STANDARDIZATION AND IMPLEMENTATION OF THE RT-PCR METHODOLOGY FOR THE DIAGNOSTIC OF CASSAVA VIRUSES

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Figure 1. In-vitro material conservation room, CIAT.

INTRODUCTION

The *in vitro* collection of the genus *Manihot* of the Genetic Resource Program (GRP) of the International Center of Tropical Agriculture (CIAT) (Fig. 1) is currently represented by 6,643 materials; which have been registered in the Multilateral System of Access and Benefit sharing of the International Treaty on Plant Genetic Resources for Food and Agriculture. These materials must be free of quarantine diseases for distribution to users worldwide. The health certification of the cassava collection material that is done in the Germplasm Health Laboratory (LSG) of CIAT focuses in evaluating quarantine type viruses (Table1). According to the latest research, in addition to evaluating other viruses (CsXV, CsCMV, CVMV), it was necessary to implement and standardize reliable molecular methods for the detection of virus associated with different diseases that occur causing symptoms in the roots and the aerial part of the plant (Calvert et al. 2008, Carvajal et al. 2014). This study presents the standardization and implementation of molecular RT-PCR diagnostic methodology using random primers for cDNA synthesis and then specific and / or generic primers, allowing us to evaluate all quarantine virus reported from a single cDNA synthesis reaction. Given that the methodology is based on a total nucleic acid extraction, the same extraction can be used for the diagnosis of DNA viruses and microorganisms, if necessary.

METHODOLOGY

This study presents the standardization and implementation of molecular diagnosis methodology RT–PCR for the evaluation of CsFSaV, CsPLV, CsNAV and CsTLV in the accessions of in vitro cassava collection of CIAT.

Different methodologies of extraction of nucleic acids, using fresh tissue of in vitro plants were evaluated, including the double-stranded RNA (dsRNA) by the method of Morris and Doods, modified (Cuervo, 1989), and ARN CTAB simple (López et al., 2006) (Fig. 2). In order to set up the minimum amount of RNA for the synthesis of cDNA and to achieve a good detection, the quality and quantity of RNA obtained was evaluated by electrophoresis (Fig. 3) and absorbance readings. The completion of the cDNA with specific primers and with random primers (Invitrogen, Burlington, ON) was compared. The conditions for the reaction in polymerase chain were established using sets of specific primers for each virus, previously designed in the Virology Laboratory of CIAT. In addition, tests were carried out to implement the use of internal controls that allowed us to confirm the existence of cDNA in each of the assessments. In this evaluation were used as positive controls, plasmids cloned with nucleic acid of each virus and tissue from affected plants, as negative control plant healthy.

Figure 2. Extraction process using the CTAB method.

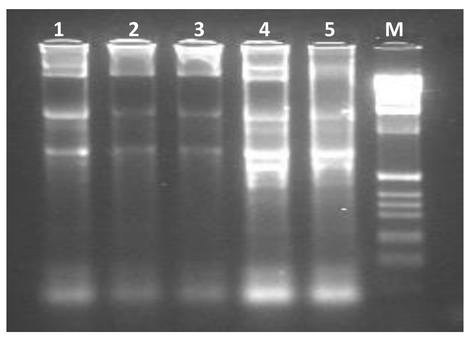

Figure 3. RNA quality in agarose gel 1%. M: molecular weight marker 1Kb plus.

Virus name	Taxonomy	Diagnostic tool	Reference
Cassava frogskin associated virus (CsFSaV)	Reoviridae/ Oryzavirus	RT-PCR	Calvert et al., 2008.
Cassava new alphaflexivirus (CsNAV)	Alphaflexiviridae/ Potexvirus	RT-PCR	Carvajal-Yepes et al., 2014
Cassava polero-like virus (CsPLV)	Luteoviridae/ Polerovirus	RT-PCR	
Cassava Torrado-like virus (CsTLV)	Secoviridae/ Torradovirus	RT-PCR	
Cassava common mosaic virus (CsCMV)	Alphaflexiviridae/ Potexvirus	ELISA, RT-PCR*	Nolt et al, 1991
Cassava X virus (CsXV)	Alphaflexiviridae/ Potexvirus	ELISA, RT-PCR*	Nolt et al., 1992
Cassava vein mosaic virus (CVMV)	Caulimoviridae/ Cavemovirus	PCR	Calvert et al., 1995

Table 1. Tools available for detection of viruses infecting cassava in the Americas. * = under development in CIAT.

RESULTS AND CONCLUSIONS

We have found that CTAB methodology for RNA extraction was the most suitable for our purpose, since a good quality and quantity of RNA was obtained. Additionally, it allows us to make evaluations for one or more pathogens with the same type of nucleic acid. The optimal amount of RNA for cDNA synthesis, in order to achieve a good detection is of 1.8 - 4 µg. For the realization of the cDNA, using random primers is the best choice because it allows the evaluation of several viruses with the same cDNA, which can be stored at -80 °C, and be used again later. It was established that the annealing temperature for amplification of CsFsAV, CsPLV and CsNAV is 55 °C, which is different from CsTLV of 45 °C. The use of internal controls is of great importance as it enables the confirmation of cDNA existence in the evaluated samples (Fig. 4 and Fig. 5). The selected primer sets are: PNad5f (5'-3'GATGCTTCTTGGGGCTTCTTKTT) and Pnadmr (3'-5'ATCTCCAGTCACCAACATTRGCATAA), with annealing temperature of 55 °C (Fig. 6) (Abad et al., 2014).

With the implementation of the RT-PCR methodology, so far 57% of the in vitro cassava collection has been evaluated, where 88% of the accessions have been negative . Table 2 shows the obtained results.

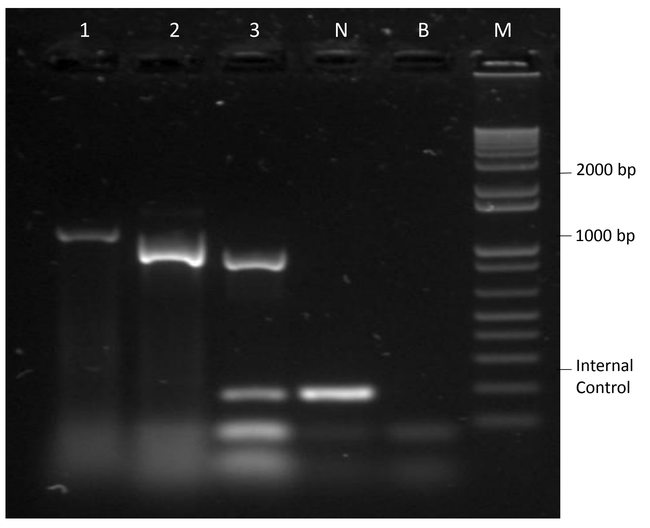

Figure 4. Multiple PCR products for the diagnosis of CsNAV (Potexvirus), CsPLV (Luteoviridae) and CsFSaV (Reoviridae) with internal controls. N: Control (-), B: White, M: molecular weight marker 1Kb plus.

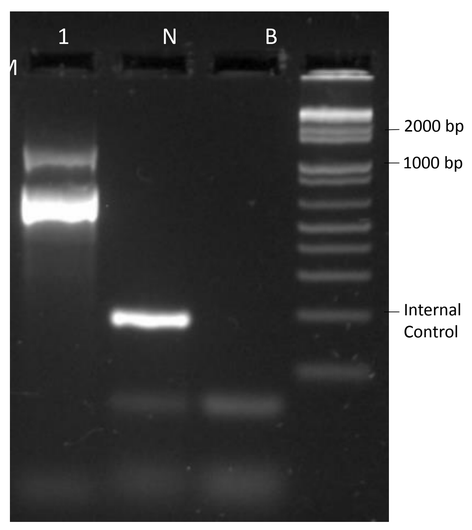

Figure 5. Multiple PCR products for the diagnosis of CsTLV (Torrado). N: Control(-), B: White, M: molecular weight marker 1Kb plus.

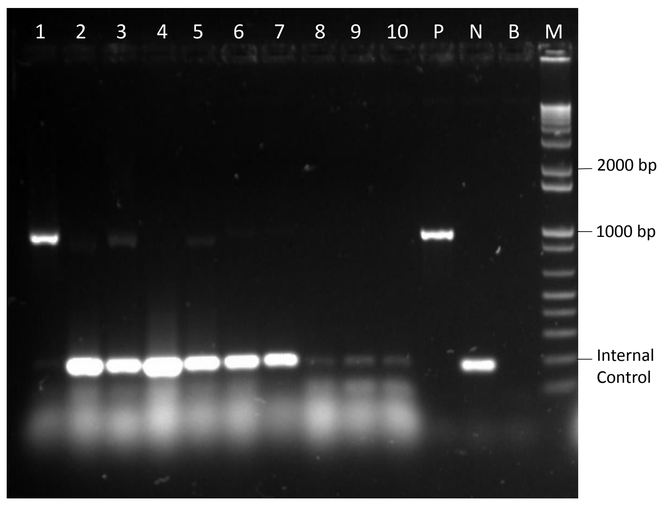

Figure 6. Multiple PCR products for the diagnosis of CsFSaV (Reoviridae) with internal controls, in agarose gel, where are observed: CsFsaV positive samples in wells 1, 2, 3, 4, 5, 6, 7. CsFSaV negative samples in wells 8, 9 and 10, P: Control (+), N: Control (-), B: White, M: molecular weight marker 1Kb plus.

Viruses	Positives Accesions %
Cassava frogsking virus (CSFV)	5.02
Cassava polero-like virus(CsPLV)	3.33
Cassava Torrado-like virus (CsTLV)	2.86
Cassava new alphaflexivirus (CsNAV)	0.75

Table 2. Preliminary results obtained.

Using this standardization the number of processes is reduced, and confidence, efficiency and sensitivity of the used technique increases, contributing to the secure exchange of cassava germplasm.

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