MOLECULAR CHARACTERIZATION OF GROUNDNUT BUD NECROSIS VIRUS (*Orthotospovirus arachinecrosis*) CAUSING NECROSIS DISEASE OF BLACKGRAM FROM KRISHNA DISTRICT OF ANDHRA PRADESH.

ABSTRACT

Orthotospovirus arachinecrosis commonly known as Groundnut bud necrosis virus (GBNV), is causal agent for necrosis disease in blackgram. GBNV poses a major challenge to blackgram production in major growing areas next to yellow mosaic disease. Symptoms of necrosis disease of blackgram include severe downward curling and twisting of leaf lamina, veinal necrosis, petiole necrosis, bud necrosis, and necrotic streaks on the stem. Infection at early growth stages of blackgram lead to plant death, while surviving plants exhibited stunted growth with a significant reduction in pod yield. GBNV-BG isolate was maintained in pure form on cowpea cv. C-152, resulted in chlorotic and necrotic local lesions on primary leaves at seven days post inoculation. The pathogenicity of GBNV on blackgram (LBG-645) was further confirmed by artificial sap inoculation. Amplification of coat protein gene of GBNV was confirmed through RT-PCR using gene-specific primers, produced an amplicon of 800 bp. Sequence analysis of coat protein gene revealed that the present study isolates OQ683310 and OR295403 shared sequence identity of 98.0 % and 99.3 % respectively at nucleotide level, with other isolates in the NCBI database. The phylogenetic analysis of the coat protein gene revealed that the isolates under this study were closely linked with GBNV tomato isolate from Tamil Nadu (MN718651 and MZ505083).

Key words: blackgram, cowpea, necrosis, *Orthotospoviruses*, GBNV, molecular characterization.

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1. INTRODUCTION

Blackgram is one of the major pulse crops with protein content that is three times higher (20-25 %) as compared to cereals. Blackgram scientifically known as *Vigna mungo* (Linn.) Hepper, belongs to the family *Leguminosae*, is widely acknowledged under different names such as urdbean, urad, mash, mashkalai, kalo daal, uzhunnu parippu, black mapte and others. Blackgram contributes around 10 per cent to India's overall pulse production, with over 90 % of its yield primarily sourced from 10 states namely Maharashtra, Karnataka, Madhya Pradesh, Gujarat, Uttar Pradesh, Jharkhand, Telangana, Odisha, Andhra Pradesh, and Tamil Nadu. In Andhra Pradesh blackgram is predominantly grown in the Krishna, Nandyal, Prakasam, Srikakulam, Bapatla, Vizianagaram, and YSR districts which together

accounted for 80.49 per cent of the state's total acreage under cultivation in 2022-23 (http://des.ap.gov.in). Biotic and abiotic stresses, coupled with subpar crop management practices, pose challenges in achieving higher blackgram yields. Viral infections are one of the significant biotic constraints for legume production, particularly in tropical and subtropical regions (Sastry and Zitter, 2014). Among several viral diseases infecting blackgram whitefly transmitted *Mungbean yellow mosaic virus* (MYMV) causing Yellow Mosaic Disease (YMD), thrips mediated *Orthotospovirus arachinecrosis* (GBNV) causing necrosis disease and an uncharacterized urdbean leaf crinkle complex are known to cause significant losses. Lately, necrosis disease infecting blackgram throughout all stages of crop growth has become a serious concern, next to Yellow Mosaic Disease (YMD).

Orthotospoviruses are known for their pestiferous nature and reported to cause substantial economic losses globally by jeopardizing the production and productivity of crops belonging to the Solanaceae, Cucurbitaceae, and Fabaceae families (Amin *et al.*, 1985; Khurana *et al.*, 1989; Krishnareddy and Singh, 1993; Kumari *et al.*, 2022). *Orthotospovirus arachinecrosis* commonly known as Groundnut Bud Necrosis Virus (GBNV) belongs to genus *Orthotospovirus*, family: *Tospoviridae* and order *Bunyavirales*. The GBNV virion is quasispherical, with a diameter ranging from 80 to 110 nm and contains a tripartite negative and ambisense RNA genome with five open reading frames. Each virus particle is composed of a granular core of nucleocapsids comprising a phospholipid membrane bounded by a lipid envelope (5 nm thick) covered with surface projections.

2. MATERIAL AND METHODS

Sap inoculation and pathogenicity test

Necrosis disease infected blackgram samples were collected from farmers' fields of Krishna district of Andhra Pradesh, and used for establishment of virus on indicator assay host cowpea (*Vigna ungiculata* cv. C-152) through mechanical sap inoculation. Healthy and clean cowpea seeds (10 to 12 seeds per pot) were sown in the plastic pots and maintained under protected conditions in green house located at RARS, Tirupati. Cowpea seedlings at cotyledonary leaf stage with uniform growth were selected for further artificial sap inoculation.

The sap transmission of GBNV was carried out by extraction of crude sap from infected blackgram leaves using 0.05 M Potassium phosphate buffer (pH- 7.0) with 0.1 % ß-mercaptoethanol in pre-chilled pestle and mortar. Seven-days old cowpea plants were pre-dusted with celite powder and the infective sap was gently rubbed unidirectionally on the adaxial leaf surface. The inoculated leaves were washed with sterile distilled water to remove excess of inoculum and abrasive agent and kept for observation. Similarly, the virus was inoculated in blackgram plants at two to three trifoliate stage (25 days old) to confirm the pathogenicity of the virus.

RNA isolation and cDNA synthesis:

The inoculum of GBNV blackgram isolate was maintained on cowpea cv. C-152. Total RNA was extracted from 100 mg infected leaves using GeneJET RNA Purification Kit from Thermo Scientific™ following manufacturer's protocol and resuspended in 40µl nuclease free water. Total RNA was quantified using nanodrop and the quality of RNA was assessed by A260/A280 ratio. RNA samples with values ranging between 2.0-2.2 was used for subsequent studies. First strand cDNA synthesis was carried out using H Minus Reverse Transcriptase (RT) enzyme (Thermo scientific). The reaction mixture containing, 1500 ng of total RNA, 1.0µl of random hexamer primer were added to 8.0µl nuclease free water. The mixture was incubated at 65°C for 5 min and immediately chilled on ice for 5 min. The final reaction mixture containing RNA, random hexamer-1.0 µl and nuclease free water-8.0µl, Reaction buffer 5X-4.0µl, Ribolock (20U/µl)-1.0µl, dNTPs10mM -2.0µl and RT enzyme-1.0µl was incubated at 25°C for 10 minutes followed by 42°C for 60 minutes. The reaction was stopped by heating the mixture at 70°C for 10 min in thermocycler. The cDNA obtained was used to amplify the genomic components of GBNV.

Amplification of genomic components of GBNV through RT-PCR:

Molecular characterization of *Orthotospovirus* was established using GBNV specific upstream (-5'ATGTCTAMCGTYAAGCAVCTHAMCG3') and downstream (5'TTACAMTTCCARMGAAGKRCHAG3') primers amplifying the coat protein gene (Gurupad and Patil., 2013). PCR reaction was performed in a 25 µl final volume of PCR mix containing 10x PCR reaction buffer, 2.5 mM of $MgCl_2$, 10 mM of dNTPs, 10 pM of gene specific forward and reverse primer, 2.5 units of Taq DNA polymerase and 100 ng of cDNA template. The RT-PCR was performed in Applied Biosystems ProFlex PCR System with the following thermal programme: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles comprising denaturation at 94°C for 45 seconds, annealing at 49°C for 45 seconds & extension at 72°C for 1 min and a final extension at 72°C for 10 minutes.

Resulted amplicon was analysed on 1.2 % agarose gels stained with ethidium bromide and viewed under gel documentation unit.

Sequence diversity and phylogenetic analysis:

The purified DNA of Coat protein/N gene were sequenced at commercial sequencing facility (Eurofins India, Bangalore). Sequence assembling, nucleotide alignment and per cent identity matrix were done with BIOEDIT software (Hall, 1999). The nucleotide sequences were deposited in the NCBI database and accession numbers were obtained. Phylogenetic analysis of the sequence was carried out by comparing the sequence to other isolates retrieved from the NCBI database with 1,000 bootstrap replicates using MEGA 7 software (version 7.0.14). Tomato Spotted Wilt Virus isolate (KY563094) was used as an out group member for rooting the phylogenetic tree of the genus *Orthotospovirus.*

3. RESULTS AND DISCUSSION

Sap inoculation and Pathogenicity test:

The blackgram plants showing characteristic symptoms of necrosis disease like necrosis of midrib and secondary veins, petioles necrosis, necrosis of the apical bud, necrosis of the unfolded leaves and twisting & downward curling of the leaf lamina were collected and inoculated on cowpea cv. C-152 plants at primary leaf stage through mechanical sap inoculation. Similarly, sap inoculation was carried out on primary host *ie.*, blackgram at two-three trifoliate leaf stage for proving pathogenicity of GBNV. The assay host cowpea cv. C-152 produced localized chlorotic spots, systemic chlorotic ring spots and necrotic ring spots (Figure 1) at seven days after inoculation. Primary leaves of cowpea with localized necrotic spots further developed premature yellowing and subsequent leaf fall leading to complete death of seedlings at 12-14 days post inoculation. Blackgram (LBG-645) plants inoculated with virus induced chlorosis and necrosis of the mid rib and marginal veins after 14 DAI, while systemic infection resulted in apical bud necrosis, petiole necrosis, necrotic streaks on stem at 16 DAI (Figure 2). Infected plant with apical bud necrosis showed reduced growth and death of plants. Symptoms induced by GBNV in cowpea and blackgram include chlorotic spots, necrotic spots, apical bud necrosis, stem necrosis and stunting of plants, were also observed by other researchers. Similarly, Jyothirmai *et al.,* (2023) observed that inoculating blackgram with GBNV resulted in symptoms such as, chlorotic and necrotic spots, necrosis and death of terminal bud and downward curling of leaves with necrotic veins similar to field symptoms. Akram *et al.,* (2013) reported that inoculation of

GBNV on 43 wild accessions of *Vigna* spp. exhibited chlorotic spots surrounded by irregular brown necrotic margins on leaves and necrosis of petioles and stems.

Figure 1. Local and systemic symptom expression of GBNV in mechanically inoculated cowpea variety C-152. A) necrotic local lesions on primary leaves; B) systemic chlorotic ring spots on young trifoliate leaves; C) premature yellowing of primary leaves.

Figure 2. Symptoms of GBNV on blackgram after sap inoculation. A) necrotic spots on trifoliate leaves after 14 days post inoculation; B) veinal chlorosis and downward curling of leaves; C) veinal necrosis and necrosis of young leaves

Molecular detection of GBNV in infected plants:

Total RNA extracted from cowpea and blackgram plants infected with GBNV-inoculum was subjected to RT-PCR using GBNV gene specific primers. Symptomatic leaves of cowpea and blackgram confirmed the presence of highly conserved coat protein gene of GBNV, with the amplicon size of 800 bp (Figure 3). The amplified DNA of coat protein fragments were sequenced and confirmed the association of GBNV with necrosis disease of blackgram. The sequences of present study are available in NCBI database under accession numbers OQ683310 and OR295403 respectively. The BLASTn search of coat protein gene revealed >95 % identity with the other available GBNV sequences in the NCBI database.

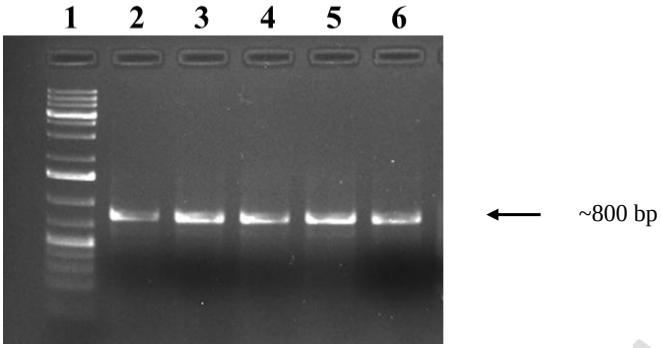

Figure 3. RT-PCR amplification of GBNV coat protein gene from infected leaves of cowpea and blackgram. Lane 1: 1 Kb plus ladder; Lanes 2-3: amplified DNA fragment from infected samples of cowpea; Lane 4-6 amplified DNA fragment from infected samples of blackgram.

Sequence identity and phylogenetic analysis:

The nucleotide and amino acid sequences of coat protein of the isolates from this study were compared with the sequences of other isolates of GBNV retrieved from the database. The sequence identity matrix of the nucleotide and amino acid sequence revealed that the GBNV blackgram isolates from Krishna district (OQ683310, OR295403) shared 92.3-99.3 % identity at nucleotide level and 93.4-100 % identity at amino acid level. GBNV-BG isolate (OQ683310) shared a maximum of 98.0 % sequence identity with the MZ505083-tomato isolate from Tamil Nadu, while, GBNV-BG isolate (OR295403) shared a maximum sequence identity of 99.3 % at nucleotide level with tomato isolate MN718651-Tomato-Tamil Nadu (Table 1). Phylogeny analysis revealed that present study isolates of GBNV were grouped into a single cluster with multiple other GBNV isolates, whereas TSWV isolate was used as an outgroup. The present study isolates (OQ683310, OR295403) were closely related to GBNV tomato isolate from Tamil Nadu (MN718651 and MZ505083) (Figure 4). The highly-conserved coat protein sequences of present study isolates show a high degree of identity with the GBNV sequences available in the database, with very little variation in the sequences. Similar results were reported by Sujitha *et al*. (2012) in onion infected with GBNV under natural field conditions, using coat protein gene specific primers. Sequence analysis showed 93-100 % and 95-100 % identity at nucleotide and amino acid levels respectively with other reported GBNV isolates. Sivaprasad *et al*. (2013) studied the sequence diversity of the coat protein of GBNV isolates infecting

groundnut, brinjal, tomato, blackgram, fieldbean, cowpea, cotton, jute, taro and calotropis. Sequence identity analysis of all GBNV isolates revealed 93-99 % identity at nucleotide level and 95–99 % identity at amino acid levels. Rajamanickam *et al*. (2020) observed 91.5-100 % sequence similarity at nucleotide level and 91.3-100 % identity at amino acid level in the coat protein gene of GBNV chilli isolate on comparison with other GBNV isolates.

Figure 4. Phylogenetic relationship of GBNV-BG isolate (OQ683310 and OR295403) from Krishna district of Andhra Pradesh with other GBNV isolates reported based on coat protein gene. Phylogenetic tree was generated using MEGA 7 software using maximum likelihood tree with 1000 replicates and TSWV (KY563094) isolate used as out group.

Virus sequences from NCBI	NUCLEOTIDE		AMINOACID	
	OQ683310	**OR295403**	**OQ683310**	**OR295403**
MZ505083-Tomato-Coimbatore-TN	98.0	98.9	97.4	98.9
MN735633-Tomato-Vengal-TN	97.8	98.9	97.4	98.9
AY472081-Tomato-Coimbatore-TN	97.5	98.6	97.4	98.9
DQ058078-Cowpea-Coimbatore-TN	97.5	98.6	97.8	99.2
MN718651-Tomato-Anandur-TN	97.5	99.3	97.1	100.0
MN735610-Chilli-Nagalapuram-AP	97.3	98.4	97.4	99.6
KX244332-Black night shade-Anandur-TN	97.2	98.5	97.4	98.9

MN735659-Chilli-Mysore-KA	97.1	98.4	97.1	98.5
KX244330-Brinjal	97.1	98.1	96.0	98.1
AY426317-Cotton	96.0	96.9	97.1	98.5
AF515819-Cowpea	95.9	96.9	96.3	97.8
MK617533-Chilli-Guntur	95.4	96.2	94.2	95.6
KX244334-Black night shade-Hessaraghatta-KA	93.1	93.9	94.2	95.6
AY512651-Carrot-Hyderabad-TG	93.2	94.3	94.5	96.0
JF281104-Pea-Shahjahanpur-UP	93.2	94.1	94.5	96.0
JQ269832-Onion-Kadapa-AP	92.3	93.2	93.4	94.9
JQ809456-Onion-Vizag-AP	92.4	93.3	93.4	94.9
HM770020-Groundnut-Coimbatore-TN	92.6	93.6	93.8	95.2
KX832987-Gerbera-KA	94.5	95.7	95.2	97.4

Table 1. Nucleotide (nt) and amino acid (aa) identities of the coat protein gene of GBNV-BG isolates (OQ683310 and OR295403) with corresponding sequences of selected isolates of GBNV from various hosts.

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CONCLUSION

In the present study, PCR based detection of GBNV virus confirmed the association of an isolate of GBNV with necrosis disease of blackgram in Krishna district of Andhra Pradesh. The pathogenicity of the GBNV-BG isolate was proved through mechanical sap inoculation on cowpea and blackgram. RT-PCR analysis confirmed the presence of GBNV in both cowpea and blackgram yielding an amplicon of 800 bp. Coat protein gene of GBNV isolates from Krishna district of Andhra Pradesh on comparision with other GBNV isolates from different crops have shown high sequence identity at nucleotide and aminoacid levels. Phylogeny analysis revealed that present study isolates of GBNV were grouped into a single cluster with multiple other GBNV isolates and closely linked with tomato-GBNV isolate from Tamil Nadu.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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