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MOLECULAR DETECTION OF BEGOMOVIRUS ASSOCIATED WITH BEAN YELLOWING DISEASE IN SRI LANKA

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ABSTRACT

Bean (*Phaseolus vulgaris* L.) is one of the most important vegetable crops commercially grown in Sri Lanka covering approximately 9000 ha. A new disease of bean showing virus-like mosaic symptoms was first reported from the Balangoda area in the Uva Province of Sri Lanka in 1999. The disease has subsequently spread to the most of the major bean-growing areas causing substantial economical losses from bean production. The disease was reported to be incited by a bipartite begomovirus transmitted by whitefly (*Bemisia tabaci*) in a semi-persistent manner. The typical succession of symptoms included diffuse yellow spots, and bright yellow mosaic patterns on leaves, which eventually become necrotic and dry on the plants. The experimentally inoculated bean plants showed similar symptoms of naturally infected plants. Leaf samples were collected from both bush and pole beans of infected and apparently healthy plants from fields, and healthy plants grown in insect proof net houses. The total DNA was extracted separately from each sample using modified CTAB method. Polymerase Chain Reaction (PCR) was carried out using a pair of begomovirus specific regenerate primers using 1:25 diluted total DNA preparations as template DNA. The core Coat Protein (CP) gene fragment of 520 bases was consistently amplified from naturally as well as the whitefly-inoculated plants. The study demonstrated the potential use of PCR with a selected primer set that is specific and sensitive for detecting begomovirus associated with Bean Yellowing Disease (BYD) in Sri Lanka.

KEYWORDS: Bean yellowing disease, Molecular detection, PCR, Begomovirus, Whitefly transmission

INTRODUCTION

Bean (*Phaseolus vulgaris* L.) is affected by many infectious diseases caused by fungi, bacteria, viruses and phytoplasmas. The natural occurrence of several viruses including common bean mosaic virus (CBMV), bean yellow mosaic virus (BYMV), cucumber mosaic virus (CMV), clover yellow vein virus (CYVV) and bean golden mosaic virus (BGMV) has been reported by different workers (Zitter and Provvidenti, 1984; Blair *et al.*, 1995).

A new bean disease termed "Bean Yellowing Disease (BYD)" was first reported in 1999 from the fields of Balangoda area in Sri Lanka and subsequently spread to all major beans-growing areas causing heavy economic losses. The disease was observed in plants of varying ages from two weeks to maturity (Polston

et al., 2009). The most common symptoms included a bright yellow mosaic pattern on the leaves, rugosity, reduced leaf size and stunted growth of the entire plant (Yimalan, 2011). Epidemics of BYD can result in 75-95 % yield loss leading to severe economic consequences (Pallemulla, 2012). No resistant bean varieties have been found from among the varieties cultivated in Sri Lanka (Yimalan, 2011).

The rolling circle amplification using Phi-29 and subsequent amplification of full genome revealed the involvement of bipartite begomovirus consisting of DNA-A and DNA-B genome components as the causal agent of BYD in Sri Lanka. Further it showed a high sequence identity to the horsegram (*Macrotyloma uniflorum*) yellow mosaic virus. Thus, the virus causing BYD in Sri Lanka has been named as "horsegram yellow mosaic virus – Sri Lanka: 2009" (Monger *et al.*, 2010). The virus causing horsegram yellow mosaic disease has been shown to be a typical old world bipartite begomovirus (Abouzid *et al.*, 2010). As the DNA-A genome showed less than 89 % identity with the corresponding sequences of all the begomoviruses reported earlier, the yellow mosaic virus (HgYMV-[IN:Coi]) was considered to be a new species of the genus *Begomovirus* (family *Geminiviridae*) (Abouzid *et al.*, 2010).

Involvement of whitefly (*Bemisia tabaci*; order: Hemiptera, family: Aleyrodidae) in transmission of BYD in Sri Lanka was reported previously (Anonymous, 2006). Begomoviruses are transmitted by whiteflies. All begomoviruses code for coat protein, which act as the protective coat of the virus particle and determine vector transmitability of the viruses by whitefly vector. Thus, the CP gene is highly conserved among begomoviruses originating from the same geographical region and adapted to transmission by local vector populations (Maruthi *et al.*, 2002).

Early and accurate diagnosis of a disease is the crucial component of any crop disease management system (McMillan *et al.*, 1988). Symptoms of BYD somewhat vary between plants (Reeder and Kelly, 2009). The reliance on symptoms is often not adequate as the viral disease may be well underway when symptoms first appear and the symptom expression can be variable. Though biological techniques of this viral disease diagnosis using whitefly vector are accurate, it is too slow and not amenable to large scale application.

Advances in molecular biology are being applied to the development of rapid, specific and sensitive tools for the detection of plant pathogens (McMillan *et al.*, 1988). Begomoviruses have been detected in plants by serological assays (Pico *et al.*, 1999) and Polymerase Chain Reaction (PCR) (Deng *et al.*, 1994; Rosell *et al.*,

1999). Polston *et al.* (2009) collected several samples of bean with characteristic symptoms of BYD from different parts of Sri Lanka, subjected to ELISA with broad spectrum antiserum for begomovirus, and found that all tested samples were positive for begomoviruses No molecular method for detection of begomovirus causing BYD has been developed to-date in Sri Lanka. The present study was thus undertaken to develop molecular technologies for diagnosis of begomoviruses-associated with BYD in Sri Lanka.

MATERIALS AND METHODS

Virus isolates and transmission tests

The samples were collected from infected bean (*Phaseolus vulgaris* L.) plants grown in experimental plots at the Horticultural Crops Research and Development Institute (HORDI) at Gannoruwa, Peradeniya, Sri Lanka. The transmission of begomovirus of bean was carried out during January 2012 using the whitefly vector *Bemisia tabaci* collected from a pure colony that had been maintained on tobacco plants at the HORDI. Whiteflies were released on infected bean plants for 24 hrs for virus acquisition. Viruliferous whiteflies were then released on to 7 day-old healthy bean seedlings maintained in insect proof cages at the rate of 10 viruliferous whiteflies per plant for 48 hrs. The inoculated plants were then sprayed with a systemic insecticide (Imidacloprid 200 g L^{-1} SL at the rate of 1 ml L^{-1}) to kill the insects. The inoculated plants under insect proof conditions were observed for symptom development.

Total DNA extraction

Total DNA was extracted from the bean leaf tissues collected from BYD-infected bean plants from HORDI,and healthy bean plants separately using the method proposed by Lodhi *et al.* (1994) with some modifications. Leaf tissue (150 mg) was homogenized with 1500 µl of pre-heated extraction buffer (65 °C for 10 min in a water bath) containing 1.4 M NaCl, 20 mM EDTA (pH=8.0), 100 mM Tris HCl (pH= 8.0), 2 % CTAB and 0.2 % β-mercaptoethanol. The mixture was incubated at 65 °C for 30 min. The supernatant was transferred to a new Eppendorf tube and equal volume of 24:1 mixture of chloroform:Isoamyl alcohol was added.

The content was centrifuged for 15 min at 10,000 rpm. The supernatant was then transferred to a fresh tube and add 300 µl chilled isopropanol and incubated for overnight at -20 °C to precipitate DNA. The content was centrifuged for 15 min at 10,000 rpm for forming a pellet. The pellet was washed with 500 µl of 70 % ethanol and dried under a fan until traces of ethanol disappeared. The pellet was dissolved

in 40 µl of $T_{10}E_{0.1}$ buffer containing 10 mM Tris HCl (pH=8.0) and 0.1 mM EDTA (pH=8.0). The DNA preparations were quantified using the spectrophotometer method. The extracted DNA and its dilutions were used for PCR amplifications as template DNA.

PCR detection of the virus

A set of begomovirus group specific universal primers [Deng A: 5'-TAATATTACCKGWKGVCCSC-3' (20 nt) and Deng B: 5'-TGGACYTTRCAWGGBCCTTCACA-3' (23 nt)] previously used by Deng *et al.* (1994) was used for the detection of begomoviruses through PCR. These primers are capable of amplifying the core CP region of begomoviruses.

The PCR amplifications were conducted in a thermo-cycler in 25 µl reaction mixture that contained 2.0 µl of total DNA extracted from the infected bean leaf tissues and diluted up to 1:25 (80-100 ng), 0.2 µl Taq DNA polymerase (5U μl^{-1}), 2.5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µl of 25 mM $MgCl_2$, 2.0 µl each primer (10 mM), 2.0 µl dNTPs mix (2.5 mM each) and sterile water to make up the volume. The mixture was subjected to one cycle of initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 45 sec and a final extension at 72 °C for 10 min. The PCR products were analyzed by 1.0 % agarose gel electrophoresis at 60V for 1 hr in 1X TBE buffer with a loading of 5.0 µl product per well. The gel was previously stained with ethidium bromide at 0.5 µg ml^{-1}. The size of the amplification products was estimated from 1 kb DNA ladder (Fermentas, GmbH, Germany). The DNA from healthy plants and double distilled water were used instead of template DNA as experimental controls.

RESULTS AND DISCUSSION

Disease symptoms and virus transmission

The initial symptoms appeared as diffuse yellow spots on the leaf (Photo 1), which later developed into a bright yellow mosaic pattern (Photo 2). Depending on the time of infection, symptoms were apparent on new flushes while earlier leaves did not show the mosaic symptoms. The younger trifoliate leaves emerged from infected plants showed downward curling after the appearance of chlorosis. Such curled leaves failed to expand properly and their surface turned stiff and leathery. The leaves with chlorosis and distortion may eventually become necrotic and dry on

the plant (Photo 3). The symptoms observed in these bean fields strongly resembled the bean viral disease caused by bean golden yellow mosaic virus, which belongs to the taxonomic family *Geminiviridae* and genus *Begomovirus*, naturally transmitted by white fly *Bemisia tabaci* (Hall, 1991). Bean golden mosaic virus was reported in common bean (*Phaseolus vulgaris*) and lima beans (*P. lunatus*) in USA (Blair *et al.*, 1995).

Photo 1. Infected bean plants showing early symptoms of isolated diffused yellow spots on leaves

Photo 2. Infected plant showing mosaic symptoms on leaves

Photo 3. Necrotic lesions developed on mosaic leaves at the later stages of disease

The disease was successfully transmitted to healthy bean plants using the whitefly vector *Bemisia tabaci* (Photo 4). The healthy bean seedlings inoculated with viruliferous whiteflies (ten adults per plant) developed symptoms similar to those of naturally-infected plants in the field. Virus-like symptoms including leaf yellowing, mottling and distortion were the initial symptoms developed on inoculated plants (Photo 5).

Photo 4. Pure culture of whiteflies (*Bemisia tabaci*) maintained on tobacco plants under insect proof cages

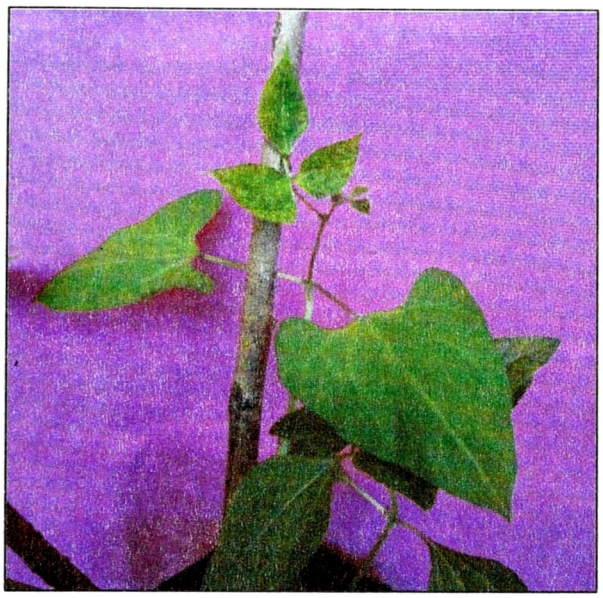

Photo 5. Tender most leaves of whitefly inoculated bean plant showing early symptoms of bean yellowing disease

Transmission of the virus to an extent of 76 to 100 % was achieved with ten adult whiteflies that were allowed 24 hr of Acquisition Access Period (AAP) and 48 hrs of Inoculation Access Period (IAP). The results were comparable with that of begomovirus-like tomato leaf curl virus (Muniyappa *et al.,* 2000), pumpkin yellow vein mosaic virus (Muniyappa *et al.,* 2003), and sunflower leaf curl virus (Govindaappa *et al.,* 2011). The TYLCV-Is that was found to spread in all regions of Spain, was also shown to be the causal agent of bean leaf crumple, which has caused severe economic losses in common bean crops (Navas-Castillo *et al.,* 1999).

PCR detection of the virus

The PCR tests carried out using a set of degenerate primers successfully amplified expected DNA fragments of 520 bp from both naturally- and whitefly-inoculated bean plants (Photo 6). This is the first report on molecular detection of begamovirus associated with bean yellowing disease infected plants in Sri Lankan laboratory. However, false negatives were observed when undiluted DNA preparations and 1:10 diluted DNA were used as a template indicating the failure of amplification of 520 bp fragment (data not shown). The amplification was resumed when 1:25 and 1:50 diluted DNA preparations were used as template DNA. The PCR inhibitors in the samples hinder the sensitivity and reliability of the PCR technique (Bertolini *et al.,* 2003).

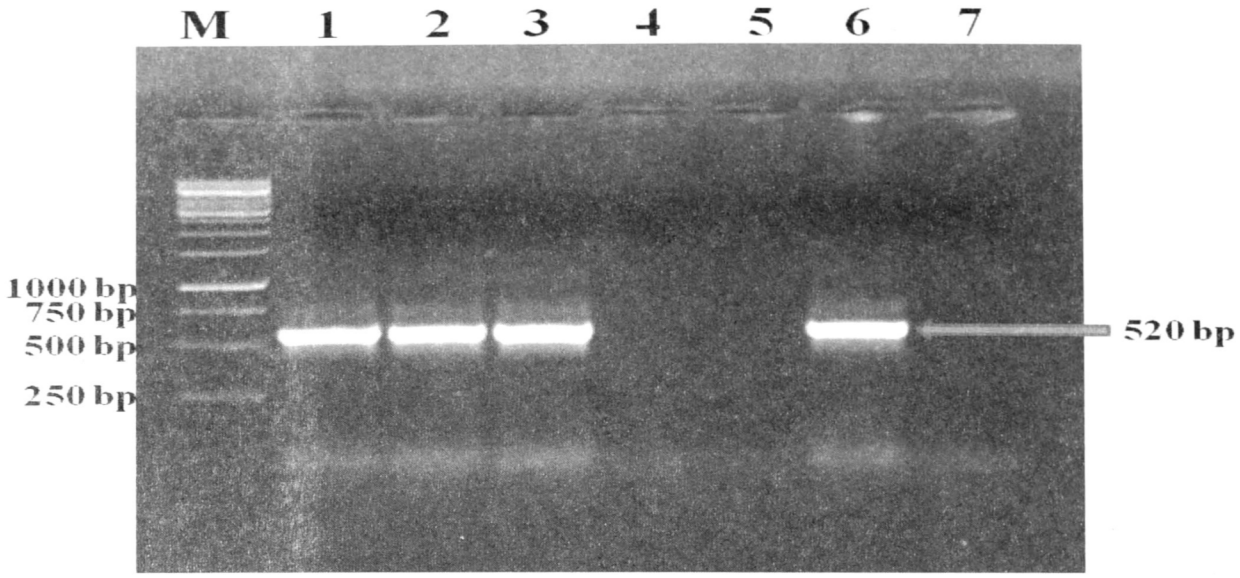

Photo 6. PCR Amplification of 520 bp region of core CP gene from different bean yellowing disease samples using begomovirus specific degenerate primers. Lane M: 1 kb DNA ladder; lane 1: Bean yellowing disease infected pole bean sample; lane 2: Bean yellowing disease infected bush bean sample; lane 3: Whitefly inoculated pole bean sample; lane 4: healthy pole bean; lane 5: healthy bush bean; lane 6: apparently healthy pole bean sample (latent infection); lane 7: Water control

Factors that inhibit the amplification of nucleic acids by PCR are present with target DNAs from many sources. The inhibitors generally can inhibit nucleic acid capture and polymerase activity for amplification of target DNA. The multiple extraction steps in DNA extraction procedures with organic solvents (phenol, chloroform), detergents (CTAB, SDS), salts (NaCl, ammonium acetate), and/or polyvinylpyrrollidone (PVP) to remove polysaccharides and polyphenolic components can inhibit enzymatic reactions (Wilson, 1997). The dilution of total DNA extracted can also lead to dilution of possible inhibitors in the sample to the desirable threshold level that does not inhibit the PCR amplification. Dilution from 1:25 to 1:50 gave PCR amplification. According to Verkooyen *et al.* (1996), 1:10 dilution of the processed clinical DNA samples was used and reduced inhibition of PCR for cervical specimens from 19 to 9.3 %. The primers used in this study have previously been used extensively for the identification of begomoviruses in a wide range of crop plants and their vector *B. tabaci* previously (Deng *et al.,* 1994; Govindappa *et al.*, 2011).

CONCLUSIONS

The Bean Yellowing Disease (BYD) in Sri Lanka was confirmed to be caused by begomovirus based on symptomatolgy, transmission studies of the virus through *B. tabaci* and PCR detection of the begomovirus-specific DNA products

from bean plants with characteristic symptoms. The PCR technique consisting of simplified extraction of total DNA, dilution of DNA up to 1:50 and use of selected primers can effectively be used for rapid and accurate detection of begomovirus in beans. In addition to the high sensitivity, PCR facilitate the analysis of sequence data for future studies. The disease assumes greater significance because of the preference of *B. tabaci* (10 adults per plant) and its efficient transmission.

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