treatment (Table 9). Among the different varieties of arhar tested, the variety German White of energised type showed maximum increase in contents of chlorophyll a, b and total followed by the inoculated type. In case of tomato, the plants belonging to energised group also showed similar effect as above but the extent of increase in total chlorophyll content was around 16% over control (Table 10). Other biochemical parameters in tomato like total free amino acids,

Figure 5. Growth pattern cotton.

precipitable sap protein, phenolics and lycopene content in mature fruit of energised tomato were also found to increase over the control by 17.7%, 59.2%, 31.8% and 20.0%, respectively (Table 10).

Seed quality

With regard to assessment of seed quality in energised arhar seeds of cv. Local, marked changes in nutritional properties were noticed. Protein content increased by 1.89% over control (Table 11). Zinc and total calorie content also appeared to be appreciably increased in such energised arhar (cv. Local) seeds (Table 12).

RNA isolation and subtractive hybridisation by suppression through PCR

The RNA was of good quality and quantity, and its extraction was homogeneous and reproducible from both the control and energised tomato plants. The extractions of this type were adequate for subsequent synthesis of cDNA in order to carry out SSH. When the product of second nested PCR was electrophoresed, a subtracted cDNA was observed which constituted only the gene fragments expressed differentially. The sweeps corresponding to cDNA of various sizes enriched mainly within the zone of 400 to 700 bp were observed.

Sequence analysis of putative differentially expressed genes

The subtracted cDNA library contained putative differentially expressed gene sequences specific to RNMV-inoculated cDNAs. The library enriched with clones

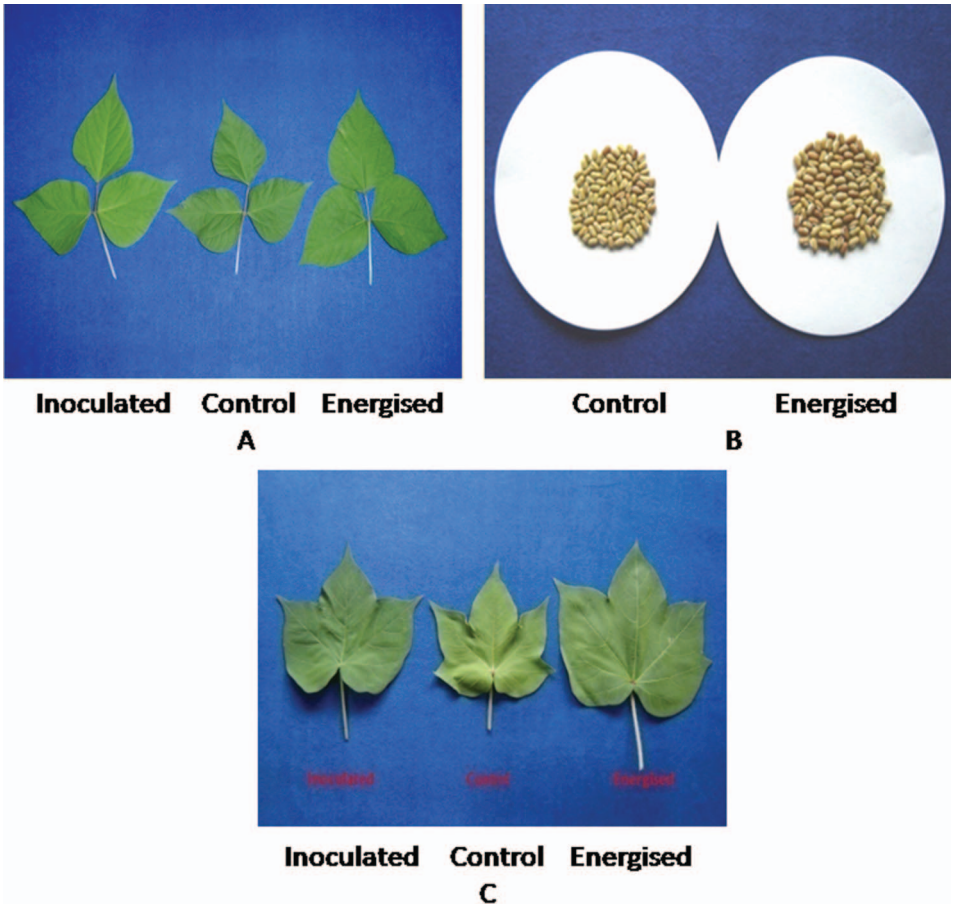

Figure 6. Leaf size (a) and seed size (b) of arhar (cv. Local) and leaf size of (c) cotton after virus treatment.

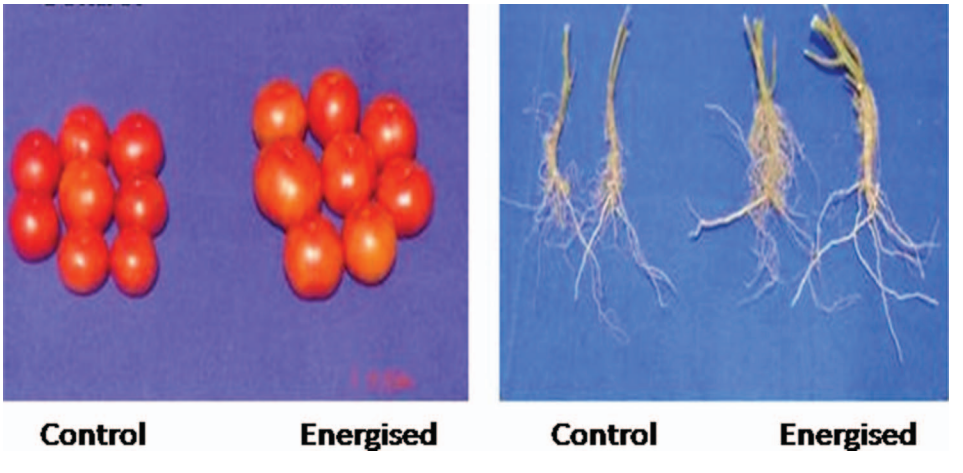

Figure 7. Fruit size (a) and root size (b) in tomato plants after virus treatment.

Table 8. Biometrical characters of rice necrosis mosaic virus inoculated tomato plants grown under field conditions at the institute farm.

S. No.	Plant characters*	Control	Inoculated	% increase over control
1	Plant height (cm)	22.37 ± 0.63	25.17 ± 0.70	12.51
2	Leaf length (cm)	13.40 ± 0.67	14.11 ± 0.54	5.29
3	Root weight (g)	5.0 ± 0.98	9.0 ± 0.67	80.0
4	No. flowers/plant	80.0 ± 2.51	91.0 ± 3.18	13.75
5	No. fruits/plant	31.0 ± 1.31	38.0 ± 1.56	22.58
6	Single fruit weight (g)	57.67 ± 0.56	74.0 ± 0.78	28.32

*Mean average of 30 plants ± S.E.

corresponding to transcripts was up-regulated by RNMV inoculation. Only 15 clones were obtained after three repetitions of the experiment at the same time. BLASTn analysis enabled us to identify these genes and to classify them as either known or novel (Table 13). Known sequences have been defined as those already present in the GenBank database. Out of 15 clones, only 11 clones were found to have related known functions and a single clone was found to be functionally unknown but with known full coding sequence. The sequences determined in this study have been submitted to the NCBI nucleotide sequence database and assigned accession numbers (FE597465-76).

Reverse northern analysis

DNA of each clone constituting the bank of differentially expressed genes was transferred to positively charged nylon membranes and hybridised with labelled cDNA from RNMV inoculated, energised plants and as well as from the control. It was observed that the 10 clones obtained in the bank of genes showed higher expression level in presence of the probe in the inoculated plant than the same from control which indicated the up-regulation of the corresponding genes in RNMV inoculated tomato plants. Whereas in case of energised plants only 4 out of 11 ESTs showed signals of up-regulation (Figure 8).

Identification of genes over-expressed in RNMV inoculated/energised tomato

Some differences in gene expression patterns were anticipated amongst the control, inoculated and energised tomato plants as morphological differences were evident during experimentation. Therefore, use of subtractive hybridisation technique was decided to adopt to identify transcripts showing enhanced expression in RNMV inoculated and energised tomato plants. Using this technique, it was possible to compare the expression of a large number of gene products simultaneously. Almost all the gene sequences isolated in the study showed close similarity with the gene sequences already present in the GenBank (Table 13). The genes encode a range of different proteins, some of which could be assigned to potential functions in photosynthesis like part of light harvesting complex (LHC), oxygen evolving enhancer protein, Rubisco, ATP synthase, etc. in inoculated plants. Other sequences showed similarity with lipid transfer protein. One EST sequence showed similarity the enzyme acetohydroxyacid synthase. One EST that codes for Mg protoporphyrin IX chelatase was found to be up-regulated. 2-oxogluterate-dependent dioxygenase

Table 9. Chlorophyll contents in leaves of different test plants upon rice necrosis mosaic virus inoculation.

S. No.	Plant*	Condition	Chlorophyll contents (mg/g of fresh tissue)					
			a	% increase over control	*b*	% increase over control	Total	% increase over control
1	*Cajanus cajan* cv. Local	Control	1.0	–	1.8	–	2.8	–
		Inoculated	1.4	40	2.7	50	4.1	46.4
		Energised	1.5	50	2.6	44.4	4.1	46.4
2	*C. cajan* cv. Hyderabad Local	Control	1.1	–	2.0	–	3.1	–
		Inoculated	1.5	36.3	2.7	35.0	4.2	35.4
		Energised						
3	*C. cajan* cv. German White	Control	0.6	–	1.1	–	1.7	–
		Inoculated	1.2	100	2.2	100	3.4	100
		Energised	1.7	183.3	3.1	181.8	4.8	182.3
4	*Vigna umbellata* cv. Local	Control	0.5	–	1.0	–	1.5	–
		Inoculated	0.7	40	1.3	30	2.0	33.3
		Energised	0.6	20	1.0	0.0	1.6	6.6
5	*Gossypium hirsutum* cv. Ganganagar Agethi	Control	0.4	–	0.7	–	1.1	–
		Inoculated	0.5	25	0.8	11.4	1.3	18.1
		Energised	0.7	75	1.2	71.4	1.9	72.7

*Mean average of four replications.

Table 10. Biochemical parameters in energised tomato plants grown under controlled glasshouse conditions.

S. No.	Parameters*	Control (mg/g)	Energised (mg/g)	% increase over control
1.	Total chlorophyll	3.38	3.90	15.2
2.	Total free amino acids	13.83	16.28	17.7
3.	Protein	0.54	0.86	59.2
4.	Phenolics	0.43	0.56	31.8
5.	Lycopene/100 g of fresh tissue of fully ripened fruit	0.05	0.06	20.0

*Mean average of four replicated samples.

Table 11. Nutritional properties of energised arhar seeds (cv. Local).*

S. No.	Seed type	Moisture (g%)	Protein (g%)	Fat (g%)	Minerals (g%)	Crude fibre (g%)	Carbohydrate (g%)	Energy (Kcal)
1	Control	8.7	21.3	1.1	4.0	6.3	58.6	330.0
2	Energised	8.2	21.7	1.2	4.0	6.4	58.5	332.0

*Mean average of 12 replicated samples obtained from National Institute of Hyderabad, India.

was also found to be upregulated in both RNMV inoculated and energised tomato plants. Up-regulation of Arabinogalactan proteins (AGPs) was also noticed in inoculated tomato plants. In addition to these, one gene with unknown function was also noted in such tomato plants.

Discussion

Present investigation highlighted that a virus, which was so long recognised as a harmful material causing retardation in growth and yield of rice has also the property to do good for some plant species of commercial importance. Like fibre crops (Ghosh 1982, 1985), this virus also induced improvement in growth of different yield-attributing characters and yield of different cultivars like arhar, rice bean, cotton and tomato as evident during the study. Not only so, in case of arhar more uniformity in plant height in both inoculated and energised plants as compared with control was noticed. Field trials with this crop at different locations showed appreciable increase in plant height, leaf area and yield with varying degrees in different varieties due to virus energisation. Yield of crops was also found to be higher in energised plants than control ones. With regard to rice bean, cotton and tomato, similar growth promotion and higher yield was noticed in both RNMV-inoculated and RNMV-energised plants as compared with those of respective control under field trial. But in case of cotton, field trial at Nagpur did not show any promise. This might be due to presence of black cotton soil where nature of soil is acidic and the virus failed to express its beneficial property. A field trial for all the crops including cotton at Barrackpore location of West Bengal, where soils are alluvial with more or less neutral pH, was done to assess the growth promotion property in all the crops due to inoculation. This clearly revealed that RNMV favours neutral alluvial type of soil than acidic one for expression of its beneficial property.

Table 12. Nutritional properties of energised arhar seeds (cv. Local)*

S. No.	Seed type	Calcium (mg/100 g)	Phosphorus (mg/100 g)	Iron (mg/100 g)	Copper (mg/100 g)	Zinc (mg/100 g)	Manganese (mg/100 g)	Magnesium (mg/100 g)
1	Control	142.0	488.0	5.03	1.87	4.34	1.03	159.0
2	Energised	139.0	475.0	4.61	1.38	4.49	0.99	146.0

*Mean average of 12 replicated samples obtained from National Institute of Hyderabad, India.

Table 13. BLAST search results of identified SSH clones from Forward subtracted library.

Clone id	Accession number	Sequence homology/match	Functional categories	*E* value
Lessubhybpsbx	FE597465	PSII 23 kda protein	Photosynthesis/Defence	9e–122
Lessubhybltp	FE597466	Lipid transfer protein	Pathogenesis/Defence	1e–29
Lessubhybahas1	FE597467	Acetohydroxyacid synthase	Defence	2e–49
Lessubhybglo	FE597468	Glycolate oxidase	Photorespiration/signal transduction	2e–84
Lessubhybatpb	FE597469	ATP synthase	Membrane transport	1e–133
LessubhyblhbcI	FE597470	Light harvesting complex II	Photosynthesis	4e–74
Lessubhybrbcs2	FE597471	Rubisco	Photosynthesis	5e–12
Lessubhybchlh	FE597472	Mg-Protoporphyrin IX chelatase	Defence	1e–06
Lessubhybrisp	FE597473	Rieske Fe-S protein	Pathogenesis/photosynthesis	1e–65
Lessubhybgad2	FE597474	2-oxoglutarate-dependent dioxygenase	Post-translational modification	0.0
Lessubhybagp	FE597475	Arabinogalactan	Plant growth and development	3e–10

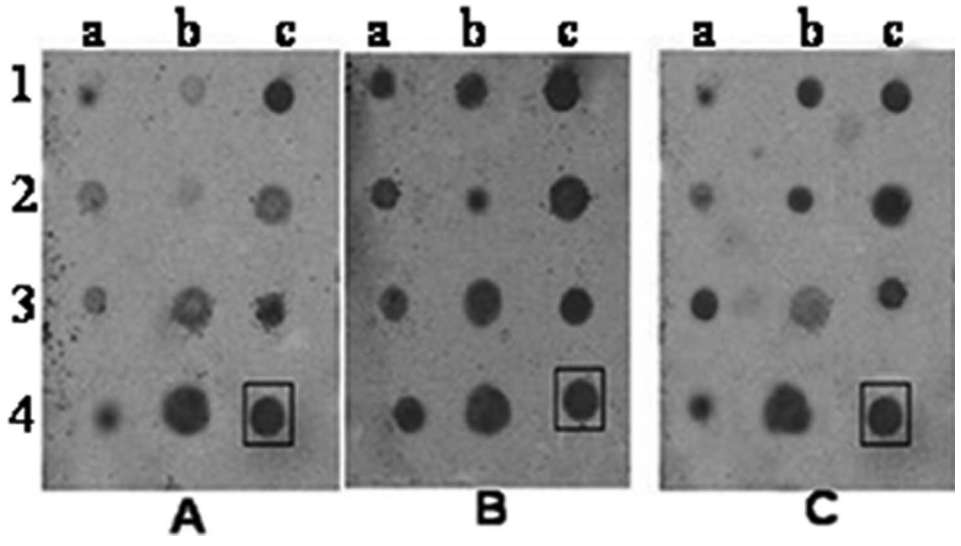

Figure 8. Reverse northern analyses.

Enormous changes in biochemical components like chlorophyll, protein in general and amino acid and lycopene content in particular in tomato were noticed in all the inoculated and energised plants. In case of arhar besides local variety, German White variety showed higher increase in chlorophyll content than the other variety. The seed protein content in energised local variety of arhar was found to 1.89% higher than control. Zinc content and total calorie content were also found to be appreciably increased in such seeds. Weight of 100 seeds obtained from such energised plants was recorded to be higher than the control. This indicated that due to RNMV inoculation, wherever growth promotion was noticed seed weight was also increased as was seen earlier with fibre crops (Ghosh 1985). In case of rice bean, along with increase in seed weight seed size also increased (Figure 4(b)).

Studies with different hosts indicated that though RNMV retards growth and yield in some monocot plants (Ghosh 1979, 1981), it acts in a beneficial way to some plants of dicot type and helps increasing yield in them. Earlier work (Ghosh 1982, 1995) revealed association of enhanced cytokinin-like material and IAA with such growth promotion. Recently, Dey et al. (2012) reported that such cytokinin-like material obtained from inoculated ones appeared to be a close derivative of zeatin having different proton–proton coupling and a molecular structure of highly substituted aliphatic group as compared with control one.

So, it is probable that in the present cases, such new types of cytokinin-like material produced in plants upon inoculation appeared to be one of the major factors responsible for the striking morphological changes in inoculated/energised plants. Stimulation of cell division along with slight increase in size in inoculated plants appeared to be the principal effect of this material perhaps in association with increased IAA (Ghosh 1982). The enhanced content of chlorophyll in the leaves of inoculated and energised plants, as evidenced during the present investigation, might have been one of the effects of the control of differentiation exerted by the cytokinin-like material through the conversion of proplastids into chloroplasts (Stetler and Laetach 1965). Furthermore, due to multiplication of RNMV within the host tissues,

as evident through serological studies (Ghosh 1982), the level of cytokinin-like material and IAA was maintained in the inoculated plants, As a result, in addition to that, the nutrients needed to keep pace with the enhanced growth and juvenility in the inoculated/energised plants were simultaneously met.

Keeping in view the property of RNMV-induced growth promotion in jute and its transmission to next generation through the seeds of inoculated plants, an attempt was made to study through molecular analysis the possibility of integration of viral genome with the host. Observation on a total of 460 amplicons obtained by STMS, ISSR and RAPD markers suggested that viral inoculation had not led to any change in the genetic background of RNMV-inoculated jute plants. Modification of gene expression through virus-induced transcriptional or post-transcriptional silencing of negative regulators of plant growth and yield appeared to be a distinct possibility (Roy et al. 2006). Hence, it is not improbable to conclude that similar situation exists in all the hosts under the present study.

For assessing the reasons for the passage of the property of such virus-induced growth promotion into the next generation, experiments at molecular level using tomato plants, grown under controlled conditions, were conducted. Some differences in gene expression patterns were anticipated amongst control, inoculated and energised tomato plants as morphological differences were evident in the study to support. Therefore, subtractive hybridisation technique was used to identify transcripts showing enhanced expression in RNMV inoculated and energised tomato plants. Through reverse northern analysis, it was noted that 12 clones showed higher expression level in presence of the probe from the inoculated plant than the same from control indicating up-regulation of the corresponding genes in RNMV-inoculated tomato plants. However, in case of energised tomato plants, only six showed up-regulation signals. This gave an indication of the presence of stronger effect of up-regulating signals in the genetic frame-work of the host and probably due to which growth promotion effects were noticed, even though not everlasting, in plants of successive generations. In present experimental plants, such effects persisted for 3 years and then failed to appear.

During analyses, the genes assigned for LHC, oxygen evolving enhancer, Rubisco and ATP synthase may account for high chlorophyll content and higher rate of photosynthesis in RNMV inoculated/energised plants. Such observation may also be true for fibre crops where due to RNMV inoculation both chlorophyll content and photosynthetic rate became higher than control (Ghosh 1995). The sequence showing similarity with lipid transfer protein is likely to have some antimicrobial properties (since this a PR protein) and involved in the plant defence to biotic and abiotic stress (Cammue et al. 1995; Garcia-Olmedo et al. 1995). Furthermore, one EST sequence which showed similarity with acetohydroxyacid synthase might have played a key role in the biosynthesis of branched chain amino acids like leucine, isoleucine and valine and those have been repeatedly reported to be a stress reliever in mammalians. Many plant resistance genes have leucine-rich repeat (LRR) domains, which are important for recognising non-self proteins in the environment and setting in motion a signal transduction pathway that ultimately leads to defensive action. Hence, it can be presumed that AHAS might have played some role in defensive mechanism in host plants by increasing the biosynthesis of leucine. Up-regulation of light harvesting complex might have resulted in the delayed degradation of chlorophyll pigments that have been enhanced due to RNMV energisation which confirms the view of Oh et al. (2000). And possibly due to this

reason, juvenility in inoculated/energised test plants was maintained for more period than control ones. One up-regulated EST that codes for Mg protoporphyrin IX chelatase acts as ABA receptor and mediates ABA signalling as a positive regulator in seed germination, post-germination growth and stomatal movement (Shen et al. 2006). The presence of ABA is reported to upregulate the CKx genes (encoding cytokinin oxidase/dehydrogenase) that are involved in cytokinin biosynthesis in maize roots (Sakakibara 2006). 2-oxogluterate-dependent dioxygenase was found to be up-regulated in RNMV energised tomato plants and this enzyme is reported to catalyse an important step of gibberellic acid biosynthetic pathway by oxidative removal of C_{20} to form C_{19} gibberellins. So, this might be accounted for increased fruit set and size in the inoculated/energised test plants (Figure 6(a)).

Up-regulation of Rieske Fe-S proteins, a subunit of the cytochrome b_6f complex furthermore suggested enhanced rate of photosynthesis possibly by increasing electronic connection between PS I and PS II reaction centres. Post-translational modification of the Rieske Fe-S protein has been reported to regulate electron transport to avoid over-reduction under stress situation. Arabinogalactan proteins, the extracellular hydroxyproline-rich proteoglycans implicated in plant growth and development, are up-regulated in energised tomato plants and hence accounted for increased plant growth due to inoculation in the study. As for the one gene with unknown function, homology comparison at nucleotide and amino acid levels in GenBank gave us some information. But this information is far from explaining the function of this gene and its relationship with growth promotion and warrants further study for drawing any inference.

So, present investigation revealed that a plant virus specific to monocot plants only showed reversible reaction when artificially applied to plants of dicot nature. Probably, after entry into the host the virus conditioned the plants in such a way that besides growth promotion and enormous changes in different metabolic activities in inoculated plants, it caused some significant influence(s) somewhere at sub-genomic level, not yet known to us, resulting this property to be transmitted to next few successive generations. Thus, it constitutes the first report of the transitory passage of the property of the virus-induced growth promotion through seeds of inoculated plants without causing any change in genetic make-up of the host. This also paves the way for improving the vertical productivity of target crops through inoculation of a virus without any adverse effect on them.

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