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Optimization of a more efficient protocol for mechanical inoculation for watermelon bud necrosis orthotospovirus and its validation with different watermelon genotypes

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ABSTRACT

Watermelon bud necrosis orthotospovirus (WBNV) is one of the emerging and most serious pathogens affecting watermelon cultivation in India. In the present study, the effect of different inoculation methods and buffer formulation on virus transmission was studied and validated in 17 watermelon genotypes. Among various inoculation methods used, maximum transmission efficiency 60–100% was obtained in 4–10 days post-inoculation (DPI) with pin-pricking and rubbing together, followed by rubbing (85–100%; 20–25 DPI) and pin-pricking (10–32%; 10–25 DPI) alone with the sodium buffer (PBS) having pH 7.0 supplemented with sodium sulfite (SS) and 2-mercaptoethanol (2-ME). Similarly, among the various abrasives used, maximum transmission efficiency 82–100% was obtained at 10–35 DPI with Celite and Carborundum together, followed by Celite (64–100%; 10–20 DPI) and Carborundum (72–100%; 20–40 DPI) alone. Among various additives used, maximum transmission efficiency 82–100% was obtained in 10 DPI with PBS supplemented with SS and 2-ME followed by PBS supplemented with SS alone (85–100%; 20–40 DPI) and PBS with no additive (76–85%; 20–35 DPI). The 15–20 days old watermelon plants were found suitable for the efficient mechanical transmission (100%) and to obtain maximum virus titre (Absorbance at 405 nm) i.e. 1.52–2.50 at 8–25 DPI. The 21–60% and 100% transmission was obtained at 4 and 10 DPI, respectively using the same buffer composition as mentioned above. For validation of optimized sap inoculation protocol for WBNV, 17 watermelon genotypes were inoculated under glasshouse conditions and the percent transmission ranged from 80 to 100%. Besides symptomatology, virus transmission was further confirmed by DAC-ELISA, RT-PCR and sequencing of the partial N gene. The O. D. at 405 nm ranged from 0.38 to 0.9 in naturally infected watermelon plants whereas in the glasshouse inoculated watermelon plants it was ranged from 0.30 to 0.73 at 15 DPI. This is the first effort on optimization of more efficient sap inoculation protocol for WBNV and it validated to 17 watermelon genotypes.

1. Introduction

Worldwide, watermelon production is affected by several fungal, bacterial, nematode and viral diseases. The most common fungal diseases of watermelon are anthracnose, *Fusarium* wilt, downy mildew and gummy stem blight (Maynard and Hopkins, 1999; Keinath, 2014). Besides fungal diseases, cultivation of watermelon is severely affected by several viral diseases in India caused by papaya ring spot virus-W (PRSV-W), watermelon mosaic virus-2 (WMV-2) and WBNV (Bhargava et al., 1975; Krishnareddy and Singh, 1993; Singh, 1993; Vani and Varma, 1993; Jain et al., 1998, 2015).

Bud necrosis disease (BND) of watermelon in India, caused by WBNV, is an emerging and serious disease affecting watermelon and continues to cause severe yield losses up to 100% (Singh and Krishnareddy, 1996; Jain et al., 1998, 2007; Kunkalikar et al., 2011). Since its first report in the experimental fields at the Indian Institute of Horticultural Research (IIHR), Bangalore during 1991-92 (Krishnareddy and Singh, 1993) it was further recorded in other watermelon growing areas of Karnataka, Andhra Pradesh and Maharashtra, which forced farmers to abandon watermelon cultivation due to 39–100% disease incidence and 60–100% estimated yield loss (Singh and Krishnareddy, 1996). WBNV belongs to the watermelon silver mottle orthotospovirus (WSMoV) serogroup IV and is a distinct member of the genus *Orthotospovirus* (formerly known as: *Tospovirus*) and family *Tospoviridae* (formerly known as: *Bunyaviridae*) (Jain et al., 1998, 2015; Adams et al., 2017). It is having quasi-spherical enveloped virion of

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80–110 nm in diameter and has tri-partite ssRNA genome segments designated as large (L), medium (M) and small (S) (Kumar et al., 2010; Li et al., 2011; King et al., 2011). In India, WBNV is transmitted by *Thrips palmi*, a melon thrips based on the species-specific markers for mtDNA and characterization of its miRNA using high-throughput deep sequencing (Rebijith et al., 2012, 2016).

Presently, incidence of BND in watermelon has been recorded from North, South and Western states of India (Pappu et al., 2009; Kunkalikar et al., 2011; Mandal et al., 2012). So far, natural infection of WBNV has been recorded from watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), muskmelon (*Cucumis melo*) and ridge gourd (*Luffa acutangula*). In recent past, it has been recorded from tomato (*Solanum lycopersicum*), chili pepper (*Capsicum annum*) (Kunkalikar et al., 2011) and chrysanthemum (*Chrysanthemum indicum*) (Holkar et al., 2017). However, its mechanical inoculation in glasshouse conditions has been practiced in watermelon, tobacco (*Nicotiana benthamiana* and *N. tabacum*), and cowpea (*Vigna unguiculata*) cvs. C-152 and Pusa Komal (Holkar et al., 2017) using the protocol developed by Mandal et al. (2001) for tomato spotted wilt orthotospovirus (TSWV) to peanut (*Arachis hypogea*).

WBNV has become a serious concern for researchers and watermelon growers and has generated an impulse for development of better management practices. Integrated disease management (IDM) approaches have been discussed and found effective against other orthotospoviruses including, groundnut bud necrosis orthotospovirus (GBNV), iris yellow spot orthotospovirus (IYSV) and TSWV (Pappu et al., 2009; Mandal et al., 2012). Adequate efforts have not been made on the IDM approaches including the identification of the resistance sources against WBNV in watermelon along with the conventional and non-conventional breeding strategies for the management of this virus. Further, to identify resistance sources, there is a need for a stable and more efficient protocol of mechanical inoculation of WBNV to watermelon. Therefore, the present investigation was carried out to optimize the mechanical transmission protocol for WBNV and its validation with different watermelon genotypes under glasshouse conditions. Further, this optimized protocol has been employed for screening of large number of watermelon genotypes that led to the identification of resistant lines both under field and glasshouse evaluations.

2. Materials and methods

2.1. Identification and maintenance of the virus isolate

2.1.1. Source of the virus isolate and electron microscopy

The present investigation was carried out using BND affected watermelon samples suspected to be infected by WBNV. Symptomatic samples were collected from the Indian Agricultural Research Institute (IARI) experimental farm and stored at −80 °C for its further use. The association of an orthotospovirus with these samples was confirmed by leaf-dip electron microscopy (Gibbs et al., 1966) and immuno- and nucleo-based assays followed by sequencing. Diseased leaf pieces (3–5 mm) cut with the help of cork borer were macerated on a clean glass slide with a flat ended glass rod in 40–50 µl phosphate buffer (0.078 M, pH 6.5) and left the finally homogenised material for a few seconds. A drop of the supernatant (15–20 µl) from the homogenised virus material was put on the carbon-coated grid (3 mm diameter, 400 mesh). Excess of the supernatant was then washed off with 180–200 µl distilled water. The carbon-coated grid was treated with 40–50 µl uranyl acetate (aqueous 2%, pH 4.2) for few seconds. Excess stain was removed and blotted dry by touching the edge of the grid with a strip of filter paper. The grid was air–dried for 1–2 min. The negatively stained grid was finally examined under electron microscope (JEM-1011) at the Advanced Centre for Plant Virology (ACPV), IARI, New Delhi. The uninoculated watermelon leaf sample maintained in glasshouse was used as healthy control.

2.1.2. DAC-ELISA

These EM positive BND affected watermelon samples were further subjected to direct antigen-coated enzyme-linked immunosorbent assay (DAC-ELISA) (Clark and Bar-Joseph, 1984) to detect the association of an orthotospovirus. The polyclonal antibodies (PAb) against the nucleocapsid (N) protein of GBNV generated at ACPV were used. The assay was performed in 96 well polystyrene microtitre plates (Costar, Sigma, USA). Measured the absorbance (O.D.) in each well at 405 nm using a Teacan Sunrise (version 1.2) ELISA reader. Compared the absorbance values of the watermelon samples with healthy control and buffer after 1 h of incubation at 37 °C. Watermelon samples showing absorbance (O.D. at 405) values more than two times than that of healthy control were considered as infected with the orthotospovirus.

2.1.3. RT-PCR conditions

The ELISA positive watermelon samples were further subjected for the specific detection of WBNV virus isolate by duplex reverse transcription - polymerase chain reaction (RT-PCR) using species-specific forward primers (for GBNV: Gs1F: 5′-ATGGTTGAAAAGAGCAAGAATG ATGC-3′ and for WBNV: Ws1F: 5′-CAAAGACTTGTTGGCTGGTGG TG-3′) and a common degenerate reverse primer (for both GBNV and WBNV GWs1R: 5′-CTTCTT(A/T)GA(A/G)TGT(AC/T)CACCATA(A/G)TCATCC-3′) designed using N gene sequences of both the viruses and were used as described by Akhter et al. (2012) and Holkar et al. (2017). Total RNA was extracted from the infected and healthy (control) watermelon samples (~100 mg) using RNeasy plant mini kit (Qiagen Inc., Chatsworth, USA) according to the manufacturer's instruction. Extracted RNA was used as template for RT-PCR along with the positive control available (WBNV N gene).

The first strand cDNA was synthesized using ImProm-II™ reverse transcriptase kit (Promega, USA). The 20 µl cDNA reaction mixture contained 8 µl template RNA (~2.5 µg), 1 µl (100 pg) reverse primer, 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP, 4 µl 5× buffer, 0.5 µl RNase inhibitor (40 $U\mu l^{-1}$) (Promega, USA) and 2.5 µl sterile distilled water and 1 µl reverse transcriptase (200 $U\mu l^{-1}$) was incubated at 42 °C for 60 min. The 100 µl PCR reaction mixture comprised 10 µl template cDNA, 10 µl 10 X PCR buffer, 3.5 µl 25 mM $MgCl_2$, 2.5 µl 10 mM dNTP, 3.0 µl (~100 pg) each of reverse and two forward primers, 1.0 µl of *Taq* DNA polymerase and 70 µl of sterile distilled water. The PCR was conducted in a thermal cycler (Biometra, Germany) with the following temperature conditions: 2 min hot start at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing for 1 min at 52 °C and synthesis at 72 °C for 1 min, and a cycle of final extension at 72 °C for 10 min. The PCR product was analysed in 1% agarose gel in Tris-acetate EDTA containing ethidium bromide (Sambrook and Russell, 2001).

2.1.4. Cloning and sequence analysis of the RT-PCR product

To confirm the virus specificity of the primers mentioned above, the RT-PCR product obtained from the field samples of watermelon was then cloned in pGEMNT Easy vector (Promega, Madison, USA), sequenced and submitted to the NCBI GenBank. The sequence of the WBNV from watermelon was compared with that of other WBNV sequences available in the database. The sequence analysis was performed using ClustalW program version 1.7 using BioEdit software (www.bioedit.software.informer.com) and sequence identity matrix was constructed.

2.1.5. Maintenance of the virus isolate

In order to maintain the confirmed WBNV isolate under glasshouse condition, initially watermelon (*Citrullus lanatus*) and tobacco (*Nicotiana benthamiana*) were used as test plants and the seedlings were raised in the glasshouse of the ACPV, IARI, New Delhi in pots (20 × 20 cm) filled with potting mixture containing farm yard manure, coco peat, vermiculite and sand in the ratio of 3:2:2:1 (w/w). The watermelon (7–8 days old; cotyledon stage) and *N. benthamiana* (30–35 days old; 4–6 leaf stage) plants were subjected to mechanical