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Immunocapture and simple-direct-tube-reverse transcriptase polymerase chain reaction (RT-PCR) for detection of *Rice yellow mottle virus*

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This study aimed at optimizing the immunocapture (IC) and simple-direct-tube (SDT) -reverse transcriptase polymerase chain reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) in order to avoid the extraction of high quality RNA required for molecular methods and avoid costs involved. *Rice yellow mottle virus* strains and phylotypes were obtained from infected rice leaf samples collected from Morogoro, Arusha and Kilimanjaro regions. The efficacy and sensitivity of IC and SDT methods was demonstrated using the aliquots from infected plant sap obtained by grinding rice leaves and binding onto PCR tube using coating buffer and in phosphate buffer saline with 0.5% Tween-20 (PBST 1X), respectively, and assayed by RT-PCR with RYMVIIIF/RYMVIIR primers. Analysis of the PCR product was performed by electrophoresis on 1% agarose gel, pre-stained with 2.5 µl of ethidium bromide (10 µg of ethidium bromide per ml of 0.5x Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer) at 100 V cm^{-1} for 30 min and visualized under UV light. The results indicate that SDT-RT-PCR and IC-RT-PCR detected RYMV in all tested infected leaf samples at the expected band size of 720 bp and had the same sensitivity as virus extraction RNA-RT PCR technique, implying that the methods can be useful for detection of wide range of RYMV strains. The negative control did not yield any amplicons. The results also show that these techniques are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. However, SDT protocol was easier and faster than IC and it was also cost-effective in terms of reagents for the detection of RYMV.

Key words: *Rice yellow mottle virus*, detection, immunocapture-RT-PCR, simple-direct-tube-RT-PCR.

INTRODUCTION

Rice yellow mottle virus (RYMV) is a variable and very damaging rice disease in Africa. A number of biotic stresses including RYMV have been reported to contribute to low yields of rice in Africa (Lamo et al.,

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2015). Yield estimates of 2.5 t ha^{-1} have been reported in Africa compared to 4.4 t ha^{-1} reported in Asia (Wilson and Lewis, 2015). Rice is an important staple food crop and is affected by RYMV disease with suspected high yield losses ranging from 25 to 100% (Kouassi et al., 2005; Luzi-Kihupi et al., 2009). The virus disease was first recorded in 1966 at Otonglo near Lake Victoria, Kenya (Bakker, 1970) and until now the RYMV is present in most of the rice-growing countries in Africa and Madagascar (Abo et al., 1998; Kouassi et al., 2005; Séré et al., 2008; Traoré et al., 2009). The virus belongs to the genus *Sobemovirus* (Tamm and Truve, 2000; Fauquet et al., 2005) and is characterized by icosahedral particles of about 30 nm in diameter (Fauquet et al., 2005) and one single strand positive sense genomic ribonucleic acid (RNA). The virus is associated with viroid-like satellite RNA ranging from 220 to 390 nucleotides (Tamm and Truve, 2000).

The RYMV is highly diverse in nature due to high rates of its mutation and recombination (Hébrard et al., 2006). The subgroup diversity of RYMV strains was determined serologically (N'Guessan et al., 2000) and molecularly by RNA sequencing using primers that target sequences encoding coat protein (Pinel et al., 2000). The studies indicated that RYMV encompasses six strains, each having a specific and restricted geographical range. Strains S1, S2 and S3 have been reported to exist in West Africa with latitudes and longitudes between 4°N and 28°N and 15°E and 16°W, respectively. Strains S4, S5 and S6 are reported to dominate in East Africa which lies between latitudes 23°N and 12°S and longitudes 22°E and 51°E (Fargette et al., 2002; Abubakar et al., 2003; Banwo et al., 2004; Kanyeka et al., 2007).

Tanzania has been reported as one of the biodiversity hotspot of RYMV. The emergence of new virulent phylotypes of RYMV in rice crop in different geographical areas is increasing and becoming a common occurrence (N'guessan et al., 2001; Kanyeka et al., 2007; Pinel-Galzi et al., 2007; Ochola et al., 2015), possibly as a consequence of the constantly growing susceptible varieties (Hubert et al., 2016). Therefore, such situations constitute a gap for the demand for reliable methods of sensitive but also rapid detection of these new RYMV phylotypes for control of RYMV in order to improve rice productivity.

Variations of enzyme-linked immunosorbent assay (ELISA) are currently used for large-scale routine testing for viruses. Recently, new ELISA methods for detection of *Rice dwarf virus* (RDV) in rice such as plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and dot enzyme-linked immunosorbent assay (dot-ELISA) have been developed (Wu et al., 2014). However, established antigen-trapping ELISA and virus amplification techniques for virus diagnosis require the presence of intact live virus, which depends on sample quality. This situation raised necessity of complementary PCR-based methods such as immuno-capture- and simple-direct-tube RT-PCR (Silva et al., 2011). One area where PCR has shown great value is the detection of viral pathogens, particularly those for which culturing is difficult or where serologically based detection systems are inadequate (Chandler et al., 1998). Polymerase chain reaction has been used successfully to detect a range of viruses including both DNA and RNA viruses (Chandler et al., 1998). However, detection of viral RNA by PCR requires reverse transcription (RT) of viral RNA prior to the reaction (Lopez et al., 2003). Rice viruses, except *Rice tungro bacilliform virus* (RTBV), are RNA viruses, and synthesis of cDNA of the viral genome by reverse transcription (RT) is necessary before the target RNA sequence is amplified (Uehara-Ichiki et al., 2013).

Several variations of RT-PCR have been developed, including nested- (Kiliç and Yardimci, 2012), one step- (Uga and Tsuda, 2005), multiplex- (Grieco and Gallitelli, 1999), real-time (Osman et al., 2007), immunocupture- (Wetzel et al., 1992) and simple-direct-tube RT-PCR (Suehiro et al., 2005). Immuno-capture-RT PCR is designed for highly specific detection of templates present in very low amounts (Wetzel et al., 1992). This method combines capture of virus particles by antibodies through the practical conditions of ELISA with amplification by RT-PCR. In this assay the virus particles are trapped onto a micro centrifuge tube or ELISA plate using virus-specific antibodies. The adsorbed virus particles are disrupted and the released viral nucleic acid is amplified by RT-PCR. Immunocapture (IC)-RT-PCR has been used successfully to detect several plant viruses such as *banana streak virus*, *beet necrotic yellow vein virus*, *cassava common mosaic virus*, *cucumber mosaic virus*, *lily symptomless virus, lily mottle virus*, *pepino mosaic virus*, *plum pox virus* and *sugarcane streak mosaic virus* (Wetzel et al., 1992; Harper et al., 1999; Hema et al., 2003; Mansilla et al., 2003; Ling et al., 2007; Zein et al., 2008; Silva et al., 2011; Ha et al., 2012; Kiliç, and Yardimci; 2012). Generally, immunocapture-RT-PCR protocol has been established for the virus sensitivity improvement in detection (Silva et al., 2011; Yang et al., 2012).

Preparation of plant extracts is a critical aspect of RT-PCR (Rowhani et al., 2004). Nucleic acid extraction from plant tissues is the most laborious, costly and time-consuming step in the detection of a virus (Rowhani et al., 2004). As the RT-PCR is effective in detecting several plant viruses, attempts have been made in this study to optimize simple and rapid techniques such as simple-direct-tube (SDT) method for detection of RYMV. Since 1 h was required to extract viral RNAs from infected tissues, an easy and rapid procedure designated SDT method was optimized for preparing viral RNA for cDNA synthesis.

The extraction of viral RNAs using SDT method may be completed in approximately 15 min and does not require the use of antiserum, filtering and centrifugation (Suehiro et al., 2005). This protocol involves grinding of plant

tissues in phosphate buffer saline with 0.5% Tween-20 (PBST 1X) and placing the extract in a micro-centrifuge tube for two minutes and allowing adsorption of virus particles to the tube wall. Simple direct tube method was successfully used for detection of *Turnip mosaic virus*, *Cucumber mosaic virus* and *Cucumber green mottle mosaic virus* in infected plants (Kobori et al., 2005; Suehiro et al., 2005). The purpose of this work was to optimize and evaluate the efficiency of IC- and SDT- RT-PCR methods for RYMV detection in infected plant materials.

MATERIALS AND METHODS

Rice yellow mottle virus strains used in this study

Infected leaves of RYMV isolates obtained from different strains used for IC and SDT-RT-PCR techniques were collected from farmers' rice fields in the cropping season, April to May 2014 in selected rice growing areas in Tanzania (Hubert et al., 2017). Isolation and characterization of RYMV isolates was done at Institut de Recherche pour le Developpment/French National Research Institute of Sustainable Development (IRD), France. For their characterization, total RNA was extracted with the RNeasy Plant Mini kit (Qiagen, Germany) (Pinel et al., 2000). The coat protein (CP) and the viral protein genome-linked (VPg) genes of the RYMV were amplified by RT-PCR (Fargette et al., 2002; Hébrard et al., 2006). The phylogenetic analysis for identification of RYMV strains and phylotypes was done to compare CP sequences using the maximum likelihood method with default parameters in SeaView software (Gouy et al., 2010). These include RYMV strains and phylotypes S4lm (Tz526), S5 (Tz429, Tz450) , S6c (Tz486) and S6w (Tz539) collected from Kilombero and Ulanga districts (latitudes 7° S and 9° S and longitudes 35°E and 37°E and at an elevation of about 300 m above sea level), Morogoro region. *Rice yellow mottle virus* phylotypes S4lv (Tz516) and S4ug (Tz601) collected from Arusha and Kilimanjaro regions, respectively, were also included. The viral extraction RNA of serological isolate of RYMV phylotype S4lm (Tz554) collected from Ulanga district, Morogoro region was used as control.

Immunocapture (IC) technique

Sterile PCR tubes were coated with 100 µl polyclonal antibodies in coating buffer (pH 9.6, 1:500 IgGs dilution) and incubated at 37°C for 4 h (Silva et al., 2011). The tubes were washed three and two times with phosphate buffer saline with 0.5 % Tween-20 (PBST 1X) and sterile distilled water (SDW), respectively. Two hundred milligrams of each infected leaf sample were ground separately in 400 µl extraction buffer (79.5g NaCL, 1.9 g KH_2P0_4, 11.36 g Na_2HP0_4.anhydrous, 1.93 g KCL, 2% polyvinyl pyrrolidine-PVP, 0.05% Tween 20 in 1000 ml SDW) and centrifuged at 7000 rpm for 7 minutes. Then, 100 µl of each sample supernatant was added accordingly to each PCR tube and incubated at 4°C for 12 h. The tubes were then washed three times with washing buffer and finally with SDW. The tubes were left to air dry for 2 min before proceeding to the RT-PCR step.

Simple-direct-tube (SDT) technique

Half a gram of infected leaves of RYMV isolates were ground using a mortar and pestle in a PBST 1X buffer at a ratio of 1:1 w/v (Suehiro et al., 2005). The crude sap (100 µl) was carefully placed into a sterile PCR tube using micropipette with a truncated tip and incubated at room temperature (25-30°C) for 15 min. The sap was removed from the tube and the tube was then washed twice with 100 µl PBST to remove any residual tissue. Then, 30 µl diethylpyrocarbonate-treated water containing 15 units of RNase inhibitor (DEPC, Sigma, Germany) was added to the tube and immediately denatured at 94°C for 1 min. The resulting solution in the tube was allowed to cool on ice for 1 min ready for RT-PCR.

Immunocapture (IC) RT reaction

The synthesis of complementary deoxyribonucleic acid (cDNA) was prepared in a final volume of 15 µl. A mixture of 1 µl antisense primer RYMV II at 100 µM and 9 µl sterile distilled water (SDW) per one sample was added into the tube containing the virus particles (Pinel et al., 2000; Silva et al., 2011). The mixture was denatured at 70°C for 5 min. The reverse transcriptase (RT)-cDNA synthesis 15 µl mixture of 9 µl SDW, 2 µl dNTPs (5 mM), 2.5 µl buffer RT x10 (Sigma, Munich, Germany) 1X final, 1 µl Moloney-Murine leukemia virus reverse transcriptase (M-MLV-RT) (Sigma, Munich, Germany) 200 U/µl and 0.5 µl RNase inhibitor 40U/µl (Sigma, Missouri, USA) were added to each denatured sample and incubated at 42°C for 1 h.

Simple-direct-tube (SDT) RT reaction

Reverse transcription was performed using cDNA synthesis from RYMV infected leaves (Temaja et al. 2012). Two scenarios were tested to determine the amount of template needed for optimal amplification results. These were categorized into: one plant sample in which high amount (7 µl) of final solution containing RNA was used, the other was a plant sample which has low amount (4 µl) of final solution containing RNA. This was done to determine a range of template that can be added to obtain optimal amplification. The reaction mixture consisted of total volume of 15 µl: 7 µl final solution containing viral RNA, 1 µl antisense primer 5′CTCCCCCACCCATCCCGAGAATT3′ at 100 µM, 1 µl of 0.2 M Dithiotreitol (DTT), 1 µl M-MLV- RT (sigma) 200 U/µl, 2.5 µl Buffer RT x10 (sigma) 1X final, 2 µl dNTPs 5 mM chacuum 2.0 mM final, 0.5 µl Rnase inhibitor 40 U/µl was incubated at 42°C for 1 h.

Extraction of RYMV RNA

Total RNA of RYMV was extracted from frozen infected rice leaves using the Rneasy Plant Mini Kit (Qiagen, Germany) (Pinel et al., 2000). Viral suspension were collected in 2 ml Eppendorf tube with sterile steel beads, frozen in liquid nitrogen and ground with high speed TissueLyser II mechanical shaker for 1 min at 30 rpm. The RTL lysis buffer was added, mixed by vortexing then incubated in water bath at 56°C for 2 min and centrifuged at 7000 rpm for 7 min. Tissues were separated by 225 µl of 100% ethanol followed by spinning at 10,000 rpm for 1 min, and then the supernatants were transferred into 2 ml Eppendorf tubes. Proteins of RYMV were removed by adding 700 µl RW1 and 500 µl RPE buffer, respectively and separately, centrifuged as above then the supernatant liquid was discarded and transferred into sterile 2 ml tubes. Ribonucleic acid was washed in 500 µl RPE buffer by spinning at 13,000 rpm for two minutes. Nucleic acids were eluted by 30 µl RNase free water directly to the spin column membrane and placed into clean sterile 1.5 ml tubes then centrifuged at 10,000 rpm for 1 min at 25°C. The obtained RNAs were stored in the freezer at -20°C for RT-PCR amplification.

PCR amplification of viral coat protein gene

A reaction of PCR to amplify the coat protein gene consisted of a

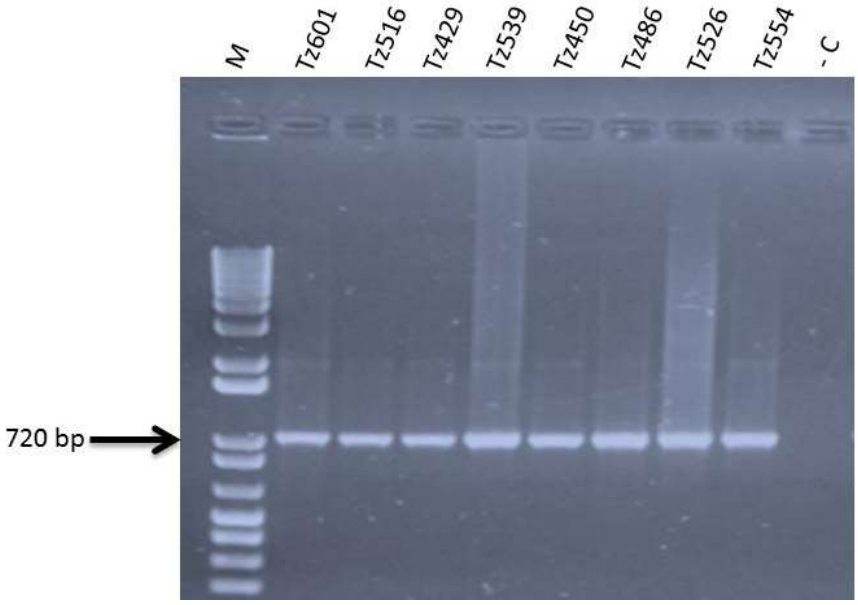

Figure 1. Immunocapture-RT-PCR amplification products obtained from *Rice yellow mottle virus* isolates of different strains S4ug (Tz601), S4lv (Tz516), S4lm (Tz526), S5 (Tz429, Tz450), S6c (Tz486), S6w (Tz539), virus extraction RNA S4lm (Tz554) and healthy plant (- C). M = DNA molecular marker (100-bp ladder). The PCR products were analyzed in 1% agarose gel.

total volume of 50 µl of a mixture (Pinel et al., 2000). The mixture included 10 µl of 5X PCR buffer, 1X final, 2 µl dNTP 5 mM chacum 2.0 mM final, 1 µl antisense primer RYMV II at 100 µM, 3 µl sense primer RYMV III at 100 µM, and 1 µl dynazyme, 30.5 µl SDW and 2.5 µl RT reaction per sample. The primer set of 5′CTCCCCCACCCATCCCGAGAATT3′ (reverse primer) and 5′CAAAGATGGCCAGGAA3′ (forward primer) were used as internal control of PCR tests to amplify the 720 nucleotide CP gene of RYMV (Fargette et al., 2002). The amplification involved three processes which were denaturation, annealing and elongation. The mixture was first heated at 94°C for 5 min to denature the sides of the double-stranded DNA. This was followed by 30 cycles whereas the mixture was heated again at 94°C for 1 min to separate the sides of the double-stranded DNA. In the same cycles, the mixture was then cooled at 55°C for 30 s to allow primers to find and bind to their complementary sequences on separated strands and elongated at 72°C for 1 min for polymerase to extend the primers into new complementary strands. The repeated heating and cooling cycles multiplied the target DNA exponentially because each new double strand separated to become two templates for further synthesis. Finally, the mixture was extended and stopped at 72°C for 10 min. The amplified PCR products were confirmed using 1% agarose gel (in 0.5X TAE buffer) electrophoresis, pre-stained with 2.5 µl ethidium bromide (10 mg/ml), and visualized under UV light.

RESULTS

The IC-RT-PCR results indicate that, all tested infected leaves of RYMV strains and phylotypes S4lv (Tz516), S4lm (Tz526), S4ug (Tz601), S5 (Tz429, Tz450), S6c (Tz486) and S6w (Tz539) yielded amplicon of the expected size at 720 bp (Figure 1). The healthy plant control (-C) gave no amplification indicating that the sample did not contain virus and that the primers did not amplify part of the plant's genome. The leaf samples infected with RYMV strain S4lm (Tz554) that RNA was extracted using RNase kit yielded amplicon at expected size of 720 bp (Figures 1 and 2).

The primers used were 3' RYMV II at 100 µM and 5' RYMV III at 100 µM in order to compare the results with the immunocapture results. The results show that the plant samples containing high amount of final solution (7 µl) yielded amplicon at expected size (720 bp) in electrophoresis analysis (Figure 2). Amplification was not obtained when low amount of template (4 µl sap) was used. The results presented in Figure 2 show that the infected leaves of different RYMV strains tested yielded amplicon of the expected size (720 bp), however, there was no amplification in the healthy control samples. Gel electrophoresis result also indicated that the strain Tz 539 of RYMV was not well amplified in SDT-RT-PCR as in the IC-RT-PCR technique. Rice leaves infected with RYMV strain S4lm (Tz554), tested by virus extraction RNA using RNase kit as a positive control was also amplified.

DISCUSSION

These results show that the IC- and SDT RT-PCR protocols optimized in this study can be used for rapid characterization of RYMV strains and are useful for

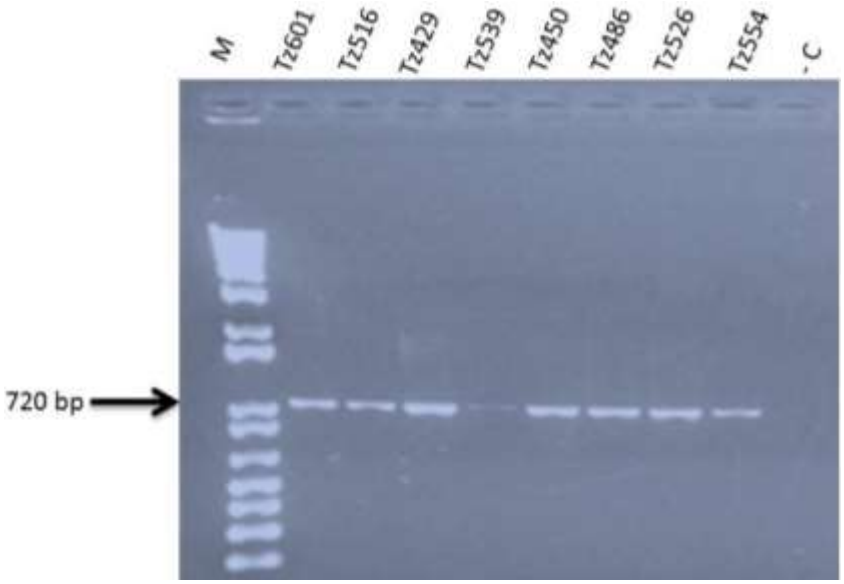

Figure 2. Simple-direct-tube-RT-PCR amplification products obtained from *Rice yellow mottle virus* isolates of different strains S4ug (Tz601), S4lv (Tz516), S4lm (Tz526), S5 (Tz429, Tz450), S6c (Tz486), S6w (Tz539), virus extraction RNA S4lm (Tz554) and healthy plant (- C). M = DNA molecular marker (100-bp ladder). The PCR products were analyzed in 1% agarose gel.

periodical surveys of RYMV. In the RT-PCR, the expected band size of 720 bp was observed in all infected rice leaves tested implying that the methods were useful and reliable for detection of a wide range of RYMV strains. This is the first report on the use SDT- and IC-RT-PCR for detection and assay of RYMV. This method has been used for detection of other plant viruses (Kobori et al., 2005; Suehiro et al., 2005; Temaja et al., 2012). The results indicate that SDT-RT-PCR and IC-RT-PCR detected RYMV in infected leaf samples similar to the virus extraction RNA-RT PCR technique.

In immunocapture, virus particles from the infected leaf isolates were trapped on the wall of an antibody coated tube and inhibitory plant extracts were removed by washing. The viral RNA released from the particles was then used as a template for cDNA synthesis using reverse transcriptase. The resulting cDNA was amplified in PCR with the RYMV conserved primers, antisense primer RYMV II and sense primer RYMV III at 100 µM (Fargette et al., 2002). Immunocapture RT-PCR technique has been utilized widely for detection of other plant viruses (Wetzel et al., 1992; Harper et al., 1999; Hema et al., 2003; Mansilla et al., 2003; Zein et al., 2008; Silva et al., 2011; Kiliç, and Yardimci; 2012; Sarovar et al., 2010; Mallik et al., 2012; Uehara-Ichiki et al., 2013). This technique has been reported to have higher sensitivity than Passive Haemagglutination Assay (PHA) and Latex Agglutination Reaction (LAR) and could detect the target virus in leaf extracts diluted from 10^{-3} to 10^{-5} (Uehara-Ichiki et al., 2013). The sensitivity of using ELISA coupled with RT-PCR has been demonstrated to be greater than just total nucleic acid extraction due to the immunocapture enrichment of samples prior to RT-PCR (Kogovsek et al., 2008; Yang et al., 2012). These findings support those of Ptacek et al. (2002) that immunocapture followed by the detection of viruses using RT-PCR is a versatile and sensitive diagnostic technique. The technique has also been used for detection of the virus in plant species or tissues that inhibit the PCR reaction and molecular detection (Ptacek et al., 2002).

In this study, the amount of sap added to the RT reaction play an important role in amplification, thus it was necessary to determine the amount of template needed for optimal RNA amplification. For instance, in this study, two scenarios were tested, one plant sample in which high amount (7 µl) of final solution containing RNA occurs and the second included plant samples with low amount (4 µl) of final solution containing RNA. This was done to determine a range of template that can be added to obtain optimal amplification. The results showed that, the plant sample containing high amount (7 µl) of final solution yielded amplicon at the size of 720 bp in electrophoresis analysis compared to low amount of final volumes, implying that amplification can be improved with high volumes as used in this study.

Gel electrophoresis results indicate that the fragments of all tested RYMV strains were amplified except Tz 539 RYMV strain band was faint in SDT-RT-PCR as compared to IC-RT-PCR technique, implying that the target amount of template may probably contained low virus concentration. The delay in fragment amplification may also possibly caused by the failure of primers to

anneal due to sequence variability. Chandler et al. (1998) suggest that the inhibitory effect of RT on PCR is mediated through the RT interactions with the specific messenger RNA (mRNA) or complementary DNA (cDNA) and that the inhibitory effect is dependent upon template concentration (or copy number). One of the problems encountered in the detection of *Grapevine leafroll-associated virus 3* (GLRaV-3) was the low concentration of virus in grapevine tissue (Acheche et al., 1999), which increased probability of inhibition from the RT enzyme on the PCR (Chandler et al., 1998).

Conclusion

The results of this study reveal that immunocapture (IC) and simple-direct-tube (SDT) -reverse transcriptase-polymerase chain reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. With these simple techniques, large numbers of plant samples detected using RT-PCR assays would be managed. Immunocapture and SDT methods may also reduce risks from cross-contaminations in sample process as the need short time in RNA preparation. The SDT protocol seemed to be easier, faster and cost-effective than IC-RT-PCR in terms of reagents for the detection of RYMV. However, the SDT-RT-PCR technique was not able to detect RYMV samples with low RNA concentration as compared to IC-RT-PCR. Studies may also be focused on detection of RYMV in insect vectors and other viruses using these techniques.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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