MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1. Plant Species

Six different plant species reported to be effective in inhibiting tomato spotted wilt virus viz., *Bougainvillea spectabilis* Willd., *Vinca rosea* L., *Casuarina equisetifolia* Forst., *Solanum elaeagnifolium* Clarke., *Morus alba* L. and *Sorghum vulgare* Pers. were selected for this study.

All these plants were collected from Tamil Nadu Agricultural University Campus, Coimbatore, India. Samples were drawn from the same plant throughout the study.

3.2 Virus Culture

A pure culture of tomato spotted wilt virus (TSWV) was established on the primary leaves of *Vigna unguiculata* (L.) Walp (cowpea) cv,C152 in an insect proof glass house. For this purpose, tomato leaves showing typical symptoms of TSWV infection (Plate 1) were collected from field. Sap was extracted from one gram of these leaves by grinding with 1.5 ml of pre-chilled 0.1 M phosphate buffer, pH 7.0 containing 0.1 % 2- mercaptoethanol in a mortar and pestle following the ice tray technique (Subramanian & Narayanasamy, 1973). The clear sap was inoculated by gentle rubbing on the primary leaves of 4 to 6 days old cowpea previously dusted with 600 mesh carborundum powder to
Plate 1. Typical symptoms of TSWV on tomato leaves.

A. Lesions as seen on the upper surface of the leaf.

B. Lesions as seen on the under surface of the leaf.

Plate 2. TSWV lesions on cowpea leaf.
serve as abrasive. Excess inoculum was washed off the leaves after 5 min. The local lesions developed after 3-5 days on the inoculated cowpea leaves were used to prepare TSWV inoculum for further studies. The virus culture was maintained on cowpea plants inside the glass house by periodical sub inoculations. A close up view of TSWV lesions on the primary leaf of cowpea is shown in plate 2. The virus was retrieved by macerating the leaf tissues of cowpea having ten local lesions with one ml of phosphate buffer as above. The clear homogenate was used as TSWV inoculum.

3.3. Assay host

Cowpea cv. C152 seeds obtained from the Pulses section, Millet Breeding Station, TNAU, Coimbatore were raised in pots in the glasshouse and used as assay host for TSWV, since it reacts with the production of local lesions in about four to five days after inoculations.

3.4 Efficacy of the different plant species

Ten per cent extracts of the fresh leaves of the six different plant species mentioned above and the fresh root powder of Bougainvillea spectabilis were prepared with distilled water separately by grinding in a mortar and pestle under cold conditions. The extracts were filtered through muslin cloth, centrifuged at 5000xg for 15 min. in a refrigerated centrifuge and supernatants collected. These extracts were tested for their antiviral activity by
spraying on the primary leaves of 4 to 6 days old cowpea (assay host) raised in pots in the glass house. After 24 h the cowpea leaves were dusted with carborundum powder and then inoculated with TSWV culture by gentle rubbing. The inoculated leaves were rinsed with water shortly after inoculation to remove excess inoculum and carborundum. The lesions developed on the cowpea leaves after 3-5 days of inoculation were compared with that of control plants to which water spray was given instead of plant extracts. Three replications were maintained for each test.

The inhibitory efficacy of each of the plant extracts against TSWV was calculated based on the formula (Kubo et al., 1990),

\[
\% \text{ inhibition} = (1-T/C)100
\]

where \( T \) = Number of lesions on the treated plants

\( C \) = Number of lesions on the control plants

3.5. Bougainvillea extract on the systemic hosts

The inhibitory efficacy of Bougainvillea leaf extract was also verified on two of the commercially important systemic hosts of TSWV viz, Capsicum annuum L. cv. Col and Arachis hypogaea L. cv. Col raised in pots inside insect proof glasshouse.

The 10 day old plants that have developed primary leaves were first sprayed with Bougainvillea leaf extract and
tested exactly as described in 3.4. Necessary controls were maintained by spraying water instead of Bougainvillea leaf extract. Three replications were maintained for each of the tests and controls. TSWV symptom development was examined from the 10th day after inoculation.

3.6. control of other viruses

The viral inhibitory efficiency of Bougainvillea leaf extract was also tested on three other commercially important plant viruses viz., Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV) and Cowpea aphid borne mosaic virus (CAMV). Each test was replicated three times. Controls were maintained for each test by spraying water instead of Bougainvillea extract.

Tobacco mosaic virus (TMV)

Leaf extract of Bougainvillea was sprayed on the leaves of *Nicotiana glutinosa* L. and inoculated with TMV culture as described for TSWV in 3.4. The lesions developed after two days on the test plants were compared with that of the control plants and per cent inhibition calculated.

TMV culture.

TMV culture was prepared by grinding 1.0 g of TMV infected leaf of *Nicotiana benthamiana* maintained in the glass house, with 3 ml of 0.1M phosphate buffer pH 7.0 as described in 3.2.
Cucumber mosaic virus (CMV)

Plants of *Cucurbita moschata* Poir. cv. Col, the systemic host of CMV were raised in pots in the glass house. The primary leaves of the plants (15 days old) were sprayed with the leaf extract of *Bougainvillea* and inoculated with CMV culture following the same method described in 3.4. CMV symptom development was examined from the 10th day after inoculation and per cent inhibition calculated.

**CMV culture**

CMV culture was prepared by grinding 1.0g of CMV infected *Momordica charantia* L. leaf (maintained in the glass house) with 3.0 ml of phosphate buffer pH 7.0 as described above. The extract was used for inoculating *Cucurbita moschata* leaves.

Cowpea aphid borne mosaic virus (CAMV)

Cowpea, *Vigna unguiculata*, cv. C152 plants were raised in pots in the glass house. This is a systemic host of CAMV. Bogainvillea leaf extract was sprayed on to the primary leaves of 5-6 days old cowpea and challenged with CAMV sap culture after 24 h as described above. CAMV symptom development was examined and noted down from 10th day after inoculation and per cent inhibition calculated.

**CAMV culture**

CAMV culture was prepared by grinding 1.0 g of CAMV
infected cowpea leaves maintained in the glass house with 3 ml of phosphate buffer, pH 7.0 as described before and the extract used for inoculation of cowpea plants.

3.7. Chemical nature of the antiviral principle

The chemical nature of the antiviral principle was analysed based on the inhibitory efficacy to TSWV of 1) Trypsin digested extracts, 2) Proteins precipitated by ammonium sulphate and 3) Acetone precipitated proteins by bioassay on cowpea plants. Necessary controls were maintained by spraying water. Three replications were maintained for each test.

All the analytical procedures were carried out in a cold room maintained at 4 °C. Centrifugations were done in a HITACHl 18 PR-52 Automatic high speed refrigerated centrifuge.

Preparation of the extract

Extract was prepared from the fresh leaves and roots of Bougainvillea following the procedure of Takanami et al. (1990) with some modifications.

Materials

10 mM phosphate buffer (PB), pH 7.2 : 28 ml of 0.2 M NaH₂PO₄ and 72 ml of 0.2 M Na₂HPO₄ were mixed and made up to 21. The pH was 7.2.

2-mercapto ethanol.

Chloroform (AR - Grade, Glaxo, India)
Waring blender - Remi Anupam mixie.

Fresh leaves and

Fresh root powder of Bougainvillea.

Method

The fresh leaves or root powder of Bougainvillea was ground in a pre-chilled blender with 10 volumes of cold PB, pH 7.2 containing 0.1% 2-mercaptoethanol. The extract was filtered through muslin cloth and centrifuged at 5000xg for 15 min. The supernatant was collected in a separating funnel. To this, equal volume of chloroform was added. Mixed and allowed to settle. The clarified aqueous and chloroform layers were collected separately. The aqueous layer was used in the subsequent steps.

From the chloroform layers obtained from the leaf and root extracts, the solvent was removed by flash evaporation at 50°C and the residues dissolved in PB, pH 7.2 and used for bioassay on cowpea plants as detailed before.

Trypsin digestion

Trypsin digestion was done following the conditions described by Wilkinson, (1986).

Materials

Leaf and root extracts of Bougainvillea - aqueous layers prepared above.

Trypsin: 1.0 mg of Trypsin (TPCK- grade, Sigma Chemical Co., USA) was dissolved in 1.0 ml of 10 mM HCl.
The leaf or root extract of Bougainvillea was frozen and lyophilized. The lyophilized powder was dissolved in Ammonium bicarbonate buffer and digested with trypsin at 37°C for 3 h. The ratio of trypsin : extract was 1:100 (w/w).

The digested leaf and root extracts were tested on cowpea plants (bioassay) for their efficacy in control of TSWV as described earlier (3.4).

**Ammonium sulphate precipitation**

**Materials**
- Leaf and root extracts (aqueous layers) of Bougainvillea
- Ammonium sulphate (AR - Grade, Glaxo, India)
- Lyophilizer
- Dialysis bag (Sigma chemical Co, USA)
- Magnetic stirrer

**Method**

To the leaf or root extract, ammonium sulphate powder was added to get 90% saturation (610 g/l) with stirring in a cold room maintained at 2-4°C. The precipitate was collected after 30 min. by centrifugation at 5000xg for 30 min. and was dissolved in PB, pH 7.2. This solution and the supernatant were dialysed separately against the PB, pH 7.2 on a
magnetic stirrer with frequent changes of the buffer for about 24-30 h. Precipitates formed during dialysis were removed by centrifugation at 10,000xg for 15 min. Portions of the two supernatants were verified for their inhibitory efficacy by bioassay as described above. Remaining supernatant were lyophilized and stored in a desiccator.

Acetone Precipitation

Materials
- Leaf and root extracts (aqueous layers) of Bougainvillea
- Chilled acetone
- Lyophilizer.

Method
To the leaf or root extract, chilled acetone was added to 40% and stirred for 30 min. The suspension was centrifuged at 5000xg for 15 min. and the resultant precipitate was lyophilized. The lyophilized powder was dissolved in PB, pH 7.2 and tested for its inhibitory efficacy on cowpea plants as described earlier.

3.8. Differential precipitation with Ammonium sulphate

The proteins present in the clarified (aqueous layer) leaf and root extracts of Bougainvillea were precipitated with different per cent saturations of Ammonium sulphate and proteins from each fraction was
tested for the inhibitory efficacy to TSWV by bioassay.

40% saturation

Materials

As given under Ammonium sulphate precipitation in 3.7.

Method

The leaf and root extracts were adjusted separately to 40% saturation by slow addition of Ammonium sulphate (243g/l) with stirring. After about 30 min. the suspensions were centrifuged and the pellets and supernatants were collected separately. The pellets were dissolved in PB, pH 7.2, dialysed and checked for their antiviral activity as described in 3.4. The supernatants collected were used for the succeeding step.

40-70% saturation

Materials

As given above

Method

To each of the above supernatants, Ammonium sulphate was added (205 g/l) with stirring to bring them to 70% saturation. After 30 min. the suspensions were centrifuged and collected the pellets and supernatants. The supernatants were used in the next step. The pellets were dissolved in PB and verified for their antiviral activity as described in 3.4.
70-90% saturation

Materials
As given above

Method
To each of the above supernatants ammonium sulphate was added to bring to 90% saturation (153 g/l). After 30 min. the suspensions were centrifuged and pellets were collected and used for bioassay as detailed in 3.4.

3.9. Purification of antiviral protein

Ammonium sulphate fractionation, ion exchange chromatographies and HPLC techniques were used to purify antiviral protein from the roots of Bougainvillea spectabilis. The details of the methods followed are given under results (4.2).

3.10. Estimation of Proteins (Bradford, 1976)

Leaf and root extracts, different ammonium sulphate saturated fractions, different fractions from CM-Sepharose chromatographies, samples of various other purification steps, extracts of different traits of Bougainvillea, extracts of different plant species already reported to contain AVPs and MAP were all estimated for their protein contents using this method.

Materials
Protein stock standard: 50 mg of bovine serum albumin was weighed accurately and dissolved in distilled water and made up to 50 ml in a standard flask.

Working standard: 10 ml of the stock solution was
diluted to 50 ml with distilled water in a standard flask. One ml of this solution contained 200µg protein.

Bradford's dye concentrate: 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 50% ethanol and to this was added 100 ml of Orthophosphoric acid. The final volume was made up to 200 ml with distilled water. Mixed and stored in the refrigerator until use.

Working dye solution: The concentrated dye was diluted one to five with distilled water, filtered and used fresh.

Beckman spectrophotometer - DU 64.

**Method**

Aliquots of sample to be estimated were taken in replicates in clean test tubes and the volume was made up to 1.0 ml with distilled water. The contents were mixed and 5.0 ml of Bradford's working dye solution was added. The contents were mixed and allowed for the colour to develop for at least 5 min. but not longer than 30 min. The absorbance was recorded at 595 nm in the spectrophotometer. Suitable blank was also conducted taking 1.0 ml distilled water in the place of the sample. The Protein standards were prepared by taking different aliquots of working standard solution ranging from 20-100 µg and developing colour with Bradford's working dye solution.

The contents of protein in the samples were estimated by comparing their absorbance values with that of the protein standards.
3.11. Estimation of sugars

Phenol-sulphuric acid method (Dubois et al., 1956)

Purified Bougainvillea antiviral protein (BAPI) obtained after CM-Sepharose Chromatography was tested for the presence of sugar moiety by this method.

Materials

BAP
5% Phenol solution
96% sulphuric acid - AR, Glaxo, India

Glucose standard: 100mg glucose (AR, Glaxo, India) was dissolved in 100ml of distilled water and mixed well. Ten ml of this was diluted to 100 ml with distilled water and used for the estimation (100 µg/ml).

Beckman spectrophotometer - DU64.

Method

One mg of purified BAPI in replicates was made up to 1.0 ml with distilled water. To this was added 1.0 ml of phenol solution and mixed. 5 ml of 96% sulphuric acid was then added to the mixture from a fast flowing pipette and agitated. The same mixing procedure was exactly practised throughout. After 10 min. the tubes were reshaken and placed in a water bath at 25-30°C for 10 min. The absorbance of the yellow-orange colour developed was measured at 490 nm. A standard curve was prepared using 10-70 µg of glucose and the colour was developed as described above.

The contents of sugars in the test samples were
estimated by comparing their absorbance values with that of standards.

3.12. Electrophoretic separation

All the chemicals used in the electrophoreses were purchased from Sigma Chemical Co., USA except HCl, acetic acid, ethanol and methanol. Marker proteins for SDS-PAGE ranging from 14.2 to 66 kDa were also purchased from Sigma chemical co., USA.

SDS-PAGE (Laemmli, 1970).

Extracts of Bougainvillea leaf, root and stalk (stem), 70-90% ammonium sulphate fractions, different fractions collected from CM- and DEAE- sepharose chromatographies, BAPI from ion exchange - HPLC, leaf extracts of different traits of Bougainvillea and pure MAP were electrophoresed by this method inorder to see the pattern of proteins, to check the purity, to know the relative molecular size of the proteins and for immuno blotting.

Materials

Acrylamide solution: 29.2 g of acrylamide and 0.8 g of bisacrylamide were dissolved in 100 ml of water, filtered and stored at 4°C in a brown bottle.

Resolving gel buffer: 18.15 g of Trizma base was dissolved in 200 ml of water and the pH adjusted to 8.8 with HCl.

Stacking gel buffer: 3 g of Trizma base was dissolved in 50 ml of water and the pH was accurately adjusted to 6.8 with HCl.
10% SDS : 10 g of SDS was dissolved in water and made upto 100 ml.

10% Ammonium persulphate : 100 mg of ammonium persulphate was dissolved in one ml of water.

TEMED (N,N,N',N'- Tetramethyl-ethylenediamine)

Sample loading buffer (2x) : 2.5 ml of stacking gel buffer, 4.0 ml of 10% SDS, 2.0 ml of glycerol and 1.0 ml of 2-mercaptoethanol were mixed. 40 mg of bromophenol blue was dissolved in this mixture and the volume was made upto 10 ml with water.

Tank buffer : 12.0 g of Trizma base and 57.6 g of glycine were dissolved in 1 litre water and 40 ml of 10% SDS was added and the volume made upto 4 l with water.

Stain solution : 200mg of Coomassie Brilliant R was dissolved in 80ml of methanol and 20ml of glacial acetic acid was added to this. The volume was then made upto 100ml with distilled water, filtered and used.

Destaining solution : 200ml of methanol and 50ml of acetic acid were mixed and the volume was made upto 500ml with distilled water.

Silver stain kit (AG-25, Sigma Chemical Co., USA)

Electrophoresis apparatus (Biotech, India)

Power pack - Hoefer - PS 500 XT

Sample preparation : The samples electrophoresed were mixed with equal volume of 2x sample loading buffer and heated in a boiling water bath for 3 to 5 minutes just before loading.

**Method**

Electrophoresis was carried out in 180 x 160 x 1.5 mm gel in a discontinuous buffer system using 14% or 17% acrylamide gels.
### Gel recipes for a 1.5 mm thick slab gels

<table>
<thead>
<tr>
<th></th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14%</td>
<td>17%</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>14.00 ml</td>
<td>17.00 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>7.50 ml</td>
<td>7.50 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.30 ml</td>
<td>0.30 ml</td>
</tr>
<tr>
<td>Water</td>
<td>8.04 ml</td>
<td>5.04 ml</td>
</tr>
<tr>
<td>Ammonium per sulphate</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
<td>0.01 ml</td>
</tr>
<tr>
<td></td>
<td>30.0 ml</td>
<td>30.0 ml</td>
</tr>
</tbody>
</table>

Separating gel was prepared as per the recipe given above. The mixture was degassed for 15-20 min. Ammonium persulphate and TEMED were added just prior to pouring the gel. This solution was poured and allowed to polymerize between two glass plates sealed at the bottom. The gel was overlaid with a film of distilled water to accelerate polymerization. After polymerization, the water layer was removed and the stacking gel was poured onto the separating gel, after placing the comb on top of the sandwich. The comb was carefully removed from the wells after polymerization of the stacking gel and rinsed with tank buffer before loading the samples. The samples were then carefully loaded into the gel wells and run at a constant current of 10 mA till the samples had completely entered the stacking gel and then at 30 mA for 4-6 h at room temperature. After the electrophoretic run, the gel was removed and
processed for Western blotting immediately or stained overnight with coomassie brilliant Blue R dye solution. The gel was then destained with destaining solution until the background of the gel was colourless and then photographed. Albumin, Bovine (66 kDa), Albumin, egg (45 kDa), Glyceraldehyde 3-PO4 dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa), Trypsinogen (24 kDa), Trypsin inhibitor (20.1 kDa) and \( \alpha \)-Lactalbumin (14.2 kDa) were used as molecular weight markers. For silver staining, the coomassie stained gel was thoroughly destained and then silver stained following the steps given in the Tech. bulletin No. P3040 of Sigma Chemical Co., USA.

**Tricine SDS-PAGE (Schagger and Von Jagow, 1987)**

Pass through fraction of DEAE-Sepharose chromatography and RP-HPLC purified BAPI were separated by Tricine gel electrophoresis.

**Materials**

Acrylamide - Bis acrylamide solution: 49.5 g of acrylamide and 3.0 g of Bisacrylamide were dissolved in 100 ml of distilled water, filtered & stored in amberlite bottle at 4°C.

Gel buffer (3.0 M Tris - HCl, pH 8.45): 36.33g of Trizma base was dissolved in 80 ml of water and pH adjusted to 8.45 with conc. HCl. To this 300 mg of SDS was added and made up the volume to 100 ml with water.

Anode buffer (0.2M Tris - HCl, pH 8.9): 12.119 of Tris base was dissolved in 450 ml of water and pH adjusted to 8.9 with conc HCl. The volume was made upto 500ml with water.
Cathode buffer (1M Tris Tricine pH 8.25 containing 0.1% SDS): 4.24 g of Trizma base, 6.27 g of tricine and 350 mg of SDS were dissolved in 300 ml of water, pH adjusted to 8.25 and volume made upto 350 ml with water.

Sample buffer : 12.1 mg of Trizma base, 17 ml of water, 2.4 ml of glycerol and 400 μl of 2-ME were mixed and pH adjusted to 6.8 with dilute HCl and volume made upto 20 ml with water. A trace amount of bromophenol blue was added.

TEMED

Stain solution }

Destaining solution }

Silver stain kit (Sigma Chemical Co., USA).

Electrophoresis apparatus

Power pack

Sample preparation : Lyophilized samples were first dissolved in distilled water. To 5 μl of the sample added 45 μl of the sample buffer & mixed.

Method

Electrophoresis was carried out in 180x160x1.5 mm gel in a discontinuous buffer system using 14% acrylamide gels.

Gel recipes for a 1.5mm thick slab gels

<table>
<thead>
<tr>
<th></th>
<th>separating gel 14%</th>
<th>stacking gel 4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide - bisacrylamide solution</td>
<td>8.00 ml</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>10.00 ml</td>
<td>3.10 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.20 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.57 ml</td>
<td>8.27 ml</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>0.20 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.03 ml</td>
<td>0.03 ml</td>
</tr>
<tr>
<td>Final volume</td>
<td>30.00 ml</td>
<td>12.50 ml</td>
</tr>
</tbody>
</table>
Separating gel and stacking gel were prepared by mixing appropriate volumes of the above solutions and were degassed for 15-20 min. prior to the addition of Ammonium persulphate and TEMED. The gel was casted as described earlier. The prepared samples were loaded on the gel as detailed earlier.

A mixture of phosphorylase b (94 kDa), Bovine serum albumin (67 kDa), Carbonic anhydrase (30 kDa), Soybean trypsin inhibitor (20.1 kDa), Ovalbumin (43 kDa) and α-Lactalbumin (14.4 kDa) were used as molecular weight markers.

The electrophoresis was performed at room temperature. The electrophoresis was started at 30V (approximately 10 mA current). When the sample had completely entered the stacking gel, the voltage was increased to 70 V. The electrophoresis took about 20 h for completion. The gel was first stained with Coomassie stain solution overnight and then destained. After the gel was photographed, it was thoroughly destained followed by silver staining using the silver stain kit, AG-25, Sigma Chemical Co., USA as described in SDS-PAGE.

3.13. Amino acid composition

Purified BAPI was analysed for its aminoacid composition.

Materials

Mobile phase :
(a) 0.15N Sodium citrate and 7% ethanol, pH 3.2
(b) 0.3N Sodium citrate and Boric acid, pH 10.0
(c) 0.2N Sodium hydroxide.

Reaction buffer: Sodium carbonate (0.384 M), Boric acid (0.210M) and potassium sulphate (0.108 M).

a) Reaction buffer 250 ml + 400 ml Sodium hypochlorite
b) Reaction buffer 250ml + 800 mg O-Phthaldialdehyde (OPA) + 3.5 ml ethanol + 1ml 2-ME and 2ml of 10% Brij solution.

Standard amino acids (PIERCE)

Sample preparation: Purified BAP\(\text{I}\) (one nanomole) from Bougainvillea root tissues was hydrolysed in a sealed tube using 6 N HCl containing 0.05 % phenol (w/v) and 0.025 % (v/v) 2-mercaptoethanol for 24 h at 110°C in vacuo. The hydrolysed sample was diluted 10 times with triple distilled water and dried in vacuo using speed vac concentrator. The dried hydrolysate was reconstituted in 100 μl of loading buffer viz., Sodium citrate buffer, pH 3.2 and used for loading.

Amino acid analyser (Shimadzu)

System controller SCL - 6B
Pump LC - 6A
Oven CTO - 6A
Peristaltic pump PRR - 2A
Data analyser CR - 4A
Fluorescence detector FLD - 6A

Method

An aliquot of 20 ul of the sample was loaded into the amino acid analyser

Post column derivatization by OPA fluorescence.

Column used: Shim - pack 1 SC - 07/S - 1504 Sodium type cation exchange.

Flow rate of mobile phase : 0.3 ml/min
Flow rate of reaction buffer : 0.3 ml/min
Column oven temperature : 55°C
Amino acid standard : 1 nmol PIERCE
Run time : 90 min.

The standard amino acid profile was obtained by
injecting one nano mole PIERCE protein amino acids. The sample chromatogram and standard chromatogram were overlaid and only the amino acid peaks of protein hydrolysate which matched with standard amino acid peaks were taken for calculation purposes. The number of residues per molecule of protein was calculated based on the molecular weight as 28,000.

3.14. Amino acid sequence analysis

Purified BAPI was used for sequencing.

Materials

RP-HPLC purified BAP
2% TFA
Sample loading device
Two part miniature column
The Hewlett Packard G1005A Protein Sequencing System.

Method

The speed vac concentrated lyophilized BAPI obtained from RP-HPLC was resuspended in 2% TFA. An aliquot of this solution was loaded onto the hydrophobic half of a two part miniature column via the sample loading device. The column was then washed with 2 ml of 2% TFA to remove any salts or buffers. The sequence analysis of the retained material was carried out by placing the column in the HP G1005A Protein Sequencer and thirty cycles of Edman sequencing chemistry was performed. The results were recorded.
3.15. High Pressure liquid chromatography

Analysis were carried out in Shimadzu HPLC system. All the solvents used were filtered through 0.45μ filter and degassed before use.

Resolution using cation exchange column

Different ammonium sulphate fractions, active fractions from CM-Sepharose Chromatography I, root extracts of Bougainvillea and *Mirabilis jalapa* were analysed using cation exchange column following the method described by Takanami *et al.* (1990).

**Materials**

Cation exchange column (TSK gel SP -5 PW, TOSO)

Shimadzu HPLC system with

- System Controller: SCL - 6B
- Pumps: A and B
- Oven: CTO - 6A
- Data analyser: CR - 24A
- UV detector: SPD - 6AV

Mobile phase:

Solvent A (10 mM Sodium phosphate buffer, pH 8.0): 94.7ml of 0.2M Na₂HPO₄ and 5.3ml of 0.2M NaH₂PO₄ were mixed and made up to 2.0 l with distilled water.

Solvent B: 10 mM Sodium phosphate buffer, pH 8.0 containing 0.18M Sodium sulphate.

Samples:

a) The root extracts of Bougainvillea and *Mirabilis* were mixed with solvent A. Filtered through 0.45μ disposable filter and 25μl of the filtrate was injected into the HPLC system.

b) The lyophilized powder of proteins pelleted from the leaf extracts of Bougainvillea by 40%, 40-70%, 70-90%
ammonium sulphate saturations were dissolved and filtered as described above and injected.

c) Different fractions (lyophilized powder) of the CM-Sepharose chromatography of Bougainvillea root proteins were treated as above and injected into the HPLC system.

Method

The column was washed thoroughly and equilibrated with solvent A. Then sample was injected and gradient run started. The anti-plant viral protein (BAP) got adsorbed onto a cation exchange column and was eluted with a linear gradient (0-0.18M) of sodium sulphate in 10mM phosphate buffer, pH 8.0. The flow rate was 1 ml / min. The time taken per analysis was 20 minutes. The peaks were detected by ultraviolet absorption at 230 nm. After the run, the column was washed thoroughly and reequilibrated before injecting the next sample.

Resolution by RP-HPLC

BAPI, MAP, reduced and alkylated BAPI and MAP were analysed by this method.

Materials

Aquapore butyl (C4) column

Mobile phase:
Solvent A - 0.1% Trifluoro acetic acid (TFA)-HPLC grade
Solvent B - 90% acetonitrile (HPLC grade): 0.1% TFA.

Samples :

a) The pooled active fractions (lyophilized powder) of BAPI collected from each of the two CM - sepharose chromatographies were dissolved individually in
0.1% TFA in distilled water, filtered and used.

b) The lyophilized powder of the pooled pass through fractions of BAPI obtained from DEAE-sepharose chromatography was dissolved and used as detailed above.

c) BAPI and MAP before and after reduction and alkylation were dissolved and injected as described above.

**Method**

The column was first thoroughly washed and then equilibrated with solvent A. 100μl sample was injected into the system. The adsorbed proteins were eluted with a linear gradient of acetonitrile: TFA: water. The flow rate was 0.7 ml/min. The peaks were detected by ultraviolet absorption at 220 nm. A linear gradient was given for a period of 25 min. after a wash for 10 min. For reduced and alkylated samples longer gradients i.e up to 65 min. were given. Fractions of elutions were collected manually and dried by Speed vac concentrator. The column was washed and re-equilibrated before injection of the next sample.

**Peptide mapping.**

Pure BAPI and MAP were reduced, alkylated, digested with trypsin and the resulting peptides separated by RP-HPLC. All the chemicals used were of Sigma grade.

**Reduction and alkylation**

**Materials**

Tris-EDTA-Guanidine hydrochloride buffer: 0.3 M Tris-HCl buffer, pH 8.3 containing 6 M Guanidine
hydrochloride and 5 mM EDTA.

Dithiothreitol
Nitrogen gas
Water bath
4 - Vinylpyridine

Sample : Purified BAPI and MAP

Method

Lyophilized protein was dissolved in Tris-EDTA-Gn-HCl buffer at the rate of 2 mg/ml buffer in a screw capped tube. The tube was gently flushed with nitrogen gas for 30 min. The solution was incubated for 30 min. to denature the protein completely. The sample was cooled, flushed with nitrogen for 15 min. DTT(1.5 mg) was added (50 fold molar excess over the cystine residues considering as five residues in the protein) and the tube was flushed with nitrogen. The tube was then capped and incubated at 37°C for 4 h to convert cystine to cysteine residues. Alkylation of the cysteine residues were initiated by the addition of 3 mole of 4-vinylpyridine/mole DTT added. The samples were incubated at 37°C for 1 to 2 h. The reduced sample was injected into RP-HPLC column as described under Resolution by RP-HPLC and collected pure reduced proteins. The collected fractions were concentrated in a speed vac.
Tryptic digestion [Wilkinson, 1986]

**Materials**

100 mM Ammonium bicarbonate buffer, pH 8.1

Tryptsin : Dissolved 1.0 mg Tryptsin (TPCK grade) in 1 ml of 10 mM HCl.

Sample : reduced and alkylated BAPI and MAP.

**Method**

The reduced and alkylated BAPI or MAP was suspended in 100 mM Ammonium bicarbonate buffer. This was then incubated with Tryptsin at 37 °C for 1-2 h. The trypsin : protein ratio was 1:100 (W/W). At the end of the digestion, the peptide mixture was centrifuged and soluble and insoluble fractions were separated.

**Peptide separation**

Peptides of BAPI and MAP were separated using C_{18} column in HPLC following the method given by Gullick (1986) with modifications as described below.

**Materials**

ODS (C18) column – HYPERSL 3µ

Solvent A : 25 mM Ammonium acetate

Solvent B : 40% Acetonitrile in 25 mM Ammonium acetate

Sample : soluble fractions of the tryptic digested BAPI and MAP

**Method**

The column was equilibrated with solvent A. Soluble fraction of the tryptic digest was injected into the HPLC system. The bound peptides were eluted with a
linear gradient of acetonitrile (Solvent B). The gradient was run for a period of 30 minutes. The flow rate was 1.0 ml/min. The eluates were monitored at 215 nm. The column was washed and reequilibrated with solvent A before injection of next sample.

3.16 Determination of pI value

pI value of BAP and MAP were determined by isoelectric focusing (IEF). The experiment was carried out using Phast system of Pharmacia. Purified BAPI, MAP, BAPI and BAP II from CM-Sepharose chromatography I were analysed in this technique.

Materials

Phast Gel IEF 3-9

pI calibration kit-Broad pI covering 3.5 - 9.3 containing eleven calibration markers.

Trypsinogen, pI marker (pI 9.3) obtained from Sigma Chemical Co., USA.

20% TCA : 20g of TCA was dissolved in distilled water and made upto 100 ml.

Stain - stock solution : Dissolved 1 Phast Gel Blue R tablet in 80 ml of distilled water and added to this 120 ml of methanol, mixed well and filtered.

Stain - working solution : one part of Stain -stock solution was mixed with 9 parts of the destain solution. Added CuSO₄ to this mixture to 0.1% (w/v). Prepared fresh before use.

Destain / Wash : 30% methanol and 10% acetic acid in distilled water.

Pharmacia - Phast System Separation and Control Units.

Pharmacia - Phast System Development Unit.
Lyophilized powders of purified BAPI, MAP and BAPI and BAP-II from CM-sepharose chromatography I were separately dissolved in 10 mM PB, pH 7.0 & used.

Method

The methods described in the 'Users Manual' of Pharmacia supplied along with the instrument was exactly followed for setting the instrument and for running, staining and destaining of the gel. The method essentially involved focusing the proteins on the gel, staining and destaining.

Gel focusing was done in three steps viz., a pre-focusing step, a sample application step and a focusing step.

The following optimized step for IEF with Phast Gel IEF 3-9 was used to program into the separation method file of the instrument system. This is exactly as given in Phast system Separation Technique File No. 100 of Pharmacia.

<table>
<thead>
<tr>
<th>SAMPLE APPL.</th>
<th>DOWN AT</th>
<th>1.2</th>
<th>0 Vh</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMPLE APPL.</td>
<td>UP AT</td>
<td>1.3</td>
<td>0 Vh</td>
</tr>
<tr>
<td>EXTRA ALARM</td>
<td>TO SOUND</td>
<td>1.1</td>
<td>73 Vh</td>
</tr>
<tr>
<td>SEP 1.1</td>
<td>2000V</td>
<td>2.5mA</td>
<td>3.5W</td>
</tr>
<tr>
<td>SEP 1.2</td>
<td>200V</td>
<td>2.5mA</td>
<td>3.5W</td>
</tr>
<tr>
<td>SEP 1.3</td>
<td>2000V</td>
<td>2.5mA</td>
<td>3.5W</td>
</tr>
</tbody>
</table>

Pre-focusing took approximately 10 min. Then 100 ng of each of the samples, pI marker mix and Trypsinogen (pI marker) were applied on to the pre-focused gel. After
the run the gel was stained and destained following exactly the method given in Development Technique File No. 200 for Coomassie staining of Phast Gel IEF 3-9 of Pharmacia. The gels were then photographed.

3.17. Absorption maxima

The absorption maxima of BAPI and MAP were measured.

Materials
Deionized water
Sample: Lyophilized powders (0.4 mg/ml) of BAPI and MAP were dissolved in deionized water and used.
Quartz cuvette.
Beckman Spectrophotometer - DU 600.

Method
BAPI and MAP samples were taken separately in a clean Quartz cuvette and scanned the absorption from 250-300nm in a Beckman Spectrophotometer. Deionized water was used as blank. The absorptions were recorded.

3.18. Molecular Weight

Materials
Lyophilized RP-HPLC purified BAPI
2% TFA

Mass Spectrometer - VG ToFSpec instrument.

Method
Lyophilized sample was resuspended in 2% TFA. An aliquot of this solution was spotted onto a stainless steel target pre-coated with a-cyano-4-hydroxy
cinnamic acid and analysed by matrix assisted laser desorption ionisation (MALDI) Mass spectrometry using a VG ToFSpec instrument fitted with a 337 nm nitrogen laser.

3.19. Mechanism of action of BAP

The mechanism of action of BAPI was analysed by studying its inhibitory effect on in vitro protein translation using Rabbit reticulocyte lysate system.

in vitro Protein translation

The method described in the Technical Manual of Promega Corporation, USA supplied along with the Rabbit Reticulocyte Lysate system was followed exactly. To check the ribosome inactivating property, BAPI was added to the reaction contents of in vitro protein translation containing Luciferase mRNA and the inhibition of translation verified with the (+)ve control reaction where no BAP was added.

Materials

BAPI

Rabbit reticulocyte lysate, nuclease treated, Promega Corporation, USA.

Amino acid mixture minus Methionine

Luciferase control RNA

\((^{35}\text{S})\)-methionine

RNasin Ribonuclease inhibitor

Nuclease free water
Method

The total reaction mixture was 50 μl. A positive control with Luciferase RNA and a negative control without Luciferase RNA were conducted simultaneously along with the experiment.

Luciferase mRNA was incubated at 67°C for 10 min and immediately cooled on ice.

The positive control reaction mixture was as follows.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit reticulocyte lysate</td>
<td>35 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7 μl</td>
</tr>
<tr>
<td>RNasin (40 u/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>lMM amino acid mixture (minus methionine)</td>
<td>1 μl</td>
</tr>
<tr>
<td>(35S)-methionine (10 m Ci/ml)</td>
<td>4 μl</td>
</tr>
<tr>
<td>Luciferase control RNA</td>
<td>2 μl</td>
</tr>
</tbody>
</table>

Total 50 μl

Contents were mixed well and incubated at 30°C for 2 h.

The experimental reaction mixture contained instead of nuclease free water, 7 μl of BAPI dissolved in nuclease free water.

A negative control was also set up in which there was no RNA added.

Estimation of TCA precipitable counts

The success of the translation reaction was tested by measuring TCA precipitable counts in the reaction. Also it was useful to know the activity of each reaction so that equal counts could be loaded on the gel run subsequently.
Materials

Translated reaction mixture from the above step.

1N NaOH / 2% H$_2$O$_2$

Water bath

25% TCA /2% casamino acids

Whatman GF/C filters

5% TCA

Acetone

Scintillation counter-Backman LS 6000 SC, USA.

Scintillation fluid

Method

After completion of the translation reaction, the contents were vortexed gently and 2 µl of the reaction mixture in each was removed and added to 98 µl of 1 M NaOH/2% H$_2$O$_2$. This was vortexed briefly and incubated at 37°C for 10 min. 900 µl of ice cold 25% TCA/2% Casamino acid was added and incubated on ice for 30 min. to precipitate the translation products.

A glass fibre filter was wet in cold 5% TCA and the precipitate was collected by vacuum filtration. The filter was rinsed 3 times in ice cold 5% TCA and once in acetone. The filter was air dried and put in a scintillation vial containing 2 ml scintillation fluid. Vials were counted in a scintillation counter. To determine total counts present in the reaction mixture, 2 µl aliquot was spotted on a glass fibre filter and counted without TCA precipitation and washing steps.
Gel analysis of translation products

The translation products were analysed on a 12% SDS-PAGE following Laemmli's (1970) method.

Materials
SDS-PAGE gels
Dimethyl sulphoxide (DMSO)
Phenyl Phenyl oxazole (PPO)
Gel drying apparatus
Autoradiography cassette with intensifying screen
XAR-2 film

Method
Aliquots of the translated samples of (+)ve control, (+)ve control with BAPI and (-)ve control containing equal counts of radioactivity were loaded in each lane of 12% SDS-PAGE gels and electrophoresed as described earlier. The gel was stained, destained and photographed to localize the marker bands.

The gel was treated with 5 volume of DMSO for 1 h. Then the used DMSO was removed and fresh DMSO added. After 30 min., the DMSO was removed and 22% PPO in DMSO added to the gel and incubated for 1 h. PPO solution was then removed and gel washed in slow running water for 30 min, when the gel turned white due to precipitation of PPO. All PPO was removed by thorough washing in distilled water. Gel was then dried in a LKB vacuum drier as per the instructions of the instrument manufacturer.
The dried gel was placed in an autoradiography cassette. XAR-5 film was placed on the gel, the cassette was closed and kept at -70°C for 2 days. Fluorograph was developed as per manufacturer's instructions and translation products visualised.

3.20. Production of antibody

Anti sera was produced by injecting BAPI into rabbits

Materials

Male rabbit (new zealand white) of 3 months old weighing 3.5 kg

Purified BAPI

Sterile injection needle (18 gauge) with glass syringes

Alcohol

Sterile absorbent cotton

Freund's incomplete adjuvent

Phosphate buffered saline (PBS), 0.1 M, pH 7.4.

Method

One mg of purified BAP I from Bougainvillea root tissues was dissolved in 1 ml of 0.1 M PBS. This was mixed with equal quantity of Freund's incomplete adjuvent and vortexed until a thick emulsion was developed which did not disperse when a drop of it was placed on the surface of a saline solution. Transferred the emulsion to a sterile syringe and removed all the air bubbles. 18 gauge needle was fitted to the syringe.

Injected 1.0 ml of the emulsion into the thigh muscle
of the rear leg of the rabbit taking precautions as described by Harlow and Lane (1988). Three weeks later a booster dose was given similarly at the back. And a week after, a second booster was given at the other side of the back as before. 10 days after this the animal was bled from the marginal ear vein and the serum prepared. Pre-immune blood was collected from the animal before injection of the antigen.

**Preparation of the serum**

The blood samples collected (before and after immunizations) were allowed to clot at 37°C for 1 h. The clot was separated from the walls of the containers by ringing with a sterile needle. The tubes were kept at 4°C overnight for the clot to contract. Next day the clear straw coloured sera were collected by centrifugation at 5,000 rpm for 30 min. at 4°C. The sera were labelled as 'pre-immune serum' and 'anti-BAPI serum' and stored with 0.02% sodium azide at -20°C in aliquots until further use.

**Specificity of the antiserum** (Ouchterlony double diffusion technique, 1978)

The specificity of anti-BAPI serum produced was checked by this method. This technique is widely used to assess the quality and cross reactivity of antigens and antibodies. It is based on the ability of antibodies to form precipitin
lines with the antigen in a layer of agar spread on a glass plate under humid conditions.

Materials

Agarose

Phosphate buffered saline (PBS) 0.1M, pH 7.2:
0.1 M Na₂HPO₄, 71.5 ml Mixed and the pH
0.1 M NaH₂PO₄, 28.5 ml adjusted to 7.2
0.85% NaCl⁺ (850mg/100 ml) 900.0 ml

BAPI and BAP II

Anti-BAPI serum and pre-immune serum

Method

One gram of agarose was dissolved by boiling in 100 ml of phosphate buffered saline. The solution was cooled in a water bath to 50°C. Glass plates were precoated in agar solution inorder to ensure a good contact between the gel and glassplate and were air dried. The plates were placed on a levelled table to obtain gels of uniform thickness and the gel solution was poured without any air bubbles. The thickness of the gel was approximately 1.5 mm. After solidification, wells were punched out as needed. The centre well was filled with 1:500 dilution of anti-BAPI serum. Other wells were loaded with purified BAPI solution. Control was maintained by substituting anti-BAPI serum with pre-immune serum. Also BAP II was separately checked with Anti-BAPI serum. The gels were incubated at room temperature for 24h in a humid chamber for the reaction to take place and the plates were photographed.
3.21. Cross reactivity of anti-BAP I serum with other AVPs

Preparation of the extract

The leaves of seven different plant species viz., *Vinca rosea* L., *Solanum elaeagnifolium* Clarke., *Besella rubra* L., *Boerhaavia diffusa* L., *Chenopodium amaranticolor* Coste and Reyn., *Sorghum vulgare* Pers. and *Mirabilis jalapa* L. which were already reported to contain anti-plant viral principle were extracted with prechilled 10 mM PB, pH 7.2 containing 0.1% ME. A 5% extract was prepared using a mortar and pestle in a cold room and centrifuged at 5000 x g for 15 min. and the supernatants collected. The concentration of proteins in these extracts were estimated using Bradford's method as described earlier.

Cross reactivity

Extracts containing 30 µg protein of each of the plant species along with *Bougainvillea* extract (used for BAP purification) and pure MAP (few µg) were tested for their cross reactivity with the anti-BAPI serum by Ouchterlony double-diffusion method as described above. After 24 h the plates were photographed.

3.22. Titer of the anti serum produced

The titer of the anti-BAPI serum was assayed by antigen excess antibody capture ELISA technique in microtiter plates (Harlow and Lane, 1988).
Materials

Purified Bougainvillea antiviral protein (BAPI)
Anti - BAPI serum
Pre-immune serum
Phosphate buffered saline (PBS), 0.1M, pH 7.2.
PBS containing 0.05% Tween 20 (PBST)
3.0% BSA in PBS (PBS-BSA) with 0.02% NaN₃
Monoclonal anti-Rabbit IgG (r-chain specific) alkaline phosphatase conjugate, 1:25,000 dilution.
p-Nitrophenyl phosphate (p-NPP substrate tablet set Sigma FAST) : one tablet dissolved in 20 ml water.
Microtiter plates.

Method

50 µl of the diluted antigen containing 1 µg BAPI was added to each of the wells of the microtiter plate.
The plates were covered and incubated in a humid atmosphere for 2 h at room temperature overnight.
The wells were emptied by flicking the plate over a waste container. The wells were then filled with PBS and allowed to stand for 5 min.
The wells were emptied and repeated the washing steps twice more.
All the wells were filled to the brim with 3.0% BSA in PBS with 0.02% sodium azide (Blocking solution) and left for 2 h or more in humid atmosphere to block the protein binding sites.
The wells were emptied and washed the plates again with PBS three times as described above.
The test sera (pre-immune and anti-BAPI sera) were serially diluted 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, 1:56200 and 1:112400 with fresh blocking solution.

50 µl of the serum was added to each of the wells starting from the 3rd well. To the first two wells 50 µl of the blocking solution alone was added for blank reactions.

The same procedure was repeated with pre-immune (control) serum.

The ELISA plates were then covered and incubated at room temperature for 2 h in humid atmosphere.

The plates were washed with PBST for 5 min, thrice to remove the unbound antibody.

50 µl of the diluted alkaline phosphatase conjugate was added to each well. The plates were covered and incubated at room temperature for 2 h.

After this all the wells were washed three times with PBST as before.

50 µl of p-nitrophenyl phosphate (substrate) was added to each well and incubated at room temperature for 30 min. during which time yellow colour was developed.

After 30 min., 50 µl of 3N NaOH was added to each well to arrest the reaction.

The intensity of the colour developed was measured by reading the absorbance at 405 nm in an ELISA reader.
3.23. BAP I in different purification steps

**Materials**

As given under 'Titer of the antiserum produced'

Samples: From different purification steps of BAP viz., root extract, 70-90% ammonium sulphate fraction, pooled active fraction of each of the two CM-Sepharose chromatographies and RP-HPLC purified BAPI.

**Method**

Samples containing 1 μg protein from each of the above mentioned were taken in the ELISA plate and tested for the presence and quantity of BAPI in the same way as described under section 3.22 with diluted anti serum (1:10,000).

3.24. BAP I in different traits and plant parts of Bougainvillea

Leaves (of six different flower colour traits) and different plant parts (of the trait used for purification) of *B. spectabilis* were taken for this study.

Two different techniques viz., SDS-PAGE and Immunoblotting were used to detect the presence of BAPI in these samples.

**SDS-PAGE**

The aqueous layer of each of the extracts of samples mentioned above containing 50 μg protein along with 5 μg of purified BAPI were electrophoresed on 14% SDS-PAGE (Laemmli, 1970) as described before.
Immunoblotting (Western)

This combines the resolution of gel electrophoresis with specificity of immunochemical reaction. Leaf extracts of different flower colour traits viz., dark purple, orange, white and dark pink (used for purification), extract of the stalk and root tissues of the trait used for purification, BAP II (protein eluted as the minor peak in CM-sepharose chromatography) and purified BAPI were used. The method followed is described below, which includes resolution of proteins on SDS-PAGE, transfer of proteins to Nitrocellulose paper, cross reactivity of proteins with anti-BAPI serum and detection of antigen-antibody complexes.

Electrophoretic separation of proteins

Aqueous extracts containing 25 μg protein of the above mentioned samples were resolved on SDS-PAGE (Laemmli, 1970) in replicates as detailed earlier.

Electrophoretic Transfer of proteins

After the electrophoresis, the proteins were transferred to nitrocellulose membranes from the gel essentially as described by Towbin et al. (1979).

Materials

Transfer buffer (Towbin buffer): Tris-glycine, pH 8.3 containing 20% methanol.

PBS, 0.1 M, pH 7.2

Wash buffer: PBS with 0.3% Tween 20.
Blocking buffer 1.0% BSA in PBS

Multi blot electrophoretic transfer unit: LKB - 205/MIDGET

3 MM Whatman filter papers

Nitrocellulose paper (NCP)

Scotch bright pads

Method

The gel, after electrophoresis was equilibrated with transfer buffer for five min. in a trough. The scotch bright pads and filter papers were also equilibrated in the transfer buffer for some time. A piece of NCP was cut to the size of the gel and was also made wet and then immersed in the buffer. On one lid of the transfer cassette, first the wet scotch bright pad was kept on which the 3 bits of filter papers were layered. The gel was then carefully kept on the filter paper. NCP was laid carefully on the gel without entrapping air bubbles between the gel and NCP. A corner of the NCP was marked to know the orientation of the gel. Another set of filter papers were kept on the NCP and the second scotch bright pad layered on the filter paper. The cassette was closed with another lid. and placed in the transfer tank filled with Towbin buffer. The sandwich was electroblotted such that the gel was towards the cathode end and NCP near the anode. The blotting was carried out over night at 20 mA in the cold room.
Visualization of proteins by Ponceau S staining

The blots were stained with Ponceau S (Harlow and Lane, 1988) to see the quality of electrophoretic separation as well as the efficiency of transfer before proceeding further.

Materials

- Ponceau S concentrate (10 x): 2% Ponceau S in 30% TCA and 30% sulphasalicylic acid.
- Tris buffered saline (TBS): 10 mM Tris-HCl, 150 mM NaCl, pH 8.0.

Method

The membrane was washed briefly with TBS to remove any traces of gel etc. and placed in a tray. One ml of Ponceau S concentrate was diluted to 10 ml with TBS and poured on to the membrane. After about 30-60 secs, the dye was removed and the NCP was washed with TBS briefly. Purple bands appeared with the background started clearing. The BAPI band was marked quickly with a pencil.

Immunoadsorption

NCP with the transferred proteins were allowed to react with the diluted antisera. The antigen-antibody complexes bound to NCP were reacted with alkaline phosphatase conjugated anti-rabbit IgG. The colour was then developed by reaction with BCIP/NBT substrate to identify the presence
of BAPI in the different samples analysed.

Materials

TBS

TBST : TBS with 0.02% Tween 20

Blocking buffer : 2% BSA in TBS containing 0.02% NaN₃

Primary antibody : a) 1:1000 dilution of the anti BAPI serum in fresh blocking solution b) 1:1000 dilution of pre-immune serum.

Secondary antibody : 1:25,000 dilution of alkaline phosphatase conjugated anti-rabbit IgG in blocking solution.

AP buffer : 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5.

AP substrate : 1 tablet of Sigma Fast BCIP/NBT buffered substrate was dissolved in 10 ml of buffer.

EDTA, 20 mM in TBS.

Method

The NCP was washed briefly with TBS and incubated with agitation in the blocking solution for an hour at room temperature. It was washed thrice in TBST for an hour with constant shaking. The NCP was then incubated with anti-BAPI serum at 37°C for 1-2 h with constant shaking and washed with wash buffer for one h with four changes. NCP was then incubated with alkaline phosphatase conjugated secondary antibody for one hour with constant shaking at 37°C and washed with wash buffer for one h with four changes. The NCP was equilibrated in AP buffer for 10 min. and blotted damp dry on a Whatman 3 MM filter paper. Immediately the NCP was transferred into 10 ml of BCIP/NBT substrate.
solution. Purple colour bands developed in reactive areas between 10 and 15 min. The NCP was washed thoroughly with water and the reaction arrested with 20 mM EDTA when there was an optimum colour development. The blot was photographed. The replica was treated in the same way except pre-immune serum was used in the place of anti-BAPI serum.

3.25. Comparison with other AVPs

The different characteristics of BAP were compared with already well characterized AVPs viz., Mirabilis (MAP) and Phytolacca (PAPs) antiviral proteins.

Comparison with MAP

Lyophilized powder of MAP received from Japan and the lyophilized powder of pure BAPI were used in the following experiments.

The inhibitory effects to TSWV of BAPI and MAP were simultaneously checked by bioassay on cowpea plants as described earlier. Necessary controls and replications were maintained and the results recorded.

BAPI and MAP were separately dissolved in deionized water and their absorption measured between 250 and 300 nm in a Beckman DU-600 Spectrophotometer and recorded.

2 µg each of BAPI and MAP were dissolved in sample buffer and loaded on 14 % SDS-PAGE gel and electrophoresed as described earlier. The gels were stained with Coomassie
stain solution as described earlier. The relative mobilities of BAP I and MAP were compared with that of the molecular weight markers loaded simultaneously and the molecular sizes calculated.

BAP I and MAP were dissolved in 0.1 % TFA and injected into HPLC-system fitted with Aquapore butyl column (C4) and eluted with a gradient of acetonitrile as described earlier. The pattern of elution of BAPI and MAP were recorded.

BAPI and MAP were reduced with DTT and alkylated with 4-Vinylpyridine as described previously and equal quantities injected into HPLC-system fitted with Aquapore butyl column. Elution was done with a gradient of acetonitrile as described before and elution profile recorded.

The reduced and alkylated BAP I and MAP were digested with trypsin and the digested fragments were separated and eluted by injecting into HPLC-system fitted with C-18 column and eluted with a gradient of acetonitrile as described previously.

One µl containing 100 ng each of BAP I and MAP in 10 mM PB, pH 7 was loaded onto prefocused Phast gel (IEF 3-9) and electrophoresed in the Phast system of Pharmacia following the procedure supplied (Separation technique file No.100) with the instrument. After the run which took about 30 min. the gel was stained with Phast Gel Blue R and destained exactly as described in the 'Development technique file No.200' - Fast coomasie staining of Phast system, Pharmacia. The gel was then photographed.
MAP was checked for its cross reactivity with anti-BAPI serum as described under section 3.21.

*Mirabilis jalapa* roots were collected from TNAU-campus Coimbatore, India and extracts prepared using 10 mM PB, pH 7.2 as described for Bougainvillea roots. The profile of elution of the root extracts of Bougainvillea and *Mirabilis* were analysed by injecting into HPLC-system fitted with cation exchange column as described before and eluted with linear gradient of 0.18 M Na₂SO₄. The chromatograms were recorded.

**Data Comparison with MAP and PAPs**

**Sequence homology**

A homology search for the sequence of the first 30 aminoacids at the N-terminal end of BAPI was made against all the anti viral proteins. The SWISS-PROT database (Release 30, September 1994) was searched to retrieve anti viral proteins.

**Homology in aminoacid composition**

The aminoacid composition of BAPI was compared with that of the publisled data of MAP (Takanami et al., 1990) and PAPs (Kung et al., 1990) to know the homology between these functionally similar proteins.