Collection and maintenance of virus isolates

Watermelon plants showing mottling and yellowing of leaves and stunting of vines symptoms were collected from the experimental field on May 2008 at IARI, New Delhi. The viruses from these samples were sap transmitted to *Nicotiana benthamiana*. On the basis of symptomatology, EM observations, ELISA using polyclonal antiserum to GBNV and WSMoV and RT-PCR using the N gene based conserved primers to *Tospoviruses* (Jain et al., 1998), the virus was designated as Watermelon bud necrosis virus (WBNV). The cultures of the isolates were maintained by regular sap inoculation to *Nicotiana benthamiana* seedlings in a greenhouse.

Electron microscopy

The leaf dip procedure (Griffin et al., 1990) was used for examining the presence of virus particle in leaf samples in a transmission electron microscope (JEOL JEM-1011) at the Advanced Center for Plant Virology, IARI, New Delhi. The carbon coated copper grids (3.5 mm diameter, 400 mesh) were prepared by layering a thin film of carbon in a vacuum evaporator (JEOL.JEE-420). The samples for electron microscopy were prepared by grinding about 5 mm$^2$ of infected leaf pieces in 3 drops of phosphate buffer (0.07M, pH 6.5) with the help of a glass rod on a glass slide. The leaf extracts were placed on the carbon-coated grids for 2-3 min and then the grids were washed with 5-6 drops of distilled water and stained with 2-3 drops of 2% uranyl acetate (pH 4.2). The excess stain was removed by blotting paper and the grids were air dried for 1-2 min before examination in the electron microscope (EM). Digital images were recorded by Gatan DV 300WI CCD camera and processing of digital image data was done using Digital Micrograph-3.4 program.

Confirmation of WBNV infection in samples by ELISA

Once received, samples were assayed by direct antigen coated indirect- enzyme linked immuno-sorbert assay (DAC-ELISA) to detect WBNV using specific antisera to GBNV/WSMoV (Clark and Bar Joseph, 1984). The antisera were obtained from the Plant Virology Unit (PVU), Indian Agricultural Research Institute, New Delhi 110012. The recipe of various buffers used for ELISA is given in Appendix. WBNV is predominantly phloem limited virus. Thus, plant parts containing phloem tissues are essential sample consider for molecular diagnosis. Therefore, the barks, petioles, veins and veinlets were taken to extract
Materials and methods

virus for ELISA test. The assay was performed in 96 well polystyrene microtitre plates (Costar, Sigma, USA). The protocol is as follows:

1. 200μl test/control extracted/ diluted from symptomatic as well as healthy citrus samples (midrib or bark) in coating buffer containing 2% polyvinyl pyrollidone (PVP, MW 40,000) (1:1 dilution, w/v) was added to each well of the microtitre plate. The plate was covered and incubates at 37°C for ½-1 h.
2. The plate was washed by flooding the wells with PBS-T for about 3 min. Washing and soaking operations were repeated thrice, then empty plate soak out residual liquid was drained on a paper towel.
3. 200μl blocking solution (heterologous protein) was added to each well and incubated at 37°C for 1h to block polystyrene well reactive surfaces. The plate was washed three times as above.
4. Then 200μl crude antiserum (primary antibody) diluted (1:4000) in PBS-TPO was added to each well and incubated at 37°C for 2h or overnight at 4°C. The plate was washed three times as above.
5. 200μl (alkaline phosphatase, ALP) antirabbit IgG (secondary antibody) diluted (1:30000) in PBS-TPO was added to each well and incubated at 37°C for 2h or overnight at 4°C. Plates were washed three times as above.
6. 200μl freshly prepared substrate (para-nitro phenyl phosphate, PNPP, 0.5–1 mg/ml) was added to each well and incubated at room temperature for 1-2 h or as long as necessary to observe reaction.
7. Enzyme-mediated colour reaction in each well was assessed by measuring absorbance at 405 nm by using a Tecan Sunrise (version 1.2) ELISA reader.
8. Samples are recorded as infected or positive whose OD values exceed the healthy sample value by 2-3 times.

Mechanical inoculation

Test plants were raised from seeds in pots under environmental control greenhouse maintaining temperature at 28°C -30°C. Young plants or cotyledonary leaf (only for cowpea) were sap inoculated with WBNV inoculum. Inoculum was prepared growing at the rate of 1.0 g of symptomic leaf tissues of *Nicotiana benthamiana* in 1.0 ml phosphate buffer, pH 7.0 containing 0.01M β-merceptoethanol and 0.2% Na₂SO₃. The inoculums was maintained on nice and applied with cotton swab on leaf predusted with carborandom.
Materials and methods

The inoculated plants were allowed in the greenhouse for symptoms development and observations were taken at 6-10 dpi. The *Nicotiana benthamiana* plants showing symptoms at 8 days post inoculation (dpi) with WBNV were used as source of infection.

**Host range**

The host range determined by sap inoculation of different plant species belonging to Chenopodiaceae, Cucurbitaceae and Solanaceae. Newly expanded true leaves of young plants were inoculated. For each plant species, five test plants were used for inoculation study. After inoculation, the plants were maintained in insect-proof chamber in the greenhouse. The host range experiment was repeated twice to see the reproducibility of the results. At 10 dpi, the leaf samples were collected and infection was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR).

**Immunosorbent electron microscopy (ISEM)**

Immunosorbent electron microscopy was conducted following Milne and Lesemann (1984). Antibody coated grids were prepared by floating the carbon coated grids for 30 min at 37 °C on a 10 μl of antisera to GBNV/WSMoV obtained from IARI, New Delhi, diluted at 1:500 dilution in phosphate buffer. The grids were washed with phosphate buffer and antibody coated grids were placed on the 10 μl drops of purified virus and allowed for 45 min at room temperature. The trapped virus was decorated with respective antibody by placing the grids again on the specific antibodies (1: 100 dilutions) for 30 min at room temperature. The grids were washed with double distilled water, stained with 2% UA and examined in transmission electron microscope.

**RNA isolation**

From infected leaf tissues, RNA was isolated by using RNeasy Plant Mini kit (QIAGEN, Maryland, USA). Approximately, 100 mg of infected leaf tissues were crushed in liquid nitrogen with the help of mortar and pestle. The powdered tissues were mixed with 450 μl RLT buffer containing beta-mercaptoethanol (10 μl/ml of RLT buffer). After vigorous vortexing, the lysate was passed through QIAshredder spin column by centrifugation for 2 min at 8000 g. The supernatant of the flow-through fraction was transferred to a new tube without disturbing the cell debris pellet. To the supernatant, 0.5 volumes ethanol was added and after mixing, it was passed through RNeasy mini column by centrifugation at 8000 g for 15 sec. The flow-through was discarded and the RNeasy column was washed with 700 μl
RW1 buffer followed by two washes with 500 µl of RPE buffer. RNA was eluted from RNeasy column with 30 µl RNase free water by centrifugation for 1 min at 8000 rpm.

### Primer designing

For the amplification of M RNA, seven pairs of overlapping primers were synthesized based on conserved nucleotide sequence in M RNA of GBNV (AY871097 and U42555) and WSMoV (DQ157768, U75379 and NC_003841) available in the GenBank were aligned using BioEdit software (version 5.0.9) and CLUSTALW (1.7) program. The primer sequence and their locations corresponding to viral M RNA are listed in Table 3.1 and Fig. 3.1.

![Fig.3.1. Schematic presentation of the strategy of M-RNA genome amplification. The number indicates nucleotide position on the genome of *Groundnut bud necrosis virus* and *Watermelon silver mottle virus.*](image)

### Reverse transcription reaction

All the complementary DNA (cDNA) of all segments of WBNV-wDel was synthesized by using an Im Prom-II™ revese Transcriptase kit (Promega Madison, WI, USA). Ten microliter of template RNA (~250 ng/µl) and 1 µl (200 ng) of reverse primer, was heated for 5 min at 65 °C and quickly chilled on ice for five minutes. After a brief centrifugation, 1 µl of 10mM dNTP and 2.5 µl of autoclaved water (HPLC grade), 4 µl of 5x buffer, 0.5 µl of RNase inhibitor (40U/µl, Promega Madison, WI, USA) and 1 µl reverse transcriptase was added and mixed gently by pipetting up and down. Finally, the mixture was incubated at 25°C for 5 min followed by 42°C for 60 min. The reaction was inactivated by heating at 70°C for 15 min.
Materials and methods

Table 3.1: Sequence of various primers used to amplify the M RNA segments of Watermelon bud necrosis virus (WBNV)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences from 5' to 3'</th>
<th>Length (nt)</th>
<th>nt position in genome</th>
<th>Pro. Size (nt)</th>
<th>Ann. Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM 55 F</td>
<td>agagcaatcgcgtgcgcccaatt</td>
<td>21</td>
<td>1-21</td>
<td>840</td>
<td>55</td>
</tr>
<tr>
<td>BM56 R</td>
<td>atgtctcaayaragcagcagatt</td>
<td>24</td>
<td>816-840</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM 59F</td>
<td>aacttctgcatactctctat</td>
<td>20</td>
<td>741-760</td>
<td>856</td>
<td>52</td>
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<tr>
<td>BM 60 R</td>
<td>gatgtctataaatctgtgaa</td>
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<td>1577-1598</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM 61 F</td>
<td>ggagagattttctctctt</td>
<td>17</td>
<td>1516-1532</td>
<td>781</td>
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<tr>
<td>BM 62R</td>
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<td>2278-2296</td>
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<td></td>
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<tr>
<td>BM 63F</td>
<td>tgaacatttcttagctatac</td>
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<td>2152-2177</td>
<td>999</td>
<td>50</td>
</tr>
<tr>
<td>BM 64R</td>
<td>caaagatcttagaagaagaact</td>
<td>23</td>
<td>3129-3152</td>
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</tr>
<tr>
<td>BM65F</td>
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<td>BM67F</td>
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<td>3660-3684</td>
<td>623</td>
<td>54</td>
</tr>
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<td>BM68R</td>
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<tr>
<td>BM57F</td>
<td>ccatctagttctcagcttt</td>
<td>20</td>
<td>4096-4115</td>
<td>801</td>
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<td></td>
</tr>
<tr>
<td>BM67WF</td>
<td>caaagatcttttctcacaaca</td>
<td>24</td>
<td>3660-3684</td>
<td>623</td>
<td>54</td>
</tr>
</tbody>
</table>

F: forward primer, R: reverse primer, nt: nucleotide, BM67wf primer was based on specific sequence of WBNV, pro: Product, Ann.: Annealing

Polymerase chain reaction (PCR)

The PCR reaction mixture consisted of 10 µl of cDNA as template, 5µl of 10x PCR buffer, 2µl of 10mM dNTP, and 2 µl each of reverse and forward primer (200 ng), 1.0µl Taq DNA polymerase (Chromous Biotech Pvt Ltd, Bangalore) and 35.8 µl of autoclaved water to makeup 50 µl of total volume. The mixture was mixed well and after a pulse spin it was placed in a PCR machine (Biometra T- Personal) with the following temperature cycles: hot start at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 sec, 45-60 °C for 1 min (specific annealing temperature for a particular combination of primers is given in Table 3.1) and 72 °C for 1-2 min. The final extension at 72 °C was allowed for 10 min.

Agarose gel electrophoresis

The PCR products were mixed with 6x loading dye (Fermentas, USA) and electrophoresis was performed in 1% agarose gel containing ethidium bromide (0.5 µg/ml). Electrophoresis was carried out at 60 V for 1-2 h. An aliquot (500 ng) of 1kb DNA ladder (Fermentas, USA) was used as molecular weight marker.
Molecular cloning

**Ligation reaction:** The PCR product was resolved on the 1% agarose gel and the specific band was isolated from gel using Promega Gel cleanup kit following manufacturer’s instruction. The eluted product was ligated in pGEM-T Easy vector (Promega, Madison, WI, USA) at 4°C for overnight. Ligation mixture consisted of 5µl of 2 x ligation buffer, 1 µl of pGEM-T Easy vector, 1 µl of T4 DNA ligase (2u/ µl) and 3 µl gel extracted PCR product.

**Preparation of competent cells:** The competent cells of *E. coli* DH5α were prepared by CaCl2 method (Mandel and Higa, 1970). Luria broth (LB, 50 ml) was inoculated with overnight grown culture of *E. coli* (1 ml) and incubated at 37°C at 200 rpm till (about 1.5 h) the bacterial growth reached an optical density of 0.30 at 600 nm. The culture was aseptically transferred to two 40 ml sterile centrifuge tubes and allowed on ice for 10 min. The bacterial cells were pelletized by centrifugation at 5500 rpm for 10 min at 4°C in Sorvall SS-34 rotor. The pellet was resuspended in 10 ml of ice cold 0.1 M MgCl2 and centrifuged at 5500 rpm for 10 min. The pellet was resuspended in 10 ml of ice cold 0.1M CaCl2 and allowed on ice for 1 h. The cells were again palletized, resuspended in 1 ml of ice cold 0.1 M CaCl2 and used for transformation after allowing at least 1 h on ice.

**Transformation:** An aliquot of 200 µl of the competent cells was added to the 5 µl of ligation mixture and incubated on ice for 1 h. The mixture was subjected to heat shock at 42°C for 2 min and immediately placed back on ice. The heat-treated bacterial cells were grown in 1 ml of LB at 37°C for 1h in a shaker incubator at 200 rpm. About 200µl of cell suspension were aseptically spread on Luria agar (LA) containing 0.1M Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (2% X-gal) and ampicillin (50 µg/ml) [IXA-plate]. The plates were incubated at 37°C for over night.

Initially, the transformants were selected based on blue and white colour of the colonies. White colonies were selected and maintained on LA plate containing IPTG, X-gal and ampicillin.

**Rapid screening of the recombinant:** For quick identification of recombinant clone, bacterial colonies were subjected to the method of rapid disruption of bacterial cell as described by Sambrook *et al.* (2001). Overnight grown bacterial colonies were picked up from the master plate with the help of autoclaved toothpick and mixed with 50 μl of 10mM EDTA, pH 8.0 in sterile microfuge tubes. To each tube, 15 μl of freshly prepared lysis solution containing 2N sodium hydroxide, 0.5% SDS and 20% sucrose was added and mixed.
well by vortexing for 30 sec. The mixture was incubated at -80°C for 10 min and then allowed to thaw at room temperature. To the mixture, 1.5 μl of 4M KCl and 5 μl of 0.4% bromophenol blue were added, mixed well and incubated on ice for 10 min. After centrifugation at 10000 rpm for 10 min, the 30 μl of supernatant was used for electrophoresis in 1% agarose gel. The colonies containing slower migrating plasmid band compare to the control (blue colonies transformed with the vector) were considered as recombinant colonies.

**Colony PCR:** The putative recombinant colonies obtained by rapid screening were further confirmed by colony PCR as described by Sambrook *et al.* (2001). The bacterial colonies grown in IXA-plate were picked up with the help of toothpicks and dissolved in 50 μl of PCR reaction mixture with the specific primers as described previously.

**Minipreparation of plasmid DNA:** The plasmid DNA from the transformed colonies was isolated by modified alkaline lysis method (Brinboim and Doly, 1979). The white colonies selected based on rapid screening and colony PCR were grown in 5 ml of LB containing ampicillin (50 μg/ml) at 37°C for overnight shaking at 200 rpm. The bacterial cells were collected by centrifugation for 2 min in sterile microfuge tube and resuspended in 100 μl of solution-I (25mM Tris- HCl, pH8.0 and 10 mM EDTA, pH8.0). An aliquot of 200 μl of freshly prepared solution-II (0.2N NaOH and 1% SDS) was added, mixed gently and allowed to lyse for 5 min at room temperature. The lysed cells were kept on ice for 5 min and mixed well with 150 μl of solution-III (ice cold 3M sodium acetate, pH 4.8) and maintained on ice for 15 min. The chromosomal DNA and bacterial debris were removed by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was collected and the DNA was precipitated by adding 0.8 volumes of isopropanol. The DNA pellet was washed with 70% alcohol, air-dried and resuspended in 20 μl of autoclaved water.

**Restriction enzyme digestion:** The purified recombinant DNA was analysed for the presence of insert by restriction enzymes digestion. The reaction mixture (20 μl) consisted of 2 μl of DNA, 2 μl of 10x buffer, 1-2 μl restriction enzyme and autoclaved water to make up the volume. The reaction was carried out at 37°C for overnight and insert size was assessed by agarose gel electrophorises.

**Sequencing and sequence analysis**

Nucleotide sequencing was done in an automated DNA sequencer ABI 3130 Genetic Analyzer at Chromous Biotech Pvt Ltd, Bangalore, India. The BLAST search was conducted
Materials and methods

in the NCBI database (http://www.ncbi.nih.gov/index.html). The nucleotide sequence data were assembled using the software program, BioEdit (version 5.0.9). The sequences of Tospoviruses used for comparison were obtained from the GenBank (Table 3.2). The relationships among tospoviruses were analysed comparing the nucleotide sequences and deduced amino acid sequence using BioEdit programme. The sequence was analysed using MEGA 4.1 package. Cluster dendrograms of the sequence data were constructed using clustal W algorithm.

Table 3.2: The sequences of Tospovirus species used for sequence comparison.

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Acronym</th>
<th>GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsicum chlorosis virus</td>
<td>CaCV</td>
<td>DQ256125</td>
</tr>
<tr>
<td>Groundnut bud necrosis virus</td>
<td>GBNV</td>
<td>NC-03620</td>
</tr>
<tr>
<td>Gloxinia tospovirus</td>
<td>HT-1</td>
<td>AF023172</td>
</tr>
<tr>
<td>Impatiens necrotic spot virus,</td>
<td>INSV</td>
<td>NC_003616</td>
</tr>
<tr>
<td>Iris yellow spot virus,</td>
<td>IYSV</td>
<td>AF214014</td>
</tr>
<tr>
<td>Melon yellow spot virus,</td>
<td>MYSV</td>
<td>NC_008307</td>
</tr>
<tr>
<td>Tomato spotted wilt virus,</td>
<td>TSWV</td>
<td>NC_002050</td>
</tr>
<tr>
<td>Tomato zonate spot virus,</td>
<td>TZSV</td>
<td>EF552434</td>
</tr>
<tr>
<td>Watermelon bud necrosis virus,</td>
<td>WBNV-Som</td>
<td>FJ694963</td>
</tr>
<tr>
<td>Watermelon silver mottle virus</td>
<td>WSMoV</td>
<td>DQ157768</td>
</tr>
</tbody>
</table>

Development of transgene construct

The complete nucleocapsid protein (NP) gene of WBNV was amplified by PCR using BM01F and BM02R primers (which contained BamHI and Xba sites) from the cloned DNA and re-cloned in sense orientation in plant transformation vector, pBI121 by replacing GUS gene by digestion with BamHI and Xba. About 500 ng each of the PCR product (NP) and the vector, pBI121 were double digested with BamHI and Xba (Fermentas, USA) at 37 °C overnight. The digested products were purified using PCR purification kit (PROMEGA) and the final product was eluted in 30 µl of autoclaved water. The ligation reaction mixture (20 µl) consisting of 10 µl of digested and purified nucleocapsid protein insert DNA (~250 ng), 5 µl of digested and purified pBI121 DNA (~100 ng), 2 µl of ligase 10x buffer, 1 µl T4 DNA ligase (Fermentas) and 2 µl of autoclaved water. The ligation mixture was incubated at 16°C for overnight. The competent cells of E. coli strain DH5α were transformed with the recombinant and the clones were selected by colony PCR as described previously.
The partial nucleocapsid protein (NP) gene of WBNV was amplified by PCR using BM49F & BM50R primers (which contained BamHI and XbaI sites) from the cloned DNA and re-cloned in antisense orientation in plant transformation vector, pBIN AR by digestion with BamHI and XbaI. About 500 ng each of the PCR product (NP) and the vector, pBI121 were double digested with BamHI and XbaI (Fermentas, USA) at 37 °C overnight. The digested products were purified using PCR purification kit (PROMEGA) and the final product was eluted in 30 µl of autoclaved water. The ligation reaction mixture (20 µl) consisting of 10 µl of digested and purified nucleocapsid protein insert DNA (~250 ng), 5 µl of digested and purified pBI121 DNA (~100 ng), 2 µl of ligase 10x buffer, 1 µl T4 DNA ligase (Fermentas) and 2 µl of autoclaved water. The ligation mixture was incubated at 16°C for overnight. The competent cells of E. coli strain DH5α were transformed with the recombinant and the clones were selected by colony PCR as described previously.

Mobilization of the transgene construct from E. coli to Agrobacterium tumefaciens by tri-parental mating

The transgene construct was mobilized from E. coli strain DH5α to A. tumefaciens strain, LBA4404 using E. coli containing pRK2013 as helper plasmid by triparental mating system (Ditta et al., 1980). A. tumefaciens LBA4404 was grown in LB containing rifampicin (50 µg/ml) at 28°C for 48 h shaking at 200 rpm. The E. coli strain DH5α containing transgene construct and helper plasmid were grown separately for 24 h in LB medium containing kanamycin (50 µg/ml) at 37°C shaking at 200 rpm. Two ml of A. tumefaciens LBA4404, 1ml of E. coli strain DH5∞ containing helper plasmid and 1ml of E. coli strain DH5α containing transgene construct were transferred to a culture tube and mixed well. An aliquot of 50-100 µl of this mixture was inoculated on pieces of sterile nitrocellulose membrane (NCM) placed on a LA plate. These plates were air dried and sealed with parafilm and incubated at 28° for 48 h. Bacterial cells that grow on NCM were diluted in 5 ml of LB medium and mixed with gentle shaking. An aliquot of 100 µl of the suspension was further diluted (ten times) in LB and 100 µl was spread on LA plate containing rifampicin (50 µg/ml) and kanamycin (50 µg/ml). The plates were incubated at 28 °C for 36 h. A single colony of transconjugant was streaked and maintained on LA plates containing both rifampicin (50µg/ml) and kanamycin (50 µg/ml). Agrobacterium colony containing transgene construct was confirmed by PCR and the confirmed colony containing transgene was stored as liquid culture with 50% glycerol at −80 °C.
Materials and methods

Preparation of Agrobacterium culture for co-cultivation

The glycerol stock of Agrobacterium culture containing the transgene construct that was stored at –80 °C was revived in LB containing rifampicin (50 μg/ml) and kanamycin (50 μg/ml) at 28 °C for 48 h shaking at 200 rpm. The culture was again inoculated in fresh LB containing rifampicin (50 μg/ml) and kanamycin (50μg/ml) and allowed to grow at 28 °C for overnight shaking at 200 rpm. This overnight grown culture was used for co-cultivation.

Plant materials for transformation

Seeds of a commercially grown cultivar, Sugar Baby of watermelon (Citrullus lanatus) were obtained from the National Seed Corporation (NSC), IARI, Pusa Campus, New Delhi-110012 and Seminis, Monsanto Holdings Private Limited, Mumbay-400093, India.

Seed sterilization and germination

Healthy and uniform watermelon seeds were soaked in 0.2% fungicide (Bavistin) overnight before sowing them in pots containing alluvial and sandy soil under 28°C temp and 56 % RH in Phytotron (in vivo). The cotyledons, hypocotyls and epicotyls excised from 6-10 days old seedlings were used as explants for regeneration and transformation studies.

Seedlings were raised under in vitro condition by rinsing the seeds with 2% sodium hypochloride and then surface sterilized with 0.2% (w/v) aqueous solution of mercuric chloride for 2 min. The seeds were subsequently rinsed with autoclaved distilled water 3 times in a laminar air flow cabinet. Sterilized seeds were cultured on half strength MS (Murashige and Skoog, 1962) salts for 4-6 days at 26 ± 2°C under dark for germination.

Preparation of explants

The explants were prepared from the seedlings raised in soil and in vitro conditions. The basal part of cotyledon, hypocotyl and epicotyl were excised (as shown in Fig. 3.3) from 6-10 days old seedlings raised in pot containing soil.
Materials and methods

These explants were taken in a beaker and covered with a muslin cloth. The explants were washed under running tap water for 30 minutes. In the next step, the explants were treated with 0.2% lavoline for 5 minutes and washed with double distilled water for 5 min. The explants were surface sterilized with 0.1% HgCl₂ for 3 min in a laminar air flow cabinet and rinsed three times with sterile double distilled water. The basal halves of cotyledon (3-4 mm which were close to the embryonic axes) and epicotyls (10 mm) were excised and used as explants. Similarly, the basal halves of cotyledon were excised from 4-6 day old in vitro-raised seedlings (Fig. 3.4A). The cotyledon basal halves were cultured with their abaxial surface touching the media. The Petri dishes were sealed with para film and cultures were kept at 25°C under low light conditions.

Culture medium

Explants were cultured on medium containing MS basal medium containing 3% sucrose and different concentrations of BAP (for cotyledon basal halves, epicotyls and hypocotyls) or BAP in combination with IAA (for cotyledon halves) and solidified with 0.8
Materials and methods

40% agar. The pH of the medium was adjusted to 5.6-5.8 with 1 N NaOH and 1 N HCl prior to autoclaving at 121°C under a pressure of 15 psi for 20 min.

Multiple shoots regeneration

Effect of hormones: The epicotyl and hypocotyl explants were cultured on MS medium containing different concentrations of BAP (0-4 mg/l) and the cotyledon basal halves on MS medium with BAP (0-3 mg/l) and IAA (0-0.5 mg/l). The data on the percent explants forming shoot and the number of shoots per explant was recorded after 4 weeks of culture.

Age of explants: The effect of the age of explants on multiple shoot induction was determined by culturing the seedling explants, cotyledon halves, epicotyl and hypocotyl excised from 5-, 6-, 7-, 9- and 10-d-old seedlings raised on MS containing 3 mg/l BAP.

Shoot elongation and rooting

The explants with stunted multiple shoots were transferred on the MS (Murashige and Skoog, 1962) basal medium for shoot elongation. Well-developed shoots (2-3 cm) were excised from the proliferating explants and transferred to the culture tube (25x10 mm) containing MS basal medium for root induction. Shoots with well-developed roots were acclimatized in liquid Hoagland solution (appendix) before their transplantation in soil.

Culture conditions

The cultures were incubated at 26 ± 2°C under 16 h photoperiod of cool white-fluorescent light (Philips, India) of 80 µEm⁻²s⁻¹ intensity. For each treatment, 22 explants were cultured and each experiment was repeated at least thrice. Cultures were observed every day and the data was recorded.

Transfer of plantlets to pots

After two days of acclimatization in liquid Hoagland solution, plantlets were transplanted in the pots containing autoclaved moistened sand. The potted plants were watered with Hoagland solution whenever required. The tray containing potted plants was covered with a transparent plastic bag to maintain higher humidity and maintained under light conditions in culture room. After 10-13 days, plants were transplanted to larger pots containing autoclaved soil. When they plants grew to a height of about 10-15 cm, they were transferred to glasshouse.

Optimization of selection system

Prior to transformation, an effective concentration of the selective agent kanamycin for the selection of transformed shoot was identified by culturing the seedling explants,
cotyledon halves, hypocotyls and epicotyls on shoot regeneration medium (MS + BA +/- IAA) containing different concentrations of kanamycin (0-200 mg/l). The cultures were maintained at 26 ± 2 °C under 16 h photoperiod of cool-white fluorescent light of intensity 80 µEm⁻²s⁻¹. The explants were subcultured on the same medium with same levels of antibiotic after every two weeks till 4 to 6 weeks. The data on the number of explants forming shoots and the number of shoots per explant were recorded after 4 weeks of culture. For each treatment, 60 explants were raised and each experiment was repeated at least thrice.

**Optimization of transformation protocol**

To establish a transformation protocol for watermelon, various parameters influencing transformation efficiency using GUS / WBNV-NP were evaluated. The explants were inoculated by immersing in *Agrobacterium tumefaciens* strain LBA 4404 (pBI121-NP) suspension (containing 10⁶ to 10⁹ cells/ml of bacteria) for 0-30 min with gentle shaking at 100 rpm. The explants were blotted dry on sterile filter paper and cultured on cocultivation medium containing MS medium with 3% sucrose and adjusted to pH (5.6-5.8) for 1-3 days at 22-28°C under light conditions. The effect of phenolic - acetosyringone (200 µM) in bacterial inoculation and cocultivation media was also tested. After cocultivation, the explants were washed with sterile distilled water, blotted dry on sterile filter paper and cultured on shoot selection medium containing suitable hormones for different explants with antibiotic cefotaxime (200-500 mg/l) / augmentin (200-300 mg/l) and standardized kanamycin concentration (100 mg/l). For each variable in an experiment, 60 explants were used and each experiment was repeated thrice.

**Genetic transformation of watermelon with A. tumefaciens harbouring a binary vector pBI 121 containing GUS and nptII genes**

*Agrobacterium culture and co-cultivation of explants*

* A. tumefaciens* strain containing transgene construct was grown in liquid LB medium containing 50 mg/l kanamycin (for plasmid selection) and 50 mg/l rifampicin (for bacterial selection) for overnight at 28°C on an orbital shaker at 200 rpm till the O.D. of bacterial suspension reached 0.6 at 600 nm.

The bacterial culture was centrifuged at 4500 rpm in a 30 ml tube (Tarson, India) for 10 min. The pellet was resuspended in liquid ½ MS medium and 200 µM acetosyringone. The explants excised and immersed in bacterial suspension for 30 min with shaking at 28°C. Agro-inoculated explants were blotted on sterile filter paper and co-cultured in Petri dish with
Materials and methods

MS medium containing acetylsyringone for 2 days under 16 h photoperiod at 26 ± 2°C. After cocultivation, explants were washed 3-4 times with sterile distilled water with vigorous stirring and blotted dry on sterile filter paper. The explants were cultured on MS basal medium containing 100 mg l⁻¹ kanamycin and 500 mg l⁻¹ cefotaxime for shoot regeneration. The explants were sub-cultured on a fresh medium containing same levels of antibiotic after every two weeks for a total 4-6 weeks. Green shoots recovered on selection medium were rooted on MS basal medium containing 50 mg l⁻¹ kanamycin and 500 mg l⁻¹ cefotaxime.

Enzyme assay

GUS activity in plant tissues was determined by a histochemical assay according to Jefferson et al. (1987). Stable expression of GUS was monitored in the calli and shoots regenerated from the Agrobacterium infected explants after 4 weeks of culture. The calli and leaves of putative transformed and non-transformed plants were immersed in freshly prepared X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution (Appendix III) after overnight at 37°C. The plant tissues were decoloured with 70% alcohol for overnight and observed under compound microscope.

Molecular analysis of transformed plants

Total nucleic acid extraction

1. 100 mg leaf tissue from the transformed and non-transformed plants were ground in a sterile mortar pestle with liquid nitrogen
2. 1 ml of CTAB buffer was added and the homogenized and transferred in a sterile eppendorf tube.
3. Tubes were incubated at 55°C for 30 min and after incubation centrifuged at 10,000 rpm for 15 min at 4°C.
4. The supernatant phase was taken, added 1/3 volume of chloroform was added and mixed gently.
5. The mix was centrifuged at 10,000 rpm for 15 min at 4°C.
6. The aqueous phase was taken and the DNA was precipitated by adding 0.1 vol. of 7.5M of ammonium acetate and 1 vol. of isopropanol.
7. Centrifuged at 10,000 rpm for 15 min at room temperature.
8. The pellet was collected and washed with 70% ethanol.
Materials and methods

9. The pellet was dried at 37°C for half an hour in dry bath and suspended in 50µl sterile water

Purification of plant DNA: The dissolved DNA was treated with 50 µg/ml RNase at 37°C for 1 h and further purified by extracting with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) to remove any trace of protein and RNase. It was followed by chloroform: isoamyl alcohol (24:1) extraction. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and two volume of chilled ethanol. The mixture was kept at -20°C for 30 min for precipitation. The pellet was recovered by centrifugation at 11000 rpm for 10 min at 4°C. The DNA pellet was washed with 70% (v/v) ethanol for 5-10 min. The pellet was air-dried and dissolved in 20 µl of TE buffer.

Quantification of DNA: Spectrophotometer reading at A\text{260} and A\text{280} were taken in NanoDrop to determine the concentration and the purity of DNA samples. The concentration of DNA was estimated using the relationship of 1.0 A at 260 nm equivalent to 50 µg DNA / ml.

PCR analysis of putative transgenic plants: Plant genomic DNA isolated from the leaf tissue was used as a template to amplify GUS gene by using specific primers (Table 3.5). PCR was carried out with Taq DNA polymerase (Chromous Biotech Pvt Ltd, Bangalore, India) in thermocycler (Biometra) using 25 of total volume (Table 3.3) and appropriate conditions (Table 3.4).

Table 3.3: Reaction mixture for PCR to amplify the DNA

<table>
<thead>
<tr>
<th>Components</th>
<th>Vol. (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (100 ng)</td>
<td>5.0</td>
</tr>
<tr>
<td>10Xbuffer</td>
<td>2.5</td>
</tr>
<tr>
<td>Primer (F)</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer(R)</td>
<td>0.5</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq pol</td>
<td>0.5</td>
</tr>
<tr>
<td>H\text{2}O</td>
<td>15.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>25.0</td>
</tr>
</tbody>
</table>
Table 3.4: Condition for PCR in Thermo cycler

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>49°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Table 3.5: Primer sequence of Gus gene used for PCR amplification

| Gus gene primers (Annealing temp. 50 °C) | Forward 5’ TGTTACGTCCTGTAGAAAC 3’ | Reverse 5’ ATTGTTTGCTCCTCGCTG 3’ |

Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al., 1989). DNA from non-transformed (control) plant was used as a negative control while the plasmid from pBI121 was used as a positive control.

**Transformation of watermelon with the transgene construct (WBNV-NP)**

*Plant transformation vector:* The disarmed *A. tumefaciens* strain LBA4404 harboring a binary vector pBI121 that contained a neomycin phosphotransferase gene (*nptII*) as a plant selectable marker and nucleoprotein gene of WBNV was used for transformation and confirmed by colony PCR.

*Inoculation and co-cultivation of explants:* *A. tumefaciens* strain LBA4404 (pBI121-NP) was grown on LA semi solid medium containing rifampicin (50.0 mg/l) and kanamycin (50.0 mg/l) at 28°C. A single bacterial colony was inoculated into 50 ml of liquid LB medium containing same levels of antibiotics and grown overnight at 28°C on a shaker at 200 rpm till
bacterial density reached at 0.7 (OD$_{600}$). Bacteria were pelleted at 4500 rpm for 10 min and pellet was dissolved in $\frac{1}{2}$ MS liquid medium (pH 5.8).

Epicotyl explants excised from 6-8 days old seedlings were precultured on MS basal medium for 2 days prior to their inoculation in the bacterial suspension (added 200 µM acetosyringone) for 30 min with gentle shaking at 100 rpm. Inoculated explants were blotted on sterile filter paper and kept on MS medium containing 200 µM acetosyringone for 2 d under 16 h photoperiod at 26 ± 2°C.

**Selection and shoot regeneration:** After co-culture, the explants were washed 3-4 times with liquid $\frac{1}{2}$ MS medium and the final wash was given with liquid $\frac{1}{2}$ MS medium containing cefotaxime (500 mg/l for 2 min.). Explants were blotted on sterile filter paper and cultured on kanamycin selection medium [MS salts and vitamins, 3% sucrose, BAP (3.0 mg/l), cefotaxime (500 mg/l) and kanamycin (100 mg/l)] for shoot regeneration. The explants were transferred on to fresh medium containing the same levels of antibiotic every 2 week for a total of 4-6 weeks until the shoots attained a height of 3-4 cm.

**Rooting of transformed shoots:** Well-developed shoots (3-4 cm in length) were excised from the regenerating explants and transferred to rooting medium, MS basal medium containing 500 mg/l cefotaxime and 25 mg/l kanamycin.

**Molecular analysis of WBNV-NP transformed plants**

**Isolation of genomic DNA:** Total genomic DNA was extracted from fresh leaves of putative transformed and non-transformed (control) plants by the CTAB (Cetyl trimethyl ammonium bromide) method (Rogers and Bendich, 1998).

**PCR analysis:** Putative transformants were screened by polymerase chain reaction (PCR) for the presence of WBNV-NP gene. The 824 bp coding region of NP gene was amplified by using oligonucleotide primers (Forward primer: 5’ TTGGATCCATGTCTACCGTTAAGCAACT 3’ Reverse primer: 5’ TTGGATCCATGTCTAACCAGCAGCAGCT 3’). The amplification reaction was carried out with initial denaturation at 94°C for 3 min and followed by 35 cycles of denaturation of 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min and final extension for 10 min at 72°C for detection of NP gene amplification. Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium
bromide staining (Sambrook et al., 1989). DNA from non-transformed (control) plant was used as a negative control while the plasmid from pBI121-NP was used as a positive control.

**Southern hybridization of PCR positive WBNV-NP transformed plants**

**Gel electrophoresis:** The PCR samples were mixed with 5 µl of 6 X gel loading dye and loaded into the wells of 0.9% agarose gel (with ethidium bromide) in 1 X TAE buffer. PCR amplification was resolved by electrophoresis at 50 volts using power supply (Amersham Pharmacia, USA) at room temperature for 2 h. DNA marker (1 kb ladder) was used for size determination of DNA bands that appeared after autoradiography.

**Transfer of DNA to the nylon membrane:** The agarose gel containing resolved DNA bands was rinsed in distilled water and immersed in denaturing solution (1.5M NaCl and 0.5M NaOH) for 30 min with gentle shaking followed by soaking in neutralizing solution (1M Tris-HCl pH 7.4 and 1.5M NaCl) for 30 min and washing in water for 10 min. After washing, a glass platform was put in a tray and a Whatman 3 mm paper wick was placed on the platform. Tray was filled with 20 × SSC. Air bubbles between the wick and glass plate were removed by gently rolling a 10 ml glass pipette. The gel was placed upside down over the wick and air bubbles between gel and wick were removed. A positively charged nylon membrane (Hybond N⁺ of Amersham) of the exact size of the gel was soaked in 2 X SSC and placed over the gel. Air bubbles between the membrane and the gel were removed. Three pieces of Whatman 3 paper of exact size of the gel were soaked in 2 X SSC and were placed over the nylon membrane. A stack of blotting papers (5 cm high) of the same size of the gel was placed on the top of the stack of filter papers along with 500 g weight. The DNA blotting was carried out at room temperature for 16 h. After the transfer, the set-up was carefully disassembled leaving the hybridization membrane in contact with the gel. The membrane was carefully removed from the gel and placed on Whatman 3 paper for air drying. After air drying, the membrane was cross-linked with UV rays for 1 min. in cross-linker (Unitec) and stored at room temperature or at 4°C until used for hybridization.

**Radiolabelled probe preparation:** The blots were hybridized with the probe to NP gene of WBNV. The radio- labeled probe was prepared by random priming method (Feinberg and Vogelstein, 1984) using a labeling kit obtained from Bangalore Genei India Pvt. Ltd. The NP gene was amplified by PCR using the specific primers and purified by a Gel Extraction Kit (Promega). The reaction mixture for probe preparation consisted of the following components:
Materials and methods

Denatured NP DNA - 5μl
Nuclease free water - 35μl
Random primer - 1μl
5 x labeling buffer - 2μl
dNTPs Mix (-dCTP) - 2μl
BSA - 2μl
αP32d CTP - 2μl
Sp.act 3000 ci/ m mol -
Klenow fragment (DNA pol.)- 1μl
Yeast RNA (100μg/ml)

The mixture was incubated for 2 h at 37°C and stored at -80°C.

Pre-hybridization: The membrane was carefully placed in a hybridization tube and 50 ml of prehybridization solution (Appendix) (Church and Gilbert, 1984) was added. Pre-hybridization was carried out in a hybridization oven with gentle rotation for 6 hrs at 65°C.

Hybridization: For hybridization, volume of pre-hybridization buffer was reduced to 25 ml. randomly primed (32P) labeled WBNV-NP probe was denatured by heating in microcentrifuge tube at 95-100°C for 5 min and cooled in an ice bath. The denatured labeled probe was added directly to pre-hybridization buffer and incubated at 65°C with gentle rotation for overnight.

Washing: WBNV-NP probe hybridized membrane was washed by following stringent conditions to remove non-specific binding of labeled probe and any trace of unincorporated (32P) dCTP. Initial washing step was carried out in 2 X SSC and 0.5 % SDS for 10 min, and repeated twice at room temperature. This was followed by washing in 1 X SSC with 0.1% SDS for 1 min at 65°C and finally a stringent washing was carried out 0.5 X SSC for 5 min and the membrane was processed for autoradiography.

Autoradiograph: Autoradiography was carried out to locate hybridized (32P) labeled probe on the blot. Hybridized nylon membrane was placed on a used X - ray film covered with Saran wrap. The membrane was placed inside X-ray film cassette (Hypercassette, UK). The blot was exposed to X-ray film (Kodak) in dark and cassette was locked tightly, wrapped over with black chart and kept at - 80°C for 3 days. The film was developed in developer (Kodak) for 1 min and fixed in fixer (Kodak) for 2 min. The
developed X-ray film was washed in running water for 10 min and air-dried at room temperature.

Fig. 3.2: Schematic presentation of transgene construction using Nucleoprotein (NP) gene of Watermelon Bud Necrosis virus (WBNV) in plant transformation vector, pBI121. The NP gene was placed between 35S promoter and NOS terminator in sense orientation by replacing GUS gene at BamHI and XbaI sites.
Fig. 3.3. Schematic presentation of transgene construction using partial Nucleoprotein (NPP) gene of Watermelon Bud Necrosis virus (WBNV) in plant transformation vector, pBIN AR. The NPP gene was placed between 35S promoter and NOS terminator in antisense orientation BamHI and XbaI sites.