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Original article

Development of recombinase polymerase amplification-based colorimetric detection assay for rapid identification of invasive cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero

Nanditha Shivakumar, Shylesha Arakalagud Nanjundaiah, Venkatesan Thiruvengadam, Manjunatha Channappa *, Shivakumara Kadanakuppe Thammayya, Kandan Aravindaram, Satya Nand Sushil

ICAR-National Bureau of Agricultural Insect Resources, Bengaluru 560 024, India

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ABSTRACT

Phenacoccus manihoti Matile-Ferrero (Hemiptera: Pseudococcidae), is an economically important invasive cassava pest responsible for the massive devastation of cassava in Asia and African continent. Initially, identifying this invasive pest posed challenges because it closely resembled native mealybug species. Additionally, the traditional morphological identification process is labor-intensive and time-consuming. Detecting invasive pests at an early stage is crucial, hence development of a rapid detection assay is essential. In the current study, we have developed a simple, rapid, sensitive, and efficient molecular detection assay for *P. manihoti* based on Recombinase Polymerase Amplification (RPA). The primers for the RPA assay were designed using unique nucleic acid sequences of *P. manihoti*, and the protocol was standardized. Specificity test demonstrated that the RPA assay could amplify DNA of *P. manihoti* only, and no amplification was observed in six other mealybug species. The specificity of assay was confirmed using SYBR green-based colorimetric detection and gel electrophoresis where positive samples showed 195 bp amplicon size in *P. manihoti* samples. The assay successfully amplified *P. manihoti* DNA in thirty minutes at an annealing temperature of 41° C in a water bath and displayed a sensitivity of 72.5 picograms per microliter. The assay's simplicity, rapidity, and high sensitivity make it a valuable tool for detecting and monitoring *P. manihoti* in quarantine stations and facilitating in development of a portable diagnostic kit.

1. Introduction

Invasive pests pose serious challenges to a nation's economic progress, agricultural output, and farmer's well-being. Other threats of invasive species include disruptions in the environment and displacement of native species. Globalization has now drastically increased the chances of entry of invasive species and India is particularly vulnerable due to its diversity in climate and weather. So far 23 alien invasive insect species have been reported in India (Singh et al., 2020). Recently the invasive cassava mealybug (*Phenacoccus manihoti* Matile-Ferrero) entered India posing a serious threat to cassava cultivation, initial report of pests done in Kerala in 2020 (Joshi et al., 2020) and subsequently spread to neighboring states caused major yield losses. Presently in India, this pest is being managed through classical biological control by field releases of *Anagyrus lopezi* (De Santis) (Hymenoptera: Encyrtidae) (Sampathkumar et al., 2021). Cassava is a major food crop in tropical countries and occupies 21.8 million hectares of area with a production of 280 metric tons. In India, the cassava area and production in 2016–17 were 2.06 lakh ha and 4.34 million tonnes, respectively. The yield reduction caused by this invasive cassava mealybug can go up to 80 % (Wang et al., 2019; Schulthess et al., 1991).

Globally twenty-four species of mealybug are known to infest cassava, in India ten species colonize this host which includes *Phenacoccus manihoti* (Matile Ferrero), *Ferrisia virgata* (Cockerell), *Maconellicoccus hirsutus* (Green), *Nipaecoccus viridis* (Newstead) *Paracoccus marginatus* (Williams and Granara de Willink), *Phenacoccus madeirensis* (Green), *Phenacoccus solenopsis* (Tinsley) *Planococcuss citri* (Risso), *Pseudococcus jackbeardsleyi* (Gimpel & Miller) and *Pseudococcus longispinus* (Targioni

* Corresponding author.
E-mail addresses: nanditha.s@jainuniversity.ac.in (N. Shivakumar), Shylesha.AN@icar.gov.in (S.A. Nanjundaiah), Venkatesan.T@icar.gov.in (V. Thiruvengadam), manjunatha.c@icar.gov.in (M. Channappa), shiva.kt@icar.gov.in (S.K. Thammayya), a.kandan@icar.gov.in (K. Aravindaram), satya.sushil@icar.gov.in (S.N. Sushil).

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Tozzetti). Morphological differentiation of mealybugs is difficult as there are high degrees of similarity between varied stages of mealybugs. Separating the nymphal stages of these mealybug species in the field is difficult using morphological characters. Hence molecular confirmation is required for accurate identification of different species of mealybugs.

The major pathways for the introduction of cassava mealybug are trade-related activities such as grain and wood shipments, long-distance trucking, aeroplanes, and ship voyages. Detecting pests at quarantine stations during international trading can avoid massive outbreaks of these pests into new areas and becoming invasive pests to that region or country. Trade-related activities are highly time bound, due to the perishable nature of commodities and other considerable interests of traders, quarantine officials are bound to certify commodities within the stipulated time. The shortage of taxonomic experts and the non-availability of equipped molecular laboratories, coupled with the absence of specialized diagnostic personnel at quarantine stations, have collectively contributed to the inadvertent entry of numerous invasive pests over the past decade. Hence, simple, sensitive, rapid, accurate detection assay is required at quarantine stations to prevent the introduction of quarantine pests.

Among the nucleic acid-based molecular diagnostic methods, PCR is a more popular and commonly used technique. However, PCR based diagnostic technique demands a costly thermal cycler, and it takes three hours or more for the complete identification of species (Manjunatha et al., 2018; Prasannakumar et al., 2021; Naganur et al., 2019). PCR also requires quality DNA for amplification (Prasannakumar et al., 2020; Sunani et al., 2019) and compounds like polysaccharides, ethanol, phenol, a few proteins, and proteinases (Rossen et al., 1992; Rådström et al., 2004), act as PCR inhibitors in inhibiting PCR reactions. Hence, attempts were made to develop an alternative, rapid, sensitive, specific colorimetric molecular detection assay for the diagnosis of cassava mealybug. In the current study, we choose Recombinase Polymerase Amplification (RPA), a novel, isothermal amplification technique to complement PCR-based detection assay (Kumar et al., 2018). RPA can amplify DNA at a relatively low temperature of 37 °C, which coincidentally happens to be the standard body temperature of a healthy human. This means that merely holding the reaction in one's hand is adequate to initiate the amplification process. Hence we have developed RPA-based single step, single tube technique, where cassava mealybug amplification and detection can be done simultaneously. Also, the technique being colorimetric can be detected by the naked eye without gel electrophoresis or gel documentation system by the addition of dyes. RPA-based detection assay could be highly useful for rapid detection of invasive at point of care or quarantine stations and thus prevent their spread in the country.

2. Materials and methods

The cassava mealybugs were sampled from six different locations and the collected samples were stored in labeled vials containing 70 % alcohol for further studies.

2.1. Morphological and molecular identification of mealybugs

The mealybug species were identified using available keys (Williams, 2004), using a Steromicroscope (SMZ800N, Nikon, Japan). After morphological identification, all the mealybug samples were surface sterilized with 70 % ethanol and washed with distilled water. DNA was isolated from all the mealybugs samples using a DNeasy Blood & Tissue kit (Qiagen, Duesseldorf, Germany) by following the manufacturer's protocol with slight modifications. DNA extraction from mealybug species is difficult because the insect has a waxy powder on the surface of the body. To remove the waxy coat of mealybugs the insect samples were soaked in chloroform for 60 min in 1.5 mL Eppendorf tube, after a period drained out the chloroform and added ultrapure water and kept overnight. The individual mealybugs of each species were taken separately and placed in 1.5 mL micro centrifuge tubes and the remaining protocols were followed as mentioned earlier. Later 1.2 % agarose gel electrophoresis was performed to check the quantity and quality of DNA through Nanodrop (DS-11 FX+, DeNovix, Delaware USA).

The isolated genomic DNA of all mealybug samples were amplified through the universal mealybug CO1 marker *i.e.,* PCoF1 (5′CCTTCAACTAATCATAAAAATATYAG3′) and LepR1 (5′ TAAACTTCTGGATGTCCAAAAAATCA3′) (Park et al., 2011) with the following conditions: initial denaturation at 95 °C for 5 mins; 35 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, elongation at 72 °C for 2 mins; and final elongation at 72 °C for 10 mins. Each reaction mixture contained 12.5 µl of 2x Emerald Amp PCR master mix (Emerald Amp GT PCR master mix, TakaRa, Japan), 1 µL of PcoF1, 1 µL of LepR1, 5 µL of the template, and 5.5 µL of molecular biology grade water to make up the volume to 25 µL. The amplified product were resolved in 1.6 % agarose gel and documented in the gel documentation system (DNR, MiniLumi, Israel). The PCR-amplified products of all the morphologically confirmed mealybug samples were subjected to Sanger sequencing in Eurofins India Private Limited, Bengaluru. Contigs were prepared for forward and reverse sequences using Bioedit software version 7.2.5, and contig sequences were analyzed using the BLAST algorithm in the non-redundant NCBI database.

2.2. RPA assay for detection of Phenacoccus manihoti

2.2.1. Designing of RPA primers

Recombinase polymerase amplification primers (RPA_PMF and RPA_PMR) were designed manually for targeting the unique genomic region reported by Wang et al. (2019) and synthesized at Eurofins India Private Limited, Bengaluru (Table 1). PCR primers (PMN F and PMN R) were also been redesigned for the same region, for comparative analysis of RPA assay with PCR.

2.2.2. Specificity test of RPA assay

The RPA reaction was done using TwistAmp (Basic kit, TABAS03KIT, USA) and a reaction mixture of volume 50 µl was constituted which contained 30 µL of rehydration buffer, 10 µL of template, 2.5 µL of forward primer, 2.5 µL of reverse primer, 2.5 µL of molecular biology grade water, and 2.5 µL of magnesium acetate and incubated at 41 °C for 30 mins, and amplification was visualized using SYBR Green I dye as well as gel electrophoresis. Polymeric chain reaction was also standardized using the primers designed from unique nucleic acid sequences. A PCR reaction mixture of volume 25 µL was prepared which included 12.5 µL of 2x Emerald Amp PCR master mix (Emerald Amp GT PCR master mix, TakaRa, Japan), 1 µL of PMNF, 1 µL of PMNR, 5.5 µL of molecular biology grade water and 5 µL of the template. The PCR protocol was standardized using a thermal cycler (Biorad, T100) and the standardized conditions were: initial denaturation at 95 °C for 3 mins; 35 cycles of denaturation at 95 °C for 30 secs, annealing at 51 °C for 30 secs, extension at 72 °C for 45 secs; and final elongation at 72 °C for 5 mins and hold at 4 °C. The amplified products were resolved in 1.6 % agarose gel and documented in the gel documentation system (DNR, MiniLumi, Israel).

2.2.3. Sensitivity test of RPA assay

The genomic DNA of *P. manihoti* was serially diluted from the original concentration of 72.5 µg per microliter up to 72.5×10^{-14} concentration of DNA, and used in both RPA and PCR assay following the standardized protocol. The sensitivity of the RPA (by SYBR Green I addition to amplified product) was observed in UV-transilluminator (DNR Bioimaging system, MiniBio-Pro, Israel) for color change, and further gel electrophoresis was done using 1.2 % agarose gel, agarose gel picture documented gel documentation system (DNR, MiniLumi, Israel).

Table 1
List of primers used in the study along with annealing temperature and amplicon size.

Sl. No.	Primers	Primers Sequences (5′-3′)	Tm (°C)	Amplicon size (bp)
1	RPA_PMF	CACGATTAAATAATTTTAGATTTTGATTACTAC	41	195
2	RPA_PMR	TAATTGAACTAAAAAAGAGGATAATCCAT	41	195
3	PMN F	TTGATAAAACAGGAATTGAGACAA	48	352
4	PMN R	CCCCATTAATTAACCAAAATTTCA	48	352

2.2.4. Validation of RPA assay

To validate the RPA assay we first collected the *P. manihoti* from the field of different locations, isolated genomic DNA, and subjected to RPA assay by following the aforementioned standardized protocol and the RPA products were tested and validated through the SYBR Green dye and gel electrophoresis.

3. Results

3.1. Morphological and molecular identification of mealybugs

Taxonomic identification of collected mealybug samples was performed using available taxonomic keys by preparation of slides and observing them in a microscope (Williams, 2004) (Fig. 1). The field collected mealybug species were confirmed as cassava mealybug (*P. manihoti*), cotton mealybug (*P. solenopsis*), citrus mealybug (*P. citri*), Jack Beardsley mealybug (*P. jackbeardsleyi*), long tailed mealybug (*P. longispinus*), Stripped mealybug (*F. virgata*), and pink hibiscus mealybug (*M. hirsutus*) with the help of specific taxonomic keys (Fig. 2) (Williams, 2004; Williams and Granara, 1992; Parsa et al., 2012). The DNA extracted from these mealybug species was further used for the development of an RPA-based detection assay.

The DNA from all these mealybugs was amplified using universal mealybug primer PCoF1/LepR1 (Table 1) with the amplified product size of approximately 650 bp (Fig. 3) and the sequences of PCR products was confirmed as respective species of mealybugs by BLAST analysis and their respective genbank accession number *viz.*, *P. manihoti* (MW039322, OK172561, OK172342, OK174324, OK173048, OK172532), *P. solenopsis* (OQ349395), *P. citri* (KY780496), *F. virgata* (KM035853), *M. hirsutus* (KY460553).

3.2. RPA assay for detection of Phenacoccus manihoti

3.2.1. Specificity test of RPA assay

The RPA has been standardized for temperature and duration of incubation using the genomic DNA of *P. manihoti*. The optimum temperature for RPA obtained was 41 °C. As for duration, the time required for amplification of DNA in RPA assay was 30 min. While the utilization of SYBR Green dye allowed to distinguish between positive and negative reactions within the fifteen-minutes timeframe, a notable improvement in the differentiation of positive and negative reactions was observed when the incubation period was extended to 30 min (Fig. 4A) Concerning specificity assay the *P. manihoti* specific RPA primers showed amplification only with *P. manihoti* genomic DNA and there was no amplification in the other mealybugs. RPA amplified products were confirmed in gel electrophoresis with an amplicon size of 195 bp. As far as SYBR Green I dye-based colorimetric assay is concerned the results had a perfect match with morphological identification results; RPA amplified *P. manihoti* samples showed a bright yellow color whereas other negative samples showed brick red color in UV transilluminator (Fig. 4A). PCR amplification of same DNA samples for redesigned primers also shown similar matching results with an amplicon size of 350 bp (Fig. 4B). Results were further confirmed by matching of Sanger sequences to the original sequences used for redesigning PCR primers.

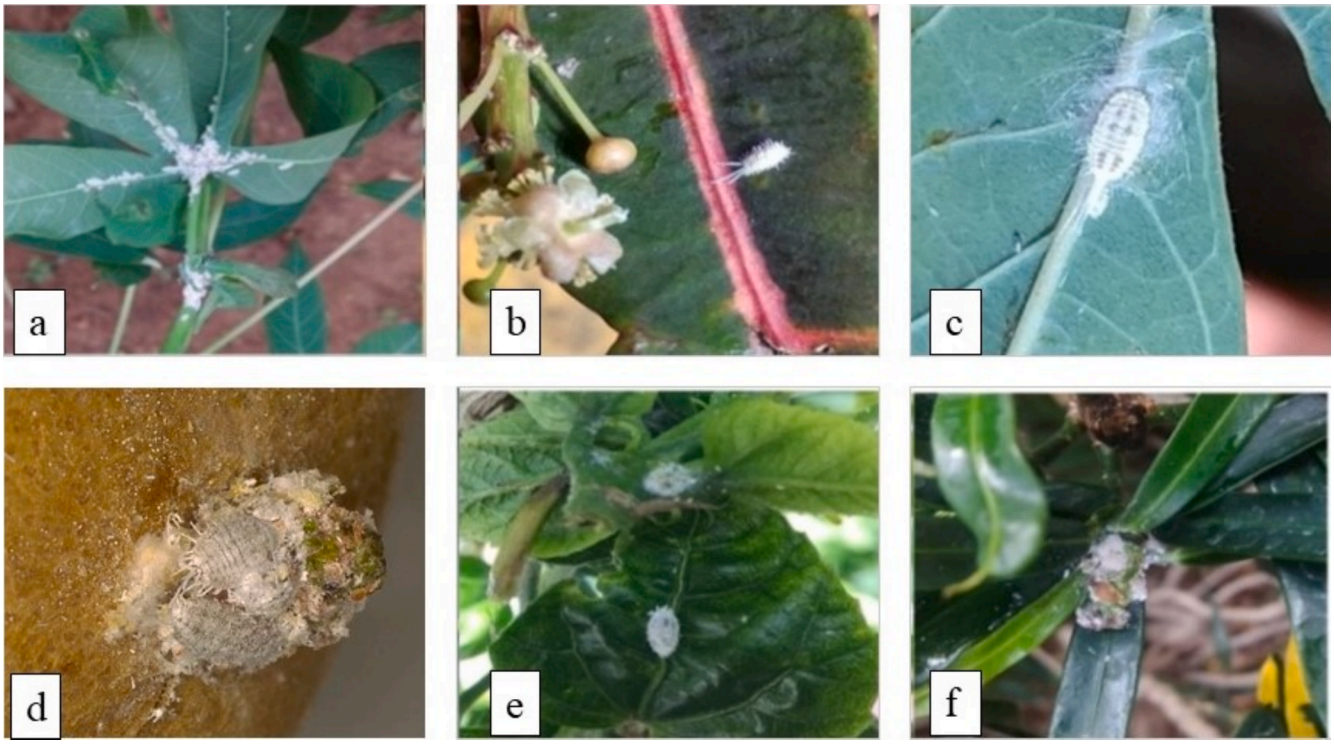

Fig. 1. Collection and identification of mealybug samples based on the integrated taxonomy using morphological characterization a) *Phenacoccus manihoti*; b) *Pseudococcus longispinus*; c) *Ferrissia virgata*; d) *Pseudococcus jackbeardsleyi*; e) *Phenacoccus solenopsis*; f) *Planococcus citri*.

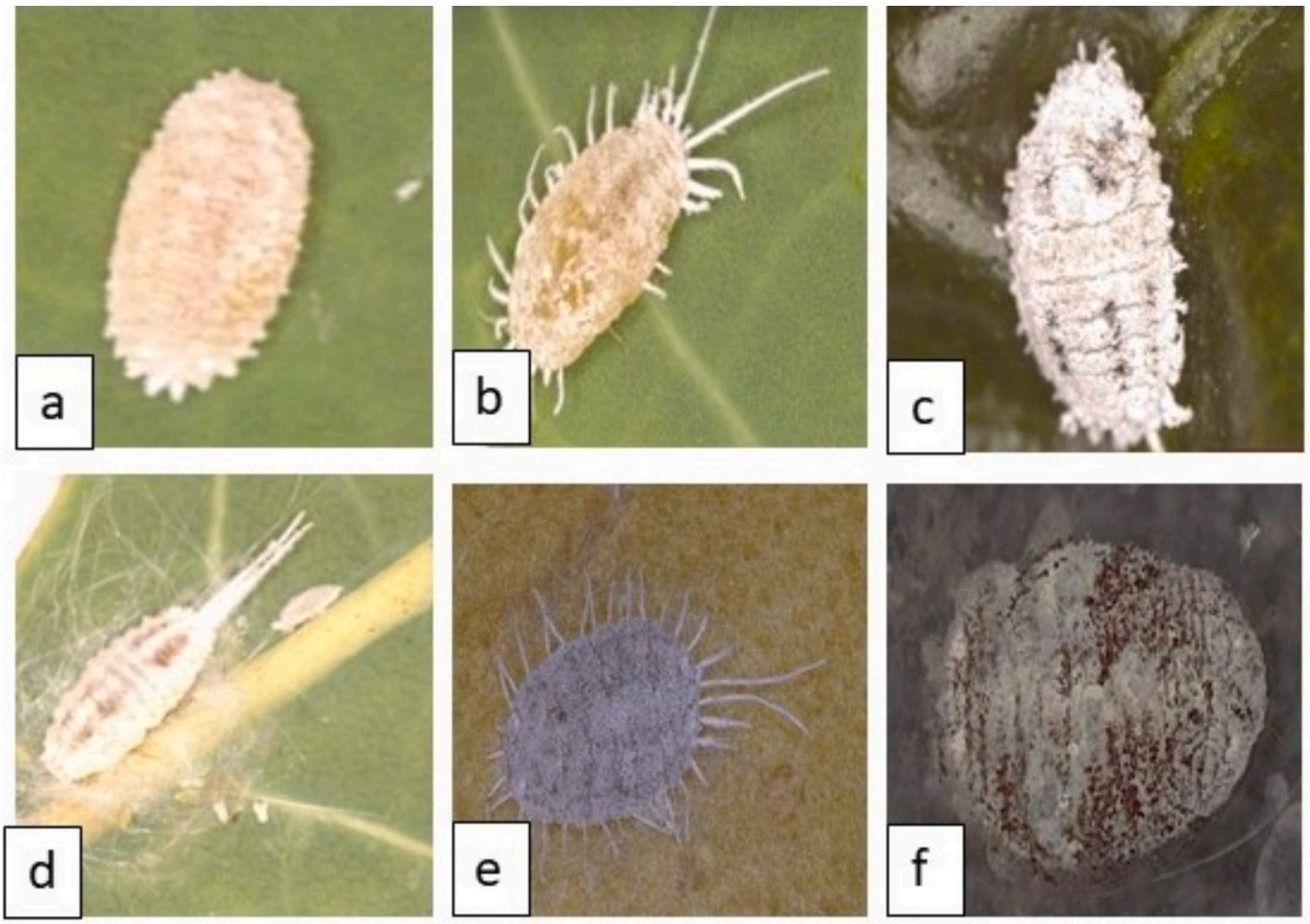

Fig. 2. Microscopic view of mealybug species characterized in the study. a) *P. manihoti*; b) *P. longispinus*; c) *P. solenopsis*; d) *F. virgata*; e) *P. jackbeardsleyi*; f) *M. hirsutus*.

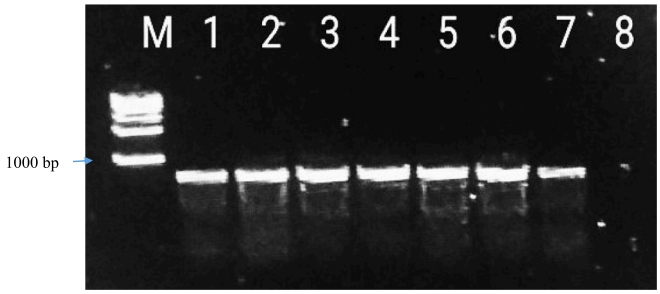

Fig. 3. PCR amplification of mealybugs with PCoF/LepR1. M – 1 kb ladder, Lane 1 – *P. manihoti*; Lane 2 – *P. citri*; Lane 3 – *P. longispinus*; Lane 4 – P. *jackbeardsleyi*; Lane 5 – *P. solenopsis*; Lane 6 – *M. hirsutus*; Lane 7 – *F. virgata*, Lane 8 – Non-template control.

3.2.2. Sensitivity test of RPA assay

The serially diluted genomic DNA of *P. manihoti* was used to test the sensitivity of RPA primers. The concentration of 72.5 pg/μL DNA was sufficient to amplify with *P. manihoti* DNA (Fig. 5A, B), hence the designed species-specific RPA primer proved to be very sensitive.

3.2.3. Validation of molecular assay

The RPA technique showed positive amplification in all the tested samples of *P. manihoti* that were collected from different locations and consisted of varied stages (Fig. 6). The primers proved to be highly specific to *P. manihoti* as there were no amplifications for the other mealybug species and non-template control.

4. Discussion

To start with the famous quote from Chinese philosopher (Confucius) "the beginning of wisdom is the ability to call things by their right names (Veale, 1974), correct identification of pests is essential for management of the same. We can draw a parallel to the realm of pest management as the correct identification of pests is imperative for their effective control. Cassava, in general, has been infected by nearly ten mealybug species however the mealybugs responsible for the major destruction of the cassava crop include *P. manihoti* and *P. herreni* (Rauwane and Ntushelo, 2020). These mealybugs can be controlled through various biological control methods like using insect parasitoids and choosing an appropriate parasitoid in biological methods requires correct identification of mealybug species. For instance, *A. lopezi* is very effective against *P. manihoti* but not against other mealybug species, unless we correctly identify species it's difficult to choose the correct parasitoids in bio-intensive pest management of mealybugs in cassava. Furthermore, the statement "prevention is better than cure" holds true in the context of pest control. It is more advantageous to preemptively address pests before they proliferate within an affected crop. To achieve this, timely identification plays a pivotal role. Early recognition of pests enables the implementation of appropriate management strategies, effectively halting their onward propagation and averting potential devastation. Hence, the necessity arises for a method that is simple, sensitive, fast, and accurate to facilitate the easy identification of the cassava

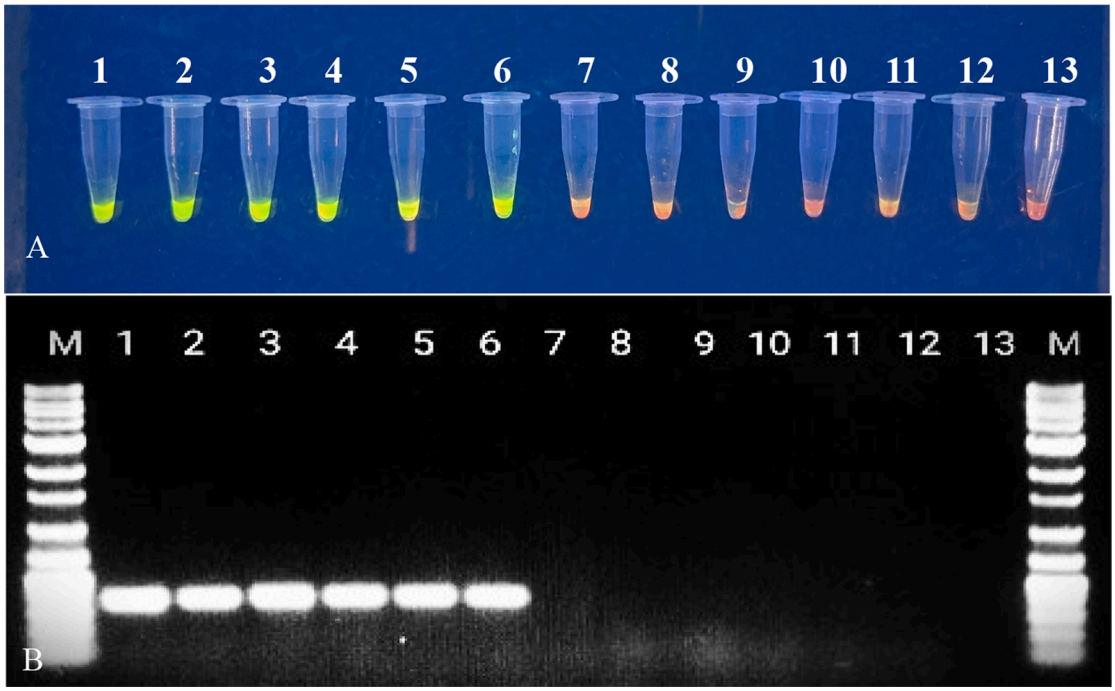

Fig. 4. A) RPA amplification of *P. manihoti* and other mealybug species. M – 100 bp Marker; Lane 1 to 6 – *Phenacoccus manihoti* collected from six different regions; Lane – 7 *Planococcus citri*; Lane 8 – *Pseudococcus longispinus*; Lane 9 – *Pseudococcus jackbeardsleyi*; Lane 10 – *Phenacoccus solenopsis*; Lane 11 – *Maconellicoccus hirsutus*; Lane 12 – *Ferrissia virgata*, Lane 13 – Non-template control, B) PCR amplification of *P. manihoti* and other mealybug species using species specific PCR primers.

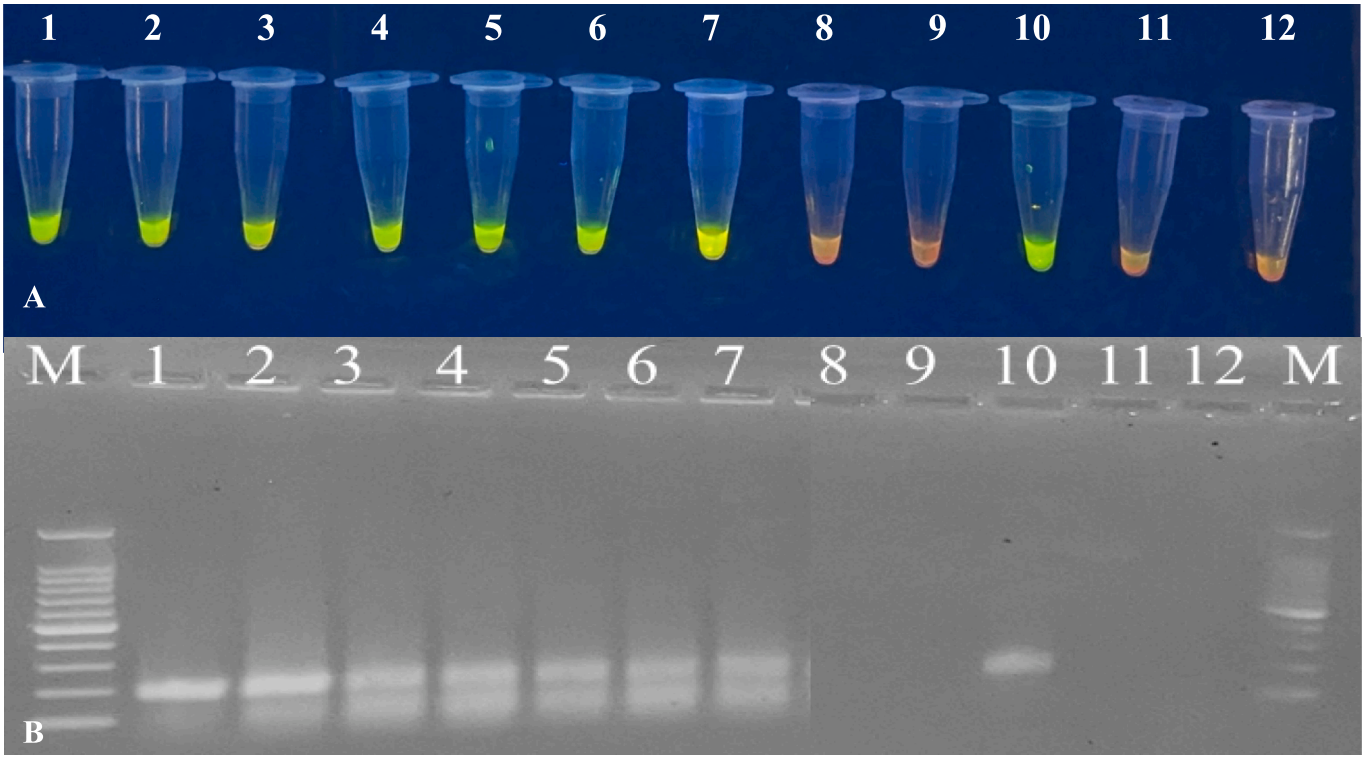

Fig. 5. Sensitivity assay of species-specific primers A) RPA sensitivity assay for species-specific primers with SYBR Green I dye; B) RPA sensitivity assay for species-specific primers with agarose gel electrophoresis; M – 100 bp Marker, Lane 1—72.5×10^{-6}, Lane 2—72.5×10^{-7}, Lane 3—72.5×10^{-8}, Lane 4—72.5×10^{-9}, Lane 5—72.5×10^{-10}, Lane 6—72.5×10^{-11}, Lane 7—72.5×10^{-12}, Lane 8—72.5×10^{-13}, Lane 9—72.5×10^{-14}, Lane 10 – *P. manihoti*; Lane 11 – negative control (*P. longispinus*); Lane 12 – non-template control.

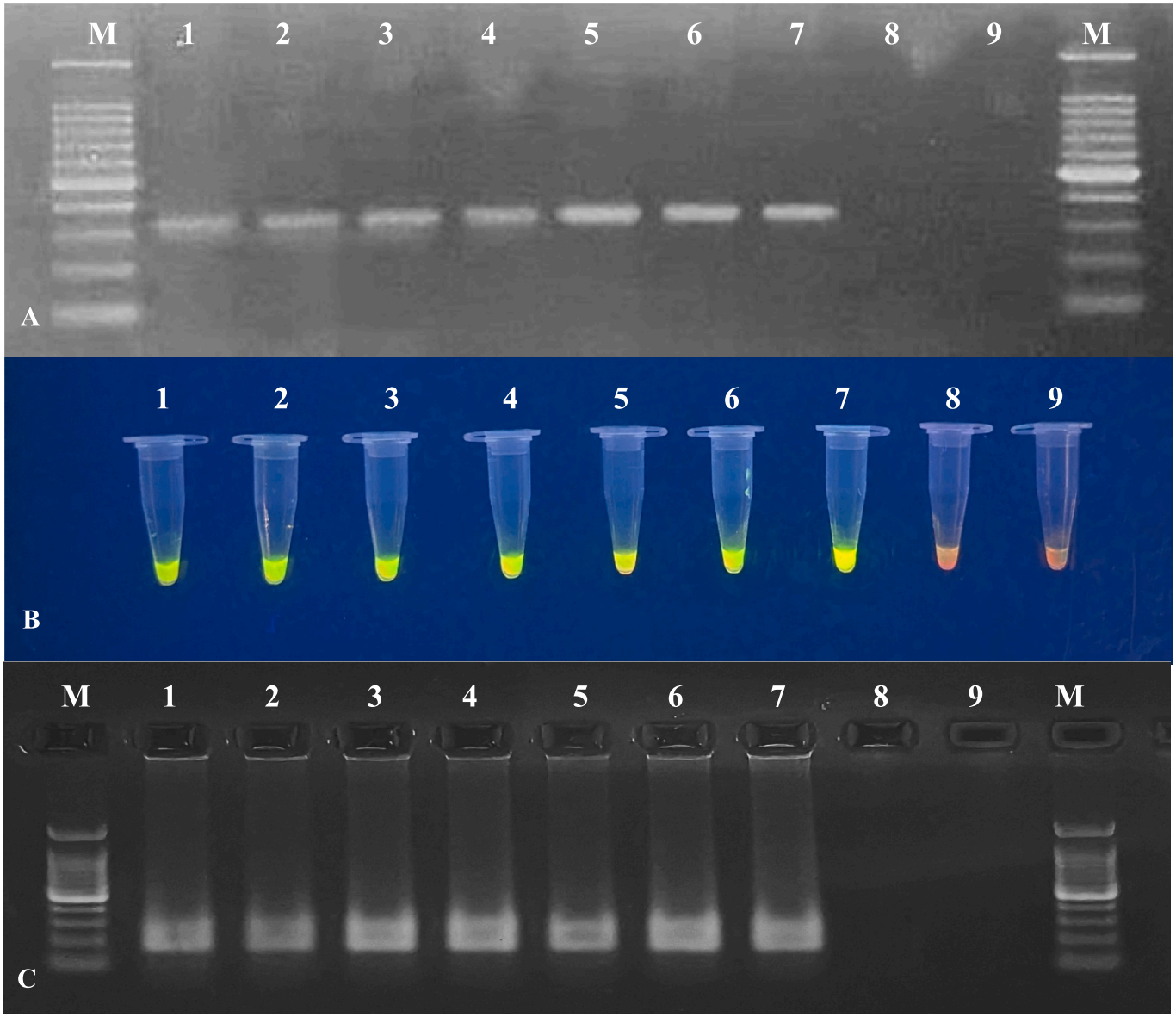

Fig. 6. Validation of species-specific marker for *P. manihoti* collected from 6 different locations. A) Validation of species specific-PCR for *P. manihoti*; B) Validation of species specific-RPA assay for *P. manihoti* with SYBR Green I, C) Validation of species specific-RPA assay for *P. manihoti* with gel electrophoresis, M- 100 bp marker; Lane 1 to 6- *P. manihoti* of different locations; Lane 7- positive control (*P. manihoti*), Lane 8- negative control (*P. longispinus*); Lane 9- non-template control.

mealybug, given its intricate recognition across diverse stages. Consequently, this study has devised a targeted RPA diagnostic assay that is exclusive to the species. The purpose of this development is to swiftly and accurately identify *P. manihoti*, a crucial step that could equip farmers with the means to curtail the pest's dissemination and select the appropriate natural enemy for its effective control. As cassava mealybug is an invasive pest, RPA-based detection assay has great advantages in quarantine centers to prevent the spread of invasive pests, most quarantine stations will not have well-established molecular labs with diagnostic experts. Additionally, taxonomic expertise on invasive pests at the port of entry is minimal; under such situations, RPA-based diagnostic will be highly helpful for accurate and fast identification of invasive cassava mealybug at the point of entry itself.

The RPA has numerous benefits compared to other techniques, especially PCR. RPA based detection of *P. manihoti* completes within 30 min, and unlike PCR, it does not demand gel electrophoresis for confirmation of amplification of DNA; hence it is very useful in laboratories with limited resources. A thermal cycler is a must in PCR for the amplification of DNA, whereas RPA protocol can be completed by holding the reaction mixture in hands, which eliminates the requirement of a costly thermal cycler; this feature of the technique enhances its utilization in the identification of invasive pests in point of entry as well as field. The recombinase polymerase amplification can be achieved in a simple water bath which is much cheaper than a thermal cycler for higher efficacy of results (Gao et al., 2021). The addition of SYBR Green I dye to the RPA amplified products makes the assay as colorimetric since SYBR Green I is an asymmetric cyanine dye known for its remarkable sensitivity. It selectively attaches to the minor groove of nucleic acids (Bruijns et al., 2016). This binding property leads to distinct color changes visible to the naked eye when forming a complex with DNA, with the color intensity varying based on the concentration of DNA present. The combination of SYBR Green I with the DNA complex is integral to the detection process. This technique, when applied to endpoint staining detection in the context of isothermal recombinase polymerase amplification, offers a range of advantages. Notably, this method is characterized by its simplicity, rapidity, and cost-

effectiveness. These qualities have led to its adoption in diverse applications including molecular diagnosis, meat product authentication, and bacterial typing (Cao et al., 2018; Singpanomchai et al., 2019). The colorimetric nature of RPA assay simplifies the technique for confirmation of results without a gel documentation system, which is again costly equipment and requires expertise to use the same. In RPA, amplification and detection of DNA can be completed in one step, one tube, which reduces the time required for confirmation of results and makes the assay quicker, and amplification and detection of DNA without thermal cycler and gel documentation makes the technique cheaper (Kumar et al., 2023). In this study, we confirm the higher specificity of the assay and we also validated results with field-collected samples, which makes to utilize techniques to identify *P. manihoti* without much taxonomic expertise; even identification can be made from developmental stages in the absence of an adult female.

Recently, the first successful demonstration of RPA technique in insects was achieved on *Thrips palmi*, a vector of tospoviruses in New Delhi, India (Priti et al., 2021). The technique was performed with crude DNA and the reaction was completed within 20 min by holding the reaction tubes in the hand. Field level diagnosis of insect pest is impossible in PCR-based technique because it requires nucleic acid extraction and higher temperature that may affect the feasibility in field conditions. Thus, RPA technique showed successful identification of *T. palmi* in field condition. Further, this technique is highly efficient in the diagnosis of plant viruses and zoonotic diseases (Li et al., 2019; Cha et al., 2020). Based on the silent features of technique, this can be performed by non-expert personnel also without any sophisticated equipment and laboratory facilities. This technique is greatly suitable to quarantine stations as well as field conditions for adopting quick management strategies (Priti et al., 2021).

The *P. manihoti* is indigenous to South America, it was accidentally introduced from South America to Congo Republic in 1973. Later its spread was reached to different Asian countries *viz.,* Thailand (2008), Cambodia (2010), Vietnam (2012), China (2014), and recently entered to India and caused outbreak in different cassava growing parts of India *viz.,* Tamil Nadu, Karnataka and Kerala (Sampathkumar et al., 2021). Though we utilized a distinctive genomic region identified by Wang *et al* (Wang et al., 2019) yet the species-specific RPA assay has significantly streamlined the process of detecting *P. manihoti*. The developed RPA assay boasts an impressive sensitivity level of $72.5x10^{-12}$. This heightened sensitivity is particularly advantageous when dealing with limited sample availability, a common scenario in quarantine stations where the number of pests in the samples can be minimal. Although PCR is widely regarded as the gold standard for pest and disease detection, the RPA assay superior sensitivity comes into play when working with samples containing minute amounts of DNA. The traditional PCR approach can encounter challenges in such cases due to its comparatively lower sensitivity. Notably, the study's findings align with this perspective, highlighting that RPA requires less DNA than PCR for successful detection.

The validation of the RPA assay involved testing it with field-collected samples. The use of species-specific RPA primers demonstrated a high level of specificity exclusively towards *P. manihoti*, with no reaction observed in non-targeted species. Consequently, the need for Sanger sequencing to validate the amplified product can be eliminated. This elimination contributes to a reduction in the cost per assay reaction. Moreover, the reliance on Sanger sequencing is often limited to well-equipped laboratories, and it consumes considerable time for confirmation. This time constraint is particularly significant as quarantine inspectors must promptly certify perishable commodities. In this context, the assay developed in this study emerges as exceptionally valuable due to its rapid detection capability, aligning well with the urgency of identifying and managing the pest effectively.

5. Conclusion

The species-specific RPA assay, developed in this study, complements to traditional methods such as morphological analysis and PCR-based techniques for detecting *P. manihoti*. As a result, this assay holds significant value for a range of applications, including pest monitoring, certification procedures at quarantine stations, and the strategic management of the cassava mealybug through the selective deployment of specific parasitoids within the framework of bio-intensive pest management. At present, our endeavors are centered on the development of a CRISPR-based detection assay, aimed at refining the technique further. Simultaneously, we are diligently working towards the creation of portable, field-deployable microdevices that can be readily employed at the point of care or port of entry of quarantine pests. These microdevices are envisioned to be instrumental in enabling rapid, accurate, sensitive, early detection and identification of the invasive cassava mealybug, thus enhancing our ability to manage its impact effectively.

CRediT authorship contribution statement

Nanditha Shivakumar: Investigation. **Shylesha Arakalagud Nanjundaiah:** Investigation, Methodology. **Venkatesan Thiruvengadam:** Conceptualization. **Manjunatha Channappa:** Conceptualization. **Shivakumara Kadanakuppe Thammayya:** Investigation, Methodology. **Kandan Aravindaram:** Supervision, Writing – review & editing. **Satya Nand Sushil:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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