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# Acibenzolar-S-Methyl (ASM)-Induced Resistance against Tobamoviruses Involves Induction of RNA-Dependent RNA Polymerase (RdRp) and Alternative Oxidase (AOX) Genes

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## Abstract

Tobamoviruses are the major viral pathogens of tomato and bell pepper. The preliminary results showed that Acibenzolar-S-methyl (ASM; S-methylbenzo (1,2,3) thiadiazole-7-carbothiate) pre-treatment to tomato and tobacco plants reduces the concentration of Tomato mosaic tobamovirus (ToMV) and Tobacco mosaic tobamovirus (TMV) in tomato and bell pepper seedlings, respectively. Pre-treatment of the indicator plant (*Nicotiana glutinosa*) with the ASM followed by challenge inoculation with tobamoviruses produced a reduced number and size of local lesions (67 and 79% protection over control to TMV and ToMV inoculation, respectively). In order to understand the mechanism of resistance the gene expression profiles of antiviral genes was examined. RT-PCR products showed higher expression of two viral resistance genes viz., alternative oxidase (AOX) and RNA dependent RNA polymerase (RdRp) in the upper leaves of the ASM-treated tomato plants challenge inoculation with ToMV. Further, the viral concentration was also quantified in the upper leaves by reverse transcription PCR using specific primer for movement protein of ToMV, as well as ELISA by using antisera against tobamoviruses. The results provided additional evidence that ASM pre-treatment reduced the viral movement to upper leaves. The results suggest that expressions of viral resistance genes in the host are the key component in the resistance against ToMV in the inducer-treated tomato plants.

Key words: acibenzolar-S-methyl, alternative oxidase, movement protein, RNA dependent RNA polymerase, systemic acquired resistance; tomato, ToMV

## Introduction

Viral diseases, especially tobamoviruses (ToMV and TMV) are becoming a menace to the farmers as well the scientists involved in the production of quality seeds. Effective viricides are lacking for the control of viral diseases of plants. Use of chemicals to control the plant pathogens, which activates SAR are gaining importance. Among the chemical inducers used for the control of plant pathogens, salicylic acid (SA)-derived chemicals and pathogen-derived elicitors have proven their efficacy and have been introduced for commercial exploitation. The comparison between resistance inducers revealed BION, a commercial formulation of BTH showed the strongest transcriptional induction of defense genes. The mechanism of induced resistance has been well studied in the past decade and well documented (Rad et al. 2005). Some of the basic characteristics of SAR are: 1) it is triggered by necrotic lesions caused by pathogens in both incompatible and compatible interactions; 2)

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it takes some time between induction and expression of protection; 3) it results in a systemic protection of the entire plant; 4) it is long lasting (several weeks or even months) and provides the plant with protection against a broad spectrum of pathogens; and 5) it is correlated with the induction of a well-characterized set of genes which directly encodes the respective protein (Neuenschwander et al. 1996). The striking advantages of using chemical elicitors are that resistance can be induced: 1) at one exactly defined time; 2) in a dosage-controlled manner; and 3) without additional effects resulting from the presence of pathogens. All these factors lead to a higher degree of comparability among studies of induced resistance (Heil et al. 2000).

Acquired resistance (AR) phenomena occur when plants became resistant to a broad range of pathogens. Ross (1961) demonstrated that pre-inoculation of Xanthi-nc tobacco plants with tobacco mosaic virus (TMV) conferred protection against some viral pathogens. After induction, protection against a subsequent infection can be restricted to the tissue treated with the inducer (local acquired resistance, LAR) or can affect plant tissues that have not been treated (systemic acquired resistance, SAR). Most studies of the SAR have sought to identify the local and systemic signaling molecules involved in its establishment and components of the signal transduction pathways leading to resistance gene expression. Enhanced disease resistance is frequently accompanied by the activation of resistance genes (Van Loon and Van Strien 1999). In case of resistance against tobamoviruses, the role of AOX and RdRp has been discussed on several occasions (Murphy et al, 1999; Xie et al. 2001).

The aim of the work described in this paper was to study the effect of ASM and Bion-M on Tobamoviruses in indicator and host plants. The work concentrates on the effect of ASM and ToMV on the expression pattern of viral resistant genes (AOX and RdRp) in tomato. The work also concentrates on the effect of ASM on the movement of ToMV in tomato.

## Materials and Methods

### Maintenance of host plants, indicator plants and viruses

Seeds of tomato cv. PKM-1, bell pepper cv. California wonder and indicator plant (*N. glutinosa*) were sown in earthen pots containing 1:2:1 ratio of sand, soil, and farmyard manure. The pots were maintained in insect-proof screen house conditions. The Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV) were maintained in bell pepper and tomato plants, respectively, in the screen house.

### Effect of inducers on tobamoviruses in indicator plants

The inducers were screened for their efficacy against ToMV and TMV on *N. glutinosa*. The primary leaves of two-month-old *N. glutinosa* plants were dipped in 100 ppm a.i. concentration of inducers (ASM and Bion-M) prepared in sterile distilled water. Three plants were maintained for each treatment. The primary leaves of control plants were dipped with sterile distilled water. After 24 h of treatment with inducers, the treated and control *N. glutinosa* leaves were inoculated with the viruses.

The standard virus inoculum was prepared by using the leaves showing mosaic symptoms harvested from the pre-maintained ToMV-infected tomato and TMV infected bell pepper plants. The leaves (1 g) were homogenized in 5 ml of the phosphate buffer (pH 7.2, 0.1 M) in a pre-chilled pestle and mortar. After homogenization, the extract was filtered through muslin cloth and the supernatant was used as the source of inoculum. The cotton swab was dipped in the virus inoculum and swabbed over carborundum pre-dusted leaves. After 10 min, inoculated leaves were washed with distilled water. The seedlings were maintained in a screen house. Observations on development of local lesions were recorded on the third day (72 h) after inoculation.

The effect of inducers on the tobamoviruses was quantified by local lesions assay on *N. glutinosa*. The number of local lesions/100 $cm^2$ was calculated by using the formula:

Number of local lesions formed on inoculated leaf x 100/Total area of inoculated leaf

The percentage inhibition of local lesion formation and size of the local lesions formed on the inoculated leaves by each treatment over the control was calculated based on the number of local lesions produced using the formula:

$$I = (C - T) \times 100 / C$$

Where, I = Percent inhibition of lesion formation over control; C = Number of local lesions in control; T = Number of local lesions in plants treated with inducers.

### Effect of inducers on tobamoviruses in host plants

Tomato cv. PKM-1 and bell pepper cv. California Wonder seeds were sown and maintained as described earlier. The obtained seedlings (20-day-old) were spray treated with Bion-M and ASM (100 ppm a.i.). After 24 h of treatment, the treated tomato and bell pepper seedlings were inoculated with ToMV and TMV, respectively, as described earlier. After 21 d, the third leaves were harvested and the virus concentration was quantified by using ELISA. The leaves (0.1 g) harvested from 15-day-old untreated or treated plants were crushed in a mortar and pestle in antigen buffer and the extract was centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant extracted from bell pepper and tomato was subjected to indirect ELISA against a-TMV and a-ToMV (kindly provided by DGISP, Denmark), respectively (Hobbs et al. 1987).

The virus concentration in the inducer-treated as well as the control host plants were quantified by subjecting the extracts of virus symptoms bearing leaves into indicator plant test (*N. glutinosa*). The number of local lesions/100 $cm^2$ and size of the local

lesions formed on the inoculated leaves was calculated as explained earlier.

### Effect of ASM treatment on viral resistant gene expression in tomato against ToMV

Tomato cv. PKM-1 seeds were sown and maintained as described earlier. The obtained seedlings were transplanted to small earthen pots. The primary leaves of seedlings (20-day-old) were dipped in ASM (100 ppm a. i.) solution for 5 s. After 24 h of treatment, the treated tomato seedlings were inoculated with ToMV. After inoculation, the leaf samples (4th) harvested at different time intervals (0, 12, 24, 48, 72, and 120 h). The harvested leaf material was snap frozen in liquid nitrogen and stored at -80 ℃.

### RNA extraction and first strand cDNA synthesis

Leaf discs (100 mg in fresh weight) from third leaf of tomato were frozen in liquid nitrogen and stored at -80 ℃. RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Maryland, USA) according to manufacturer's instructions. RNA samples were digested with DNase (DNA-free, Ambion, Austin, TX, USA) to remove the traces of DNA contamination and 1 μg equivalent RNA was reverse transcribed by using RETRO Script Kit (Ambion) as per manufacturer's instructions.

### Reverse transcription-PCR for RdRp, AOX, and β-Actin

For analyzing the RdRp and AOX gene expression, reverse transcription PCR (RT-PCR) was used to amplify the transcripts of the target genes as well as the constituely expressed reference gene, β-actin. The primers for RdRp (accession number Y10403), AOX (accession number AY524818) and β-actin (accession number U60481) were designed by using the Primer3 software. The primers used in our experiments were summarized in Table 1.

PCR reactions were carried out in a 50 μl mixture containing 1μl of cDNA, 2.5 units of Taq DNA polymerase, 125 μM of each dNTPs, and 0.2 μM of primer (1μl) (Sigma) of each of target and reference gene. The cycle numbers were optimized to ensure that amplication of reference gene and the gene of interest remained with in the exponential amplification range, thereby giving an accurate representation of transcript abundance. Amplication was carried out according to the following temperature profile: 94 ℃ for 2 min for denaturation, followed by 30 cycles for 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 1 min, with a final elongation at 72 ℃ for 10 min. The relative amount of each obtained PCR products were readily quantified by direct scanning of the ethidium bromide stained 1.4% TAE-agarose gels (Infinitti, Vilber Lourmat, France).

PCR products (50 μl) were also purified using Minielute™Gel extraction kit (Qiagen, France) according to manufacturer`s instructions for sequencing. PCR products were sequenced by the dideoxynucleotide method using the BigDye Terminator ver. 3.0 Kit (Applied Biosystems) from both strands and multiple sequence alignment was performed using Multalin software (France) and deposited to Genbank (Accession numbers EU408340, EU408341, and EU408342).

The increase in fold gene expression was calculated by using the formula:

$$\frac{\dfrac{\text{Quantity of target gene}}{\text{Quantity of reference gene}}}{\dfrac{\text{Quantity of target gene in DW treatment at 0 h}}{\text{Quantity of reference gene}}}$$

### Semi-quantification of ToMV by RT-PCR

The tomato cv. PKM-1 was maintained as described earlier. The tomato plants were spray treated with ASM (100 ppm a.i.) (whole plant). The virus inoculum was prepared as mentioned above. The ToMV was inoculated to basal leaf of the plants along with control (untreated). The fourth leaves were harvested at different time intervals (0, 5, 10, 15, and 20 days post-inoculation, dpi). The leaves were snap frozen in liquid nitrogen and stored at -80 ℃. RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. RNA samples were digested with DNase (DNA-free, Ambion, Austin, TX, USA). Specific primers were designed for movement protein of ToMV with primer 3 software (Table 1).

Reverse transcription was performed using RETRO Script Kit (Ambion) as per manufacturer's instructions. Reverse transcription was carried out in Master Cycler Gradient (Eppendorf, France). The RT mixture contained 2 μl of reverse primer (50 μM), 1μg equivalent RNA, 5 μl of nuclease-free water, 2 μl of RT buffer, 2 μl of DNTPs, 1 μl of RNase inhibitor, and 1 μl of Reverse transcriptase (RETRO Script Kit, Ambion). RNA was denatured by initial denaturation at 75 ℃ for 3 min. The RT

**Table 1.** Details of primers used in the study.

| Sl. No. | Name of the gene | Primers designed | Amplicon (bp) |
|---|---|---|---|
| 1 | RNA dependent RNA polymerase | Forward, 5'-TTCTCTCTGCGGAAGTGGTT-3'<br>Reverse, 5'-GATCCGAGGAGCACCAAATA-3' | 491 |
| 2 | Alternative oxidase | Forward, 5'-ACACGAGTTGTCATGGGTCA-3'<br>Reverse, 5'-ACATCAGTGGGGAAACGAAG-3' | 491 |
| 3 | β-Actin | Forward, 5'-GGATCTTGCTGGTCGTGATT-3'<br>Reverse, 5'-ATCATGGATGGCTGGAAGAG-3' | 267 |
| 4 | ToMV- MP | Forward, 5'-TGAAAATGAATCATTGTCT-3'<br>Reverse, 5'-CATCTTCAATCAAATTATC -3' | 623 |

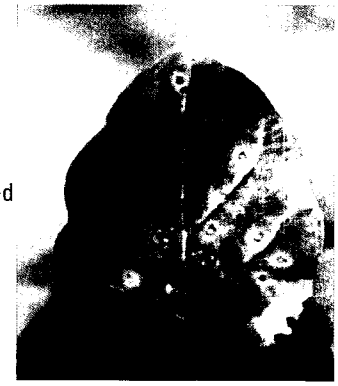


**Fig. 1.** Effect of ASM on the Expression level of RdRp in tomato seedlings inoculated with ToMV. Gels shown are representative of three replicates tester per treatment. Data points on graphs represent average-fold increase in percent fluorescence intensity across three replicate samples ± SE.

mixture was added to the denatured RNA. The reaction was carried out at 42 °C for 60 min and the process was terminated at 92 °C for 10 min.

The cDNA synthesized from the RT reaction was taken for the amplification of movement protein of ToMV. The PCR reactions were carried out in a 50μl mixture containing 1μl of cDNA, 2.5 units of Taq DNA polymerase, 125 μM of each dNTPs (1 μl), 1 μl BSA (0.1%), 5 μl of buffer with $MgCl_2$ (Sigma), and 0.2 μM of primer of forward and reverse primers. PCR amplication was carried out according to the following temperature profile: 94 °C for 3 min for initial denaturation, followed by 40 cycles for 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with a final extension at 72 °C for 5 min. The obtained PCR product was electrophoresed on the 1% agarose-TAE gel. The bands were readily quantified by direct scanning of TAE-agarose gels (Infinitti, Vilber Lourmat, France). To support this data, the ToMV concentration was also quantified by ELISA using antibody of ToMV at different time intervals as explained earlier.

### Data analysis

For data analysis the statistical computer application package SPSS 10.0 was employed. The data generated were average of three independent experiments. Data were subjected to analysis of variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; $P = 0.05$).

## Results

### Effect of inducers on tobamoviruses in indicator plants

The pre-treatment of inducers (ASM and Bion-M) followed by challenge inoculation of TMV or ToMV to *N. glutinosa* produced a fewer number of local lesions with smaller-sized lesions while more number of larger-sized local lesions were noticed in untreated leaves. The ASM and Bion-M pretreatment of leaves challenge inoculation with TMV produced 67 and 62% protection over the control leaves, respectively (Table 2). Similarly,

**Table 2.** Effect of inducers on Tobacco mosaic virus (TMV) infection on *Nicotiana glutinosa*

| Treatment | No. of local lesions / 100 cm² | Size of local lesions (in mm) | Inhibition in local lesions / 100 cm² (%) | Reduction in local lesion size (%) |
|---|---|---|---|---|
| Control | 60 ± 0.87[a] | 5 ± 0.27[a] | - | - |
| Bion-M | 23 ± 0.75[b] | 3 ± 0.23[b] | 62 | 40 |
| ASM | 20 ± 0.58[c] | 2.7 ± 0.3[b] | 67 | 46 |

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ($P = 0.05$).

**Table 3.** Effect of inducers on Tomato mosaic virus (ToMV) infection on *Nicotiana glutinosa.*

| Treatment | No. of local lesions / 100 cm² | Size of local lesions (in mm) | Inhibition in local lesions / 100 cm² (%) | Reduction in local lesion size (%) |
|---|---|---|---|---|
| Control | 67 ± 0.47[a] | 6 ± 0.21[a] | - | - |
| Bion-M | 18 ± 0.35[b] | 4 ± 0.29[b] | 73 | 33 |
| ASM | 14 ± 0.28[c] | 3 ± 0.27[c] | 79 | 50 |

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ($P = 0.05$).

**Table 4.** Reduction in virus concentration in inducer treated bell pepper plants challenge inoculated with TMV (as indexed by indicator plant test).

| Treatment | No. of local lesions / 100 cm² | Size of local lesions (in mm) | Inhibition in local lesions / 100 cm² (%) | Reduction in local lesion size (%) |
|---|---|---|---|---|
| Control | 71 ± 0.57[a] | 6.2 ± 0.47[a] | - | - |
| Bion-M | 33 ± 0.43[b] | 4.1 ± 0.17[b] | 53 | 33 |
| ASM | 23 ± 0.33[c] | 3.9 ± 0.27[c] | 68 | 37 |

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ($P = 0.05$).

**Table 5.** Reduction in virus concentration in inducer treated tomato plants challenge inoculated with ToMV (as indexed by indicator plant test).

| Treatment | No. of local lesions / 100 cm² | Size of local lesions (in mm) | Inhibition in local lesions / 100 cm² (%) | Reduction in local lesion size (%) |
|---|---|---|---|---|
| Control | 79 ± 0.37[a] | 5.9 ± 0.57[a] | - | - |
| Bion-M | 37 ± 0.47[b] | 4.2 ± 0.29[b] | 53 | 29 |
| ASM | 27 ± 0.75[c] | 3.7 ± 0.27[c] | 66 | 39 |

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ($P = 0.05$).

the treatment of ASM and Bion-M resulted in 79 and 73% protection in ToMV inoculated plants over the control, respectively (Table 3). ASM and Bion-M treatment resulted in 2.7- and 3-mm-sized local lesions when compared to 5 mm in the control in TMV inoculated leaves. Treatment with Bion-M followed by inoculation of TMV revealed 40% reduction in the size of the local lesions while ASM treatment and challenge inoculated plants showed 46% reduction in local lesions. Similarly, ToMV produced 3- and 4-mm-sized lesions in ASM and Bion-M treated leaves in comparison with 6 mm in the control. Treatment of ASM and Bion-M followed by inoculation of ToMV showed 50 and 33% reduction in the size of local lesions, respectively (Fig. 1).

## Effect of ASM and Bion-M treatment on disease development

The inoculation of leaf sap obtained from the tomato and bell pepper seedlings treated with ASM and Bion-M, followed by challenge inoculation of ToMV and TMV, respectively, to *N. glutinosa* produced fewer number of local lesions along with the reduced local lesion sizes compared to the control. ASM was comparatively more effective than Bion-M in reducing the local lesions. In *N. glutinosa* plants inoculated from the sap of tomato (ToMV) and bell pepper (TMV), a reduction in the local lesion formation by 66 and 68%, respectively, was clearly evident (Tables 4 and 5).

Further, the ASM and Bion-M treatments on tomato and bell pepper seedlings, followed by challenge inoculation of ToMV and TMV, respectively, showed significant reduction in the viral concentration in comparison with control seedlings as evident from the results of indirect ELISA carried out by using the antibodies of ToMV and TMV, respectively. The control plants showed absorbance of 0.415 and 0.397 for TMV and ToMV, respectively (Table 6). Bion-M treatment showed 0.117 and 0.134 absorbance at 410 nm in TMV and ToMV inoculated treated plants, respectively, whereas ASM treatment showed further reduction in absorbance of 0.110 and 0.125, respectively, for TMV- and ToMV-inoculated plants. The seedlings also showed reduced severity in the symptom expression, which also correlates with the ELISA and indicator plant test results (Table 6). Based on the results obtained in indicator plants and host plants, the ASM alone was used for the further experiments.

**Table 6.** Reduction in virus concentration in inducer treated host plants challenge inoculated with tobamoviruses (Indirect ELISA) (Absorbance at 410 nm).

| Inducers used | TMV | ToMV |
|---|---|---|
| Virus inoculated +ve Control | 0.497 ± 0.47$^{a}$ | 0.410 ± 0.32$^{a}$ |
| Virus control -ve control | 0.057 ± 0.37$^{d}$ | 0.051 ± 0.57$^{e}$ |
| Control plants (Untreated) | 0.415 ± 0.49$^{b}$ | 0.397 ± 0.61$^{b}$ |
| Bion-M treated | 0.117 ± 0.42$^{c}$ | 0.134 ± 0.37$^{c}$ |
| ASM treated | 0.110 ± 0.33$^{c}$ | 0.125 ± 0.37$^{d}$ |

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ($P = 0.05$).

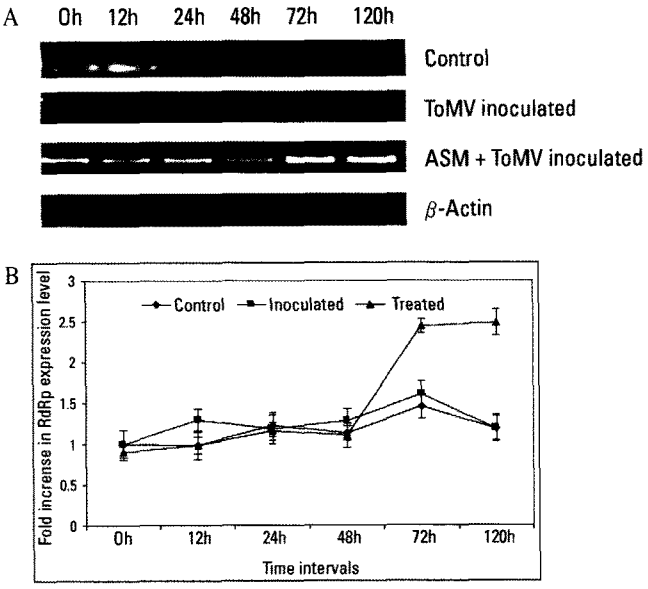


**Fig. 2.** Effect of ASM on the Expression level of AOX in tomato seedlings inoculated with ToMV. A) Gels shown are representative of three replicates tester per treatment. B) Data points on graphs represent average-fold increase in percent fluorescence intensity across three replicate samples ± SE.

## Effect of ASM treatment on RdRp and AOX genes expression

A differential expression of RdRp and AOX genes was clearly evident in tomato plants pre-treated with ASM and challenge inoculated with ToMV (Fig. 2). The expression levels of RdRp gene remained constant in all the time points in distilled water controls and ToMV-inoculated untreated plants. Consequent to ASM treatment followed by challenge inoculation of ToMV, an increase in RdRp transcripts was noticed at 72 h with an increase of over 2.5-fold and the trend remained throughout the study. On the other hand, a slight increase in the AOX transcripts was noticed at 120 h post-inoculation of ToMV alone. However, the expression levels remained unchanged in distilled water-treated plants. In ASM treated plants, upon inoculation with ToMV, enhanced expression of AOX gene was evident at all the time points. A maximum increase of 2.9-fold was noticed during 72 h post-inoculation (Fig. 3).

## Semi-quantification of ToMV

RT-PCR using specific primers for movement protein gene showed the translocation of ToMV to the upper 4$^{th}$ leaf on 20 d after inoculation of the pathogen to the first true leaf in the con-

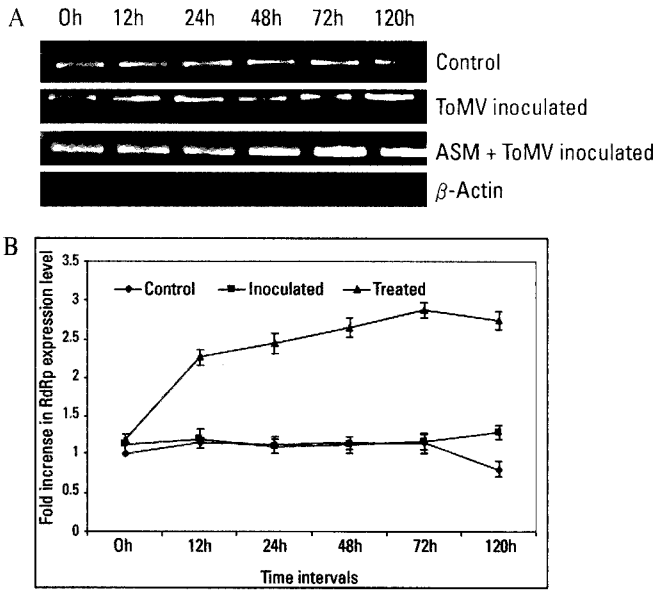


**Fig. 3.** Effect of ASM on Virus (movement protein) in tomato plants inoculated with ToMV. A) Gels shown are representative of three replicates tester per treatment. B) Data points on graphs represent average-fold increase in percent fluorescence intensity across three replicate samples ± SE.

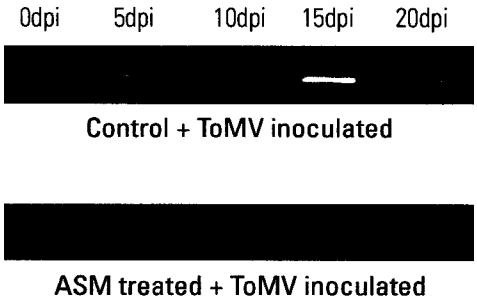


**Fig. 4.** Semi-quantification of ToMV by RT-PCR dpi means days post-inoculation.

trol tomato plants (Fig. 4). In contrast, the tomato plants pretreated with ASM and challenge inoculated with ToMV to the first leaf showed less band intensity over controls. These observations correlated with the ELISA results wherein, the absorbance at 410nm was 0.479 in controls at 20 dpi, while it was 0.137 in ASM treatments (Table 7).

## Discussion

We have demonstrated that treatment with the ASM and Bion-M induces the resistance in the host plants as well as in indicator plants against tobamoviruses as evidenced by the reduction in the number of local lesions by the treatment of *N. glutinosa* leaves with inducers. The results also confirmed

**Table 7.** Effect of ASM on viral concentration in tomato plants inoculated with ToMV (Indirect ELISA).

| Time intervals (Days) | Absorbance at 410nm | |
|---|---|---|
| | Control + ToMV | ASM + ToMV |
| 0 dpi | 0.047 ± 0.47$^{e}$ | 0.049 ± 0.12$^{d}$ |
| 5dpi | 0.117 ± 0.49$^{d}$ | 0.054 ± 0.24$^{d}$ |
| 10dpi | 0.268 ± 0.37$^{c}$ | 0.079 ± 0.27$^{c}$ |
| 15dpi | 0.357 ± 0.33$^{b}$ | 0.112 ± 0.36$^{b}$ |
| 20dpi | 0.479 ± 0.57$^{a}$ | 0.137 ± 0.47$^{a}$ |

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ($P = 0.05$).

reduction in the size of the local lesions. In general, the reduction in size of the local lesions implicates to the induced resistance against plant viruses. Further, the treatment of host plants with ASM and Bion-M showed less viral concentration in comparison with controls as evident by the results of indirect ELISA and indicator plant tests. This confirms that ASM induces resistance against tobamoviruses in both indicator and host plants, which reacts differentially after inoculation with tobamoviruses. Actigard (ASM) has been effective in reducing the incidence of TSWV on tobacco, especially in combination with the insecticide imidacloprid (Pappu et al. 2000). Actigard (ASM) reduced incidence of tomato spotted wilt virus by as much as 76% along with other chemicals and cultural practices (Momol et al. 2002). Applications of ASM reduced the incidence of tomato plants infected with TSWV by 28% in each of two seasons with high disease pressure, but had no significant effect on TSWV in a season with mild disease pressure (Momol et al. 2004).

In our experiments, increased RdRp was induced only in the ASM treated- and ToMV-inoculated plants. Induced RdRp could protect plants from the viral infection by synthesizing the small cRNAs by using viral RNA as templates and target their degradation. The cRNA synthesized by inducible RdRp activity may also interfere with the replication and/or translocation of the viral RNAs, resulting in the reduced synthesis of viral components important for virus proliferation and symptom development. Further, tobacco lines deficient in the inducible RdRp activity showed more severe disease types and higher levels of viral RNAs (Xie et al. 2001). Apparently, the inducible RdRp reduces accumulation of viral RNAs at least partly by enhancing the turnover of viral RNAs. *Arabidopsis thaliana* RdRp1 (AtRdRp1) has been implicated to promote turnover of viral RNAs through a number of possible mechanisms. The inducible AtRdRp1 could play a role in the extension of viral siRNAs, thereby sustaining the production of siRNAs and degradation of newly synthesized viral RNAs (Yu et al. 2003). Furthermore, AtRdRp1 may recognize certain sequences of full-length viral RNAs as templates for synthesis of cRNAs that could interfere with viral RNA replication, translation, or both leading to

enhanced production of small viral RNAs relative to full-length genomic RNAs (Yu et al. 2003). Based on this observation, our results provides additional evidence that the inducible RdRp by treatment with ASM has a major role in resistance against ToMV.

The results of our experiments showed a higher level of AOX (2.9-fold increase) was induced by the treatment with ASM and ToMV, indicating the possible role in viral resistance. Along with these, a slight increase in AOX transcripts was noticed in 120 hours post-inoculation of ToMV alone. The resistance of tobacco plants against TMV is known to occur through salicylhydroxamic acid (SHAM)-sensitive pathway (Chivasa et al. 1997). Pretreatment of plants with cytochrome pathway inhibitors predominantly induces AOX gene expression, which is similar to the situation induced by SA treatment. Further, several studies indicate that viral resistance (SA-induced resistance in susceptible plants and N-gene-mediated resistance responses) is antagonized by SHAM, a well-known inhibitor of AOX activity (Naylor et al. 1998). Hence, it has been suggested that an important role for SA in plant viral resistance is to induce AOX and that resistance responses are dependent upon AOX activity and hence compromised by SHAM. SA can stimulate the inhibition of all three main stages in virus infection: replication, cell-to-cell movement, and long distance movement (Singh et al. 2004).

ASM treatment showed reduced ToMV concentration as evidenced by the results of RT-PCR (for ToMV movement protein gene) and indirect ELISA. SA induces resistance to TMV by inhibiting the TMV RNA and coat protein along with viral RdRp in TMV susceptible tobacco (Chivasa et al. 1997). Similarly, SA induces resistance against PVX and Turnip vein clearing virus (TVCV) in tobacco and *Arabidopsis*, respectively (Naylor et al. 1997; Wong et al. 2002). SA-induced resistance to TMV results from the inhibition of both virus replication and movement of the virus through the plasmodesmata linking adjacent cells. The inhibition of replication occurs in the meshophyll cells, while restriction of cell-to-cell movement occurs in the epidermal cells (Murphy and Carr 2002). Further, SA can inhibit the systemic spread of CMV through the plant. This is because the long distance movement of CMV through the phloem is inhibited in plants after SA treatment (Naylor et al. 1998). These results obtained by various groups shows that SA treatment inhibit the replication, cell-to-cell movement, and long distance movement of plant viruses. The reduction of viral concentration in the 4$^{th}$ leaf in our present study may be due to one of the similar mechanisms described above.

The results of the present study clearly show that ASM has the potential to induce resistance against tobamoviruses especially against TMV and ToMV and the mechanisms involves RdRp and AOX gene induction.

## Acknowledgements

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