

Potential of LAMP assay as a tool for ecological monitoring and on-site detection of *Bipolaris oryzae* in rice

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Abstract

One of the most significant rice seed-borne pathogens, *Bipolaris oryzae* causes serious yield losses worldwide every year. Traditional seed health testing involves incubation for up to a week, but molecular diagnosis using PCR-based assays requires sophisticated equipment and is not amenable to on-site testing. Loop-mediated isothermal amplification (LAMP) is ideal for field-level diagnosis since it offers quick, high-specific amplification of target template sequences at a single temperature. The exon 2 of the Glycosyl Transferase Family 90 protein of *B. oryzae* was targeted for designing the LAMP primers. The LAMP assay proposed was incredibly sensitive, detecting the *B. oryzae* template's presence at concentrations as low as 10 fg at 60 °C in 30 min. The specificity of the test was assessed using genomic DNA retrieved from 22 different phytopathogens. The assay was employed to biologically monitor the existence of this pathogen by testing it on ten weed species in rice fields infected with *B. oryzae*. The pathogen was detected in 8 weed species except *Brachypodium distachyon* and *Phalaris minor*. The assay was used to detect the presence of *B. oryzae* in seeds from 13 different rice varieties. Seed samples from two cultivars tested negative while the remaining cultivars were confirmed to harbor the pathogen. Using this novel assay, *B. oryzae* can be detected rapidly in rice seeds and farm soil. The proposed LAMP assay has the potential to be employed in seed health laboratories, plant quarantine stations and on-site detection of *B. oryzae* in seeds and plants.

Introduction

Rice (*Oryza sativa* L.) is a vital dietary demand for more than half of the globe's population. Worldwide, annual production of improved rice is about 480 million metric tonnes. It is cultivated almost everywhere in the Indian subcontinent and is a vital agricultural product. Despite being India's primary producer of rice, having the largest area (46.38 million hectares), and ranking second in terms of production, a debilitating trend is that the country's productivity per hectare ranks fifty-one and has remained stable over time; however, no discernible advancement has been reported [1, 2].

Farmers endure yield and financial losses every year as a result of biotic and abiotic stress on their crops. Among the many factors limiting the production of rice, diseases caused by fungi, nematodes, viruses and bacteria result in significant financial losses [3, 4, 5]. Seed-borne diseases are known to contribute to the loss of crop yields [6] and have been considered potentially destructive to many varieties of rice crops grown in various locations in the country [7]. Over 56 fungal diseases are known to infect rice worldwide, of which 41 are known to be seed-borne. These infections have been shown to significantly reduce the quality of rice seeds [8, 9]. Depending on the agroecology, disease severity and crop sensitivity, seed-borne infections in rice cause yield losses of 50–80 per cent [10, 11]. These pathogens promote seed discoloration, seed rot, impaired seed germination and seedling vigour and impede the plant throughout its early development stage. Seed-borne fungal infections are quite complicated to manage because the fungal hyphae establish themselves and become dormant [12]. Pre- and post-emergence mortality of grains is caused by seed-borne fungus, which also has an impact on seedling vigour and viability, which

reduces the capacity to germinate and results in morphological heterogeneity in the plant [13, 14]. Also, crop production and quality are highly reliant on seed quality since seed typically influences yield.

Brown spot disease has a serious impact on the rice production. Brown spot is caused by *Bipolaris oryzae* (Breda de Haan) Shoem, which is the most significant seed-borne constraint to rice production in all rice-growing nations around the globe [15]. In terms of economic significance, this disease ranks second to blast disease and causes significant declines in cereal yields during both rainy and summer seasons. Brown spot has been observed to cut down production in Asia by 6 to 90%. The disease was first documented in West Bengal in 1943 and was a contributing factor to India's "Great Bengal Famine," [16] which caused the death of nearly two million people due to impoverishment [17].

The most prevailing technique for detection of seed borne pathogen include morphological observation of pathogen colonies after incubating rice seeds on blotter or agar plates [16] and/or detection of pathogen-specific DNA fragments based on the conventional polymerase chain reaction (PCR) or real-time PCR [18, 19, 20, 21]. To differentiate *B. oryzae* from other fungi, morphological recognition needs a skillful method. The ability to differentiate *B. oryzae* from another fungus through morphological identification is imperative. The procedure requires a week or more if a pathogenicity test is needed for further confirmation. To distinguish *B. oryzae* from *B. sorokiniana*, [22] designed a SCAR (sequence characterization amplification polymorphism) primer. Although PCR-based tests considerably shorten the testing period, they necessitate comparatively expensive thermocycler and imaging systems [23, 24, 25]. Rice farmers will need to make timely disease diagnoses on-site, choose pathogen-free seeds for sowing, as well as make early choices on seed treatment for fungus-contaminated seeds. Therefore, it is critical to developing a quick and simple seed test technique for identifying *B. oryzae*.

Loop mediated isothermal amplification (LAMP) technique is used to circumvent the issue encountered with the traditional PCR [26]. This method was initially established by [27] and was adopted for the quick detection of plant pathogens because of its high specificity, sensitivity and efficiency to perform in isothermal conditions in the field [28]. LAMP amplification is incredibly efficient, and may result in the accumulation of 10^9 copies of the target sequence in less than an hour without heat cycling. Moreover, the LAMP enzyme exhibits less sensitivity to inhibitors during amplification processes and is not strictly dependent on expensive thermal cyclers and sophisticated equipment [29]. However, inexpensive equipment such as water baths or heating blocks is adequate to maintain temperature during the reaction period, making LAMP a more cost-effective approach than PCR, particularly real-time PCR [30].

Under this circumstance, the present study was undertaken to develop a quick detection assay for the detection assay for the detection of seed borne *B. oryzae* suitable for on-site or

in farm applications in the future.

Results

PCR assay

In a PCR assay, thirty-five isolates of *B. oryzae* collected from diverse locations were amplified using the ITS 1 and 4 primers. The sequenced products were compared with other

B. oryzae sequences available in the NCBI database. A similarity search was done in NCBI using the BLAST tool. The nucleotide sequence of the study isolate's ITS region revealed 100% identity with other *B. oryzae* isolates and shared significantly very less similarity with other *Bipolaris* isolates (Supplementary Table 1).

Designing of LAMP primers

Five distinct primer sets were predicted for unique sequences using the Primer Explorer V5 software tool and employed in developing a LAMP assay specific for *B. oryzae*. The primers were designed to facilitate *B. oryzae*-specific amplification and demonstrated 100% identity and query coverage for *B. oryzae*. The primers were selected following a review of the thermodynamic criteria specified by the manufacturer of the Primer Explorer for the selection of LAMP primers (Table 1). The G values of the 3' end were determined during the design of LAMP primers and were less than - 4 Kcal/mole. In silico analysis of the five designed primer sets revealed that only one primer pair had a stable DNA construct with no primer-dimer formation. Eventually, a set of six primers with high species specificity and sensitivity that targeted the exon 2 of glycosyl transferase family 90 protein of *B. oryzae* sequences were selected for future investigation. For the outer primers (forward F3 and reverse-B3), there was a probability for the development of weak to moderate secondary structures. For the inner primers (FIP and BIP), there was the potential for the development of moderate to strong secondary structures. However, the loop primers (LF and LB) had no to very weak secondary structures (Supplementary Table 2). Two to three guanine or/and cytosine bases were clamped over the primer's 3' end. The following six primers annealed to distinctive sites in 1725 bp of the particular exon 2 of the glycosyl transferase family 90 protein target sequence, confirmed *in silico* hybridization of the primers. Primer binding locations are clearly marked in Fig. 2 for elucidation

Table 1
Sequence of oligonucleotide primer sets used for amplification of target sequence in exon 2 of the Glycosyl Transferase Family 90 protein in this study

Primer name	Primer type	Sequence 5'→3'	Primer length (nt)	GC (%)	GC clamp	3'ΔG
BoG-F3	Forward outer	CACTGATCCGTCCCAACG	18	61	2	-5.94
BoG -B3	Backward outer	GTATTGGTACCGCGCCAA	18	56	3	-3.39
BoG -FIP (F1c-F2)	Forward inner	CAACTTCCACTCCCGAGAGCTT-CGCTCAGCACTTCACAGC	40	61	2	-5.49
BoG -BIP(B1c-B2)	Backward inner	ACCCGGATTACAACGTGAACGC-GGCAGCTTCTTGAGCCATT	41	55	2	-5.79
BoG -LF	Forward loop	CGGATGAGAAGAGCGGTAGCAATC	24	54	1	-4.91
BoG -LB	Backward loop	CACCGATGTCATAACCGAGTCT	22	50	1	-4.59

Optimization of LAMP assay reaction components

The LAMP critical components (MgSO_4 , betaine, *Bst* polymerase, dNTPs and HNB dye) which affect the effectiveness of the detection were optimized (Table 2). In particular, Magnesium ions greatly influence primer annealing, strand dissociation, polymerase activity, product specificity as well as working of LAMP primers [31, 32, 33, 34, 35]. The colour shift of the LAMP reaction mixture after incubation period is mainly due to Mg^{2+} ions chelation by dNTPs [36]. The LAMP response was strongly impacted by the MgSO_4 concentration. Colour change was established as the major benchmark for the optimization of MgSO_4 concentration. The development of sky-blue color during the LAMP investigation points to a positive reaction in the concerned samples (Supplementary Fig. 1), whereas the development of violet color points to a negative reaction. *B. oryzae* LAMP reaction products were amplified with six different concentrations of MgSO_4 : 0, 2, 4, 6, 8 and 10 mM. The MgSO_4 at 6 mM conc. exhibited a deep sky blue colour in the LAMP reaction. The intensity of the sky blue colour decreased with either an increase or decrease from the optimal MgSO_4 concentration. The intensity of sky blue decreased at concentrations of 2 mM and 4 mM. Additionally, the LAMP assay was insufficient to generate colour at concentrations of 0 mM, 8 mM, and 10 mM, demonstrating the magnitude of the significance of MgSO_4 for the LAMP assay. The outcomes have been verified employing 2% agarose gel electrophoresis. A noticeable ladder-like pattern could be seen in the presence of 6 mM MgSO_4 , but no such pattern could be seen in the samples containing 0 mM, 8 mM, or 10 mM MgSO_4 (Supplementary Fig. 1a).

Table 2
Standardization of reagents used in LAMP assay

S. No.	Reagent	Stock Conc.	Concentration of Tested Reagents/ 1 μ L Reaction Volume				
1.	MgSO ₄ (New England BioLabs)	100 mM	2mM	4 mM	6 mM	8 mM	10 mM
2.	Betaine (Sigma Aldrich)	5M	0.6 M	0.8 M	1.0 M	1.2 M	1.4 M
3.	dNTP Mix (Genei)	10 mM	0 mM	0.6 mM	0.8 mM	1.2 mM	1.4 mM
4.	<i>Bst</i> DNA polymerase Large Fragments (New England BioLabs)	8U	0 U	2 U	4 U	6 U	8 U
5.	HNB dye	400 μ M	80 μ M	100 μ M	120 μM	148 μ M	300 μ M
The values (bold) indicated optimal reagents concentrations selected for LAMP assay.							

Betaine in LAMP assay reduces the impact of DNA-protein electrostatic interactions by eliminating the composition dependency of DNA melting temperature [37]. Also, it will accelerate and change the temperature of the DNA melting transition by disrupting the DNA duplex and weaken the secondary structure formation of primer [38, 39]. LAMP reaction products were amplified with six different concentration of betaine: 0, 0.6, 0.8, 1.0, 1.2 and 1.4 M. Betaine at 1.4 M concentration generated a characteristic ladder-like pattern and the intensity of reduced at concentration of 1.0 and 1.2 M, however, no such ladder like pattern were observed in other concentrations viz., 0, 0.6 and 0.8 M implying negative results (Supplementary Fig. 1b).

The LAMP reaction will be negatively impacted by the abundant dNTPs concentration [40]. This phenomenon is most likely caused by the sequestration of free Mg²⁺ ions by an overabundance of negatively charged nucleotides (dNTPs). Five different dNTPs concentrations were tested in the current investigation. When the dNTPs quantity was stood from 0.6 mM to 1.4 mM, the amplification improved substantially which was evidenced by stronger intensity of bands on 2% agarose gels (Supplemental Fig. 1c).

Bst DNA polymerase is the pivotal enzyme in LAMP isothermal amplification technique. *Bst* DNA polymerase was evaluated at five different concentrations using the LAMP assay and amplification was recorded at all the concentrations except at 0 U. However, the best amplification was found at a concentration of 6 U *Bst* polymerase (Supplementary Fig. 1d).

The fundamental endpoint system employed in this investigation was attained by the dye Hydroxy naphthol blue (HNB). During the LAMP reaction, the magnesium ions interact with the pyrophosphate

produced by dNTPs. A precipitate develops, shifting from magenta color of the reaction mixture to blue to signifying a positive reaction. An assortment of HNB dye concentrations was investigated, and the findings showed that concentrations less than 120 μ M had an effect on the optical detection of LAMP results but had no effect on the ladder-like pattern on 2% agarose gel. However, higher concentrations had an impact on the gel pattern of the LAMP assay products and visual representation (Supplementary Fig. 1e).

Optimization of LAMP assay reaction conditions

Since the temperature of the reaction mixture was crucial for the stringency of the annealing process, the LAMP assay was functional within a broad range of temperatures. Temperatures between 60 and 65°C were critical for *Bst* DNA polymerase to function effectively. The most intense color of reactions occurred across a broad temperature range of 58.3 to 65.9°C. The low incubation temperature encourages primers to bind to non-target regions. A short range of temperatures (56°C to 70°C) (Supplementary Fig. 2a) was used to accurately assess the colour intensity using template DNA from the *B. oryzae* to find the optimum temperature for the assay. The assay performed well at two temperatures (56 and 60°C). However, the intensity of colour development was reduced at 65°C and negative results were observed at the temperature of 69 and 70°C. The outcomes were also evaluated on a 2% agarose gel.

In order to identify pathogens in a short period of time, the duration of the incubation period must be optimized, which was notably crucial in LAMP-based assays. According to [41], a prolonged incubation period of the LAMP reaction mixture led to false positive results. Incubation time was optimized by assessing different durations from 10 to 150 min. A distinct sky blue color development was observed at an incubation period of 30 min, whereas, the intensity of the sky blue colour development was significantly reduced when an increase or decrease in the incubation period was used. Negative results were observed in the LAMP products incubated at an incubation period of 90 and 150 min. The results were again reconfirmed with 2% agarose gel (Supplementary Fig. 2b).

Thus, conspicuous amplification and colorimetric reaction was seen at a temperature of 60°C. As a conclusion, *B. oryzae* was effectively detected at 60°C for 30 min under appropriate parameters.

Limit of detection (LoD) of LAMP assay

The standout feature of the LAMP assay was its minimal DNA requirement, which was adequate to detect the target pathogen within 20–60 min. With the aid of 10-fold successive dilutions of *B. oryzae* DNA (100 ng to 1 fg), the lowest detection limit of the devised LAMP assay was determined. The lowest achievable detection limit for *B. oryzae* per reaction during 30 minutes incubation was up to 10 fg, which was validated by a colour change from violet to sky blue and reconfirmed by a typical ladder-like pattern on 2% gel electrophoresis (Fig. 3; Table 3). This experiment was performed three times.

Table 3
Sensitivity of LAMP assay for the detection of *B. oryzae*

S. No.	Concentration of genomic DNA template	LAMP assay
1.	100 ng	+
2.	10 ng	+
3.	1 ng	+
4.	100 pg	+
5.	10 pg	+
6.	1 pg	+
7.	100 fg	+
8.	10 fg	+
9.	1 fg	-

Specificity of the LAMP assay

The specificity of the primers was evaluated using the *B. oryzae* isolate Bo1 and non-target DNA samples from 22 different phytopathogens listed in Table 5. The LAMP assay effectively detected *B. oryzae*, as evidenced by a colour shift in the reaction solution from violet to sky blue, and did not react simultaneously with non-target phytopathogens, which remained violet. Electrophoresis on 2% agarose gels stained with ethidium bromide was used to confirm the specificity of the *B. oryzae* LAMP evaluation. However, DNA from other non-target phytopathogens did not produce a characteristic ladder-like pattern in reactions with *B. oryzae* genomic DNA. The observations pointed out the outstanding selectivity of the newly developed LAMP primers encoding the glycosyl transferase family 90 protein for detecting *B. oryzae* (Fig. 4; Table 4).

Table 4
Specificity of LAMP assay for the detection of *B. oryzae* targeting Exon 2 of glycosyltransferase family
90

S. No.	Organism	NCBI genbank Accession number	Host	Result
1.	<i>Bipolaris oryzae</i>	MZ995432	Rice	+
2.	<i>Bipolaris sorokianiana</i>	OP303316	Wheat	-
3.	<i>Bipolaris cynodontis</i>	OP303345	Bermuda grass	-
4.	<i>Sarocladium oryzae</i>	OM841519	Rice	-
5.	<i>Sarocladium attenuatum</i>	OP099851	Rice	-
6.	<i>Magnaporthe oryzae</i>	OP303344	Rice	-
7.	<i>Rhizoctonia solani</i>	OP412773	Rice	-
8.	<i>Ustilaginoidea virens</i>	MZ157263	Rice	-
9.	<i>Exserohilum rostratum</i>	OM891473	Rice	-
10.	<i>Simplicillium obclavatum</i>	OP501088	Rice	-
11.	<i>Pyricularia grisea</i>	ON116174	Cumbu	-
12.	<i>Exserohilum turcicum</i>	ON524816	Maize	-
13.	<i>Macrophomina phaseolina</i>	ON945550	Groundnut	-
14.	<i>Sclerotium rolfsii</i>	ON945552	Groundnut	-
15.	<i>Sclerotinia sclerotiorum</i>	ON025544	Cabbage	-
16.	<i>Colletotrichum gloeosporioides</i>	ON799265	Mango	-
17.	<i>Colletotrichum scovillea</i>	ON182070	Chilli	-
18.	<i>Colletotrichum truncatum</i>	ON182072	Chilli	-
19.	<i>Fusarium oxysporum</i> f. sp. <i>ubense</i>	OM103052	Banana	-
20.	<i>Phytophthora infestans</i>	ON705720	Potato	-
21.	<i>Puccinia arachidis</i>	OL437026	Groundnut	-
22.	<i>Uncinula necator</i>	MK637521	Grapes	-
23.	<i>Pantoea dispersa</i>	OP378111	Rice	-
24.	Nuclease free water	-	-	-

Table 5
Validation of LAMP assay for the detection of different isolates of *B. oryzae*

S. No.	Isolate code	Location	Latitude	Longitude	Result
1.	Bo 1	Thirukovilur, Kallakurichi	11.9687°N	79.2086°E	Positive
2.	Bo 2	Vedaranyam, Nagapattinam	10.3774°N	79.8495°E	Positive
3.	Bo 3	PBS, CBE	11.0137°N	76.9354°E	Positive
4.	Bo 4	Naiyanarkovil, Ramanathapuram	9.5456° N	78.6945° E	Positive
5	Bo 5	Thiruvennainallur	11.8570°N	79.3682°E	Positive
6.	Bo 6	Wetland, Coimbatore	11.0031°N	76.9249°E	Positive
7.	Bo 7	Vellimalaipattinam, CBE	10.9779°N	76.7610°E	Positive
8.	Bo 8	Gopalapattinam, Ramanathapuram	9.4896 °N	78.6497°E	Positive
9.	Bo 9	Vasanthakrishnapuram, Villupuram	12.0455°N	79.2232°E	Positive
10.	Bo 10	Thiruvannamalai	12.2286°N	79.0665°E	Positive
11.	Bo 11	Bhavanisagar	12.2286°N	79.0665°E	Positive
12.	Bo 12	Mayiladuthurai	10.9010°N	79.1472°E	Positive
13.	Bo 13	Orayur, Trichy	10.8378°N	78.6483°E	Positive
14.	Bo 14	Pollachi	10.6609°N	77.0048°E	Positive
15.	Bo 15	Chinnasalem, Salem	11.6342°N	78.8785°E	Positive
16.	Bo 16	Vayalur	10.8240°N	78.6230°E	Positive
17.	Bo 17	Perugamani, Trichy	10.8708°N	78.5943°E	Positive

S. No.	Isolate code	Location	Latitude	Longitude	Result
18.	Bo 18	K. Sathanoor	10.8360 °N	78.6591 °E	Positive
19.	Bo 19	Kumaramangalam	10.7403 °N	78.6804 °E	Positive
20.	Bo 20	Pudhur, S	11.2700 °N	79.7213 °E	Positive
21.	Bo 21	Ganapathiagaram	10.9061 °N	79.1602 °E	Positive
22.	Bo 22	Rayampettai,Tanjore	10.8871 °N	79.1230 °E	Positive
23.	Bo 23	Aduthurai	11.0139 °N	79.4810 °E	Positive
24.	Bo 24	Athukudi	11.1729 °N	79.7078 °E	Positive
25.	Bo 25	Sivapuri, Chidambaram	11.3838 °N	79.7092 °E	Positive
26.	Bo 26	Kallanai, Tanjore	10.8554 °N	78.9412 °E	Positive
27.	Bo 27	Govindhanallucheri	10.9374 °N	79.2237 °E	Positive
28.	Bo 28	Thiruvidaimaruthur	10.9979 °N	79.4572 °E	Positive
29.	Bo 29	Chellapatty, Madurai	9.94700 °N	77.8899 °E	Positive
30.	Bo 30	Bhavanisagar	11.4739 °N	77.1465 °E	Positive
31.	Bo 31	Dindugal	10.4091 °N	78.0410 °E	Positive
32.	Bo 32	Kodikulam, Madurai	9.9569 °N	78.1607 °E	Positive
33.	Bo 33	Samayapuram	10.9076 °N	78.7065 °E	Positive
34.	Bo 34	Perambalur	11.2300 °N	78.8799 °E	Positive
35.	Bo 35	Palaiyur, Thiruvarur	10.6648 °N	79.4507 °E	Positive

S. No.	Isolate code	Location	Latitude	Longitude	Result
36.	Nuclease free water	-	-	-	Negative

Validation of LAMP assay

The initial validation of the LAMP assay technique was performed on the *B. oryzae* culture sample. The LAMP test was preliminary developed for the *B. oryzae* pathogen based on color development to validate the collaborative screening of reaction with thirty-five distinct isolates obtained from different locations of Tamil Nadu, India. Additionally, it was verified by encountering the samples through 2% agarose gel electrophoresis. The tubes containing template DNA and HNB indicator dye could be seen to have a vivid sky blue tint. As with the tubes holding nuclease-free water, the violet colour showed that *B. oryzae* was not detected (Fig. 5; Table 5).

Further, the developed assay was validated using artificially *B. oryzae* inoculated rice seed samples. The results revealed that those seeds which were inoculated with pathogen showed colour development whereas, those which were surface sterilized didn't develop colour resulting in negative reaction. Similar negative results were seen in nuclease-free water, which served as the negative control (Fig. 6). In case of field samples, 32 field samples were collected from different farmers' fields testing the assay at field level. Using uninfected samples as a healthy control, the LAMP test was verified for field specimens in each location. The panicle containing discolored seeds showed a positive reaction (deep sky blue colour), whereas the healthy panicle without infection showed a negative reaction (violet colour) (Supplementary Fig. 3).

When weed samples were evaluated utilizing *B. oryzae* LAMP primer sets, rice brown spot infected leaf sample and all weed species except *Brachypodium distachyon* and *Phalaris minor*. generated positive outcomes. However, the degree of amplification varied among the weed species. No amplification was observed in NTC (Fig. 7; Table 6). Rice seeds from 11 of 13 rice varieties have showed positive reaction for *B. oryzae* (Fig. 8; Table 7). Two varieties namely, Co 55 and TRY 43 did not exhibit amplification along with NTC.

Table 6
Validation of LAMP with weeds collected from
paddy fields

S. No.	Weeds	Results
1.	<i>Oryza sativa</i>	+
2.	<i>Echinochloa crusgalli</i>	+
3.	<i>Brachypodium distachyon</i>	-
4.	<i>Typha latifolia</i>	+
5.	<i>Leersia hexandra</i>	+
6.	<i>Phalaris minor</i>	-
7.	<i>Cyanodon dactylon</i>	+
8.	<i>Digitaria sanguinalis</i>	+
9.	<i>Eleusine</i> spp	+
10.	<i>Paspalum scrobiculatum</i>	+
11.	Nuclease free water	-

Table 7
Validation of LAMP assay with different
varieties of rice.

S. No.	Rice seed variety	Results
1.	Co 39	+
2.	Co 51	+
3.	Co 52	+
4.	Co 53	+
5.	Co 54	+
6.	Co 55	-
7.	ADT 37	+
8.	ADT 43	+
9.	RNR 15048	-
10.	TN 1	+
11.	TPS 5	+
12.	TRY 43	-
13.	ASD 16	+
14.	Nuclease free water	-

In case of soil samples, soil collected from *B. oryzae* infected field showed positive reaction with the development of deep sky blue colour whereas, the non-template control produced violet color indicated the absence of target DNA (Fig. 9).

Discussion

Seeds are passive vectors of plant pathogens over international boundaries and may even be accountable for the invasion of exotic pathogens to new locations or the resurgence of bygone diseases [42]. Modern agricultural research has consistently emphasized the importance of seed health for the envisioned plant population and triumphant harvest. One of the most significant impediments to rice production and productivity is lack of healthy rice seed [43].

The International Seed Testing Association (ISTA) and the Seed Health Committee formed at the end of the 1920s had an obsession with seed health with the overarching objective of standardizing and developing diagnostic procedures for seed pathogens [44]. Early detection and appropriate identification of causative organisms are critical challenges in the management of seed-borne diseases. Seed health tests and seed treatments were commonly used to eliminate seed-borne inoculum [42]. Furthermore,

pathogen-free seed was suggested for disease management, and as a result, seedlots were frequently checked for pathogen presence by regulatory organizations and seed manufacturers. In order to implement early control measures like seed treatment, seed certification and quarantine legislation, it is essential to identify the seed-borne inoculum to lower the probability of disease outbreaks.

It is universally acknowledged that both major and minor fungal diseases of rice were disseminated via seeds. Brown spot is a serious seed-borne and seed-transmitted disease of rice that impacts rice producers all over the world. It is caused on by *Bipolaris oryzae* Shoemaker (*Helminthosporium oryzae* Breda de Hann, teleomorph *Cochliobolus miyabeanus*). Uncertainty over the significance of seeds as a source of primary *B. oryzae* inoculum in brown spot epidemics has recently been brought to light [45]. The number of *B. oryzae* infected seeds in a rice seed lot may alter disease establishment; the more infected seeds present, the greater the likelihood of an epidemic via seed borne inoculum [16]. It is evident that the consequences of diseases vary depending on the geographic location of the rice crop (upland, lowland, rainfed, irrigated, tropical, subtropical, etc.), cultural conditions, and crop management as well as cultivation techniques. The threshold level for a disease should, however, be zero at a location where the pathogen is not present. However, there is insufficient evidence that *B. oryzae* seed-borne inoculum has any effect on brown spot epidemics or rice yield. It did, however, reduce seedling emergence [46]. The ability to predict pathogen tolerance criteria in seeds is crucial [47, 48d]. This knowledge may be gained by understanding the effects of seed-borne inoculum on epidemics.

The diagnosis of seed-borne fungal infections depends on traditional isolation and culture methodologies. However, due to physical similarities, it is challenging to differentiate *B. oryzae* from other *Bipolaris* species and *Exserohilum* spp. by microscopic examination alone, which is be complicated and need professional expertise. As of now, the ISTA (International Rules for Seed Testing) approved procedure requires blotting a sample of 400 rice seeds, placed uniformly over filter paper (90 mm) infused with distilled water. After 7 days of incubation at $26 \pm 2^{\circ}\text{C}$ in 12h cycles of light and darkness, each seed was inspected and confirmed by stereoscopic and the proportion of infected seeds was documented. As a result, an apparent, swift and precise seed health testing approach might make a significant contribution in eliminating contaminated seed batches and therefore reducing the risk of outbreaks of this disruptive disease. Any company claiming to assure the absence of pathogens in rice seed is concerned with financial loss due to misdiagnosis. The seedling blotter assay has been widely used in seed health testing for seed borne diseases; however, substantial seed sample quantities are required to assure the test result's reproducibility. The tests demand the use of sizable, expensive rooms with controlled environments.

Routine identification tests are often carried out using PCR-based techniques in well-equipped laboratories. These advanced technologies are time-consuming, nevertheless, as they increase the difficulty of on-site detection and extended the time span between sampling and the findings [49]. Furthermore, the PCR-based technique has a few shortcomings, one of which is its susceptibility to inhibitors, which may impact specificity and may result in false negative results. These inhibitors consist of an array of little-explored compounds, such as polyphenolics, polysaccharides, melanin, humic or

tannic acids, and rice seed proteins [23, 50]. The relevance of early disease detection is demonstrated by the development of machine learning-based computational approaches for the diagnosis of plant pathogens [51].

To counteract this, it is indispensable to identify seed-borne *B. oryzae* for disease surveillance and management tasks. This requires quick and accurate detection of a suitably low level of a pathogen. The LAMP test has exemplary attributes due to the fact that enables rapid, specific, and simple field screening and is perhaps less vulnerable to inhibitors [29]. Because of its great sensitivity, rapidity, and simplicity of result visualization, LAMP-based assays have been consistently documented in the case of phytopathogens. This test is straightforward and doesn't call for any unique skills, tools, or knowledge. To carry out the LAMP reactions, only the primers, reagents, and temperature-controlled equipment are needed. Furthermore, the outcomes are easily depicted with the naked eye. Also, LAMP products can be detected through gel electrophoresis with the production of ladder-like pattern in positive samples [52].

[53] recently developed a LAMP test for the early detection of sheath rot pathogen (*Sarocladium oryzae*) in rice seed. [54] reported the detection of *Magnaporthe oryzae* and

S. oryzae in rice seeds. [55, 56] developed a LAMP test for detecting *U. virens* and *Fusarium* spp. in rice seeds. Several plant pathogens have been detected using LAMP assay under field circumstances [57, 58].

In light of these most recent results, we developed and evaluated a LAMP assay for rapid and simple detection of *B. oryzae*, a seed-borne fungal pathogen causing brown spot of rice. Three key factors have to be taken into account to detect *B. oryzae*: (1) the existence of several *Bipolaris* species associated with brown spot [59] (2) the presence of alternate hosts near the rice fields and (3) the presence of the pathogen in the seeds, leaf tissues and soil. Consequently, any diagnostic assay must be precise and sensitive enough to detect the target pathogen among a group of related microorganisms encountered in tissues, seeds or environmental samples.

Optimizing the MgSO_4 concentration is important for the LAMP primers to function properly. According to [60, 61, 62], the concentration of 6.0 mM MgSO_4 used in the present study was the most effective. Higher Mg^{2+} concentrations in the reaction mixture can cause false positive results, decrease primer specificity by binding to the wrong template, stabilize double-stranded DNA to prevent complete denaturation and reduce product yield [63, 64].

Betaine (N,N,N-trimethylglycine), an amino acid analogue with both positive and negative charges, was employed as a common additive in isothermal nucleic acid amplification assay since it reduced the melting template (T_m) of ds DNA [65]. It was discovered that betaine at 1.4 M concentration resulted in positive amplification. It has been reported that betaine improves amplification specificity by reducing secondary structure formation and base stacking, improving strand separation, amplification efficiency and acting as a molecular barrier to prevent hybridization between the primer and template [66, 67, 68].

As the "brain box" of the LAMP technique, *Bst* DNA polymerase is the key enzyme in the LAMP assay and it is highly imperative to optimize the concentration. It has excellent strand displacement, polymerase activity and thermal stability. It can amplify templates swiftly, efficiently and accurately under consistent temperature circumstances, achieving the entire LAMP assay in about an hour. In the current investigation, *Bst* polymerase at 6 U concentration was optimal for detecting *B. oryzae*. The results of this study were consistent with those of [69, 70, 71].

The concentration of dNTPs regulates LAMP amplification by sequestering Mg^{2+} ions, which increases the frequency of proximal DNA strand separations and so accelerates the commencement of LAMP amplification [72, 73]. However, excessive nucleotide addition by dNTPs reduces the quantity of free Mg^{2+} to levels that impede DNA polymerase activity. The current study demonstrated that 1.4 mM dNTP concentration was ideal for *B. oryzae* detection by LAMP assay. The result of the present investigation was found to be parallel with those of [61, 74, 75].

Aerosol deposition of positive samples into controls might represent one consequence of false outcomes [76]. This is readily prevented by introducing pre-reaction dye to the LAMP reaction solution. As a possible nontoxic replacement for other dyes, including berberine, HNB dye has recently been demonstrated to be a pre-reaction dye that may be used to colorimetrically visualize LAMP amplicons. Several methodologies, including the visual evaluation and gel electrophoresis, can be employed to assess the results. In this instance, the pre-adding of 120 μ M HNB to the LAMP reaction solution had no impact on the effectiveness of amplification, and a positive response was simply identified by the easy discrimination of a colour change from violet (negative) to light blue (positive). Cross-contamination occurs in post-reaction dye applications in field circumstances because of the possibility for cis and trans priming of primer, which can result in nonspecific detection and erroneous positive/negative results [77].

Although the LAMP reaction may be seen with the naked eye using GeneFinderTM fluorescent dye, these colorimetric approaches require additional procedures including the addition of dye to test for colour changes after the reaction [78]. As a result, this may reduce the usefulness of LAMP assay for on-site applications and increase the risk of cross-contamination [79]. Thus, pre-addition of HNB dye to the reaction tube precludes any potential cross-contamination.

The detection of plant pathogens at the species level can be achieved through LAMP assay. The LAMP test is extremely selective and can only detect the pathogen of interest. The LAMP reaction is a series of three steps: primary LAMP product synthesis, cycling amplification and elongation. The six to eight regions in the target DNA were addressed by four to six LAMP primers distinguishing the LAMP assay as a specific and sensitive technique [27]. LAMP primers were created by focusing on species-specific genes. As a result, the LAMP test is extremely selective and exclusively detects the pathogen of interest. The assay was extremely specific, demonstrating positive amplification for *B. oryzae* but no amplification for other *Bipolaris* species such as *B. sorokianiana* and *B. cynodontis*, both of which are recognized as being associated with rice leaf spot [59]. Surprisingly, only *B. oryzae*, out of all the species of *Bipolaris*, was strictly selective in the assay. In comparison to the formerly developed assay for this pathogen by [60],

which had a lower sensitivity (100 fg/l), the assay's high sensitivity (10 fg/l) will play a substantial part in detecting even minute quantities of pathogen.

Though there aren't many molecular detection techniques for *B. oryzae*, any PCR or qPCR-based assay will certainly take significantly longer to detect. [80] have disclosed a qPCR-based molecular assay for the detection of *B. oryzae*. [81] previously employed hyperspectral reflectance to identify *B. oryzae* in rice. Even the LAMP-based test established by [60] required a least of 1 hour to detect *B. oryzae*.

To establish the LAMP assay, naturally infected and healthy leaf and seed samples were obtained from the field and examined for *B. oryzae* detection. The LAMP test was performed on all gathered samples and the *B. oryzae* infected samples produced a deep sky blue colour, while the healthy ones remained violet. Similar to this, [60] confirmed the LAMP test for the early detection of *B. oryzae*-infected rice leaf under field conditions.

It is important to note that there are some rice varieties such as CO 53, CO 51, TRY 4, CO 43, CR1009, VGD 1, CO 54, Dhanrasi and ADT 53 which were resistant to brown spot but are not very popular in the major rice-growing regions of Tamil Nadu [82]. In this investigation, we also attempted to determine if the test can identify the pathogens in a few of the existing rice varieties, including a few widely cultivated ones. The LAMP assay established in this investigation detected the presence of *B. oryzae* in the majority of the seed varieties examined. The pathogen was not detected in Co 51, which has resistance to rice brown spot [83]. Furthermore, CO 55 seed indicated the lack of *B. oryzae*. To avert sudden outbreaks of this disease, it is also advisable to seed health testing in seed lots provided to farmers for cultivation.

To determine whether the disease is thriving on weed hosts in a specific location, bio-surveillance of such hosts was performed. Common weed species infected by *B. oryzae* include *Echinochloa crusgalli*, *Typha latifolia*, *Brachypodium distachyon*, *Leersia hexandra*, *Phalaris minor*, *Cyanodon dactylon*, *Digitaria sanguinalis*, *Eleusine* spp., and *Paspalum scrobiculatum* [84]. Using the LAMP assay, we were capable to detect the presence of

B. oryzae in all 10 weeds tested at the Paddy Breeding Station, TNAU, Coimbatore, except for two species, *Brachypodium distachyon* and *Phalaris minor*. These weeds growing around brown spot-infected rice fields provide this pathogen refuge all over the year and serve as primary inoculum during the crop season [85, 86]. Corresponding to this, the pathogen may also persist in plant remnants and soil found in fields [87]. Diagnostic assays that are compatible with environmental samples such as soil samples from agricultural fields, will undoubtedly pave the way for robust, early and cost effective disease management [88]. The LAMP assay employed in the study was also highly capable of detecting the pathogen in soil.

Since the LAMP approach is astonishingly simple, accurate and reliable, it is impeccable for the development of diagnostic tools for the accurate and rapid identification of *B. oryzae* in seeds as well as for disease outbreak predictions. This may be an appropriate method for both on site and the laboratory detection of *B. oryzae*. By identifying *B. oryzae*-infected seeds at seed testing labs and quarantine

stations, this assay will go a long way in facilitating in the sustainable management of rice brown spot disease.

Material and methods

Isolation of fungal isolates

Thirty-five *B. oryzae* isolates were isolated from rice grain samples collected at randomly from farmer's fields, storage godowns and research institutes across Tamil Nadu, India, documented properly and taken to the laboratory for further investigation. Each isolate was cultured on potato dextrose agar (PDA) medium for 5–7 days at a temperature of $27 \pm 2^\circ\text{C}$. To extract DNA from fungal mycelia, cultures were raised in a 250 mL conical flask with 100 mL of autoclaved potato dextrose broth. After inoculation into flasks, the mycelial discs of the fungal isolates were grown for duration of 7–10 days at $27 \pm 2^\circ\text{C}$. The mycelial mat was removed and dried on filter paper from the broth. Total genomic DNA was extracted using the modified CTAB technique [89]. The concentration and purity of each genomic DNA were evaluated using a Nano-drop Spectrophotometer ND-1000 (Nanodrop, Wilmington, DE) and the quality of all extracted DNA was assessed by measuring its absorbance at 260 and 280 nm. DNA concentrations were further verified using agarose gel electrophoresis. Ten microliters of pure DNA were run on an agarose gel with 0.8% (w/v) concentration and 0.1 g/mL of ethidium bromide. The Biorad Gel Doc was used to visualize the DNA. The DNA was preserved at -20°C for future use.

PCR detection and sequencing

A 25 μL reaction mixture including 1 μL of genomic DNA, 12.5 μL of 2X PCR Master mix (smART Prime Cat No. 280311) and 2.5 μL of each forward and reverse primer was employed for the PCR investigation. (ITS 1F and ITS 4R). Nuclease-free water was added to bring the total volume to 25 μL . DNA amplification was carried out in an Eppendorf thermocycler (Germany) with the following cycling conditions: initial denaturation at 95°C for

2 min; 35 cycles of denaturation at 95°C for 30 s; annealing at 55°C for 30 s; elongation at 72°C for 60 s; and terminal elongation 72°C for 10 min. A 1.2% agarose (Himedia) gel stained with 0.1 $\mu\text{g/mL}$ ethidium bromide was used in gel electrophoresis to separate the DNA bands. The DNA bands separated on the agarose gel were visualized under UV gel documentation system. On an agarose gel, the sizes of the amplicons were assessed by contrasting them with a 100 bp DNA ladder (3422A Takara). The amplified PCR product was sequenced by Sanger's dideoxy technique at Biokart India Pvt. Ltd. (Bangalore, India). In order to determine the variability of

B. oryzae isolates sequences, they were compared to other *B. oryzae* ITS region sequences deposited in the NCBI database. They were contrasted with other *B. oryzae* ITS region sequences deposited in the NCBI database to ascertain the variability of *B. oryzae* isolates.

Designing of LAMP primer

The assessment of a putative target gene with high nucleotide diversity among related species is a significant phase in LAMP primer design. Exon 2 of glycosyl transferase family 90 protein of the *B. oryzae* was targeted for to generate the LAMP primers. LAMP oligonucleotide primer sequences were designed using the sequences obtained from NCBI GenBank (Accession number XM_007686669.1) with the web interface application Primer Explorer V5 (<http://primerexplorer.jp/e/>) available at Eiken Chemical Co., Ltd. Six LAMP primers that were chosen for the current investigation were presented in Table 1 duly following parameters provided in the Primer Explorer Manual. Figure 1 depicts LAMP primer's structure and its complement target DNA used in this work. In addition, glycosyl transferase family 90 protein sequences of *B. oryzae* (XM_007686669.1), *B. oryzae* (XM_007685947.1), *B. oryzae* (XM_007684438.1), *B. oryzae* (XM_007688708.1), *B. maydis* (XM_014226032.1),

B. sorokianiana (XM_007703176.1), *B. victoria* (XM_014700148.1) and *B. zeicola* (XM_007718954.1) were acquired from the NCBI website (<http://www.ncbi.nlm.nih.gov>) to verify the specificity of primers against other four *Bipolaris* spp. (Fig. 2). Developed primers were screened implementing the Oligo Evaluator™ (Sigma Aldrich) for secondary structure development (hairpins, palindromes, repeats, dimers, cross dimers and runs), primer dimerization (primer-dimer), % GC and GC clamp (<https://www.sigmaaldrich.com>) [90]. The specificity of the LAMP primers was tested *in silico* using BLASTn by comparing the primer sequences with the NCBI GenBank nucleotide and genome databases [91].

LAMP optimization

The *B. oryzae* isolate Bo1 (MZ995432.1) was used for optimization purposes. Different LAMP reagents such as MgSO₄, betaine, *Bst* polymerase, dNTPs and HNB dye concentrations were optimized. LAMP conditions such as reaction temperature and incubation time were also optimized. The optimal temperature was assessed from a gradient of temperature assay from 56°C to 70°C. The LAMP products were incubated at different time period from 10 to

150 min. The final LAMP reactions were accomplished in a 25 µL LAMP reaction mixture that contained 2.5 µL of 10x Thermopol reaction buffer (New England Biolabs, Ipswich, MA, USA), 1.4 M betaine (Sigma-Aldrich, St. Louis, MO), 1 µL of 6 mM MgSO₄ (New England Biolabs, Ipswich, MA, USA), 1.4 mM of 3.5 µL dNTPs (Genei, Bangalore, India), 1 µL of 0.2 mM outer primers F3 and B3, 1.4 mM of 1 µL inner primers FIB and BIP, 0.4 mM of 1 µL loop primers, 1 µL of 6U *Bst* DNA polymerase (0.24 U; New England Biolabs, Ipswich, MA, USA), 1 µL Hydroxy Naphthol Blue (HNB) (120 µM; Sigma- Aldrich, St. Louis, MO), 1.5 µL of DNA from *B. oryzae* Bo1 culture (~ 10 ng) and nuclease free water were added to make volume upto 25 µL. LAMP reaction with nuclease free water instead of DNA served as negative control. The LAMP assay reactions were executed out in an Eppendorf thermal cycler for 30 min at a constant temperature of 60°C, and the reaction was subsequently halted by heat inactivating the *Bst* polymerase at 80°C for 2 min.

LAMP products were visualized through colorimetric reactions after termination. The quantity of amplification product was evaluated by 2% agarose gel electrophoresis stained with 0.1 µg/mL ethidium bromide.

Limit of detection (LoD) of LAMP assay

LoD assays were performed using ten-fold serial dilutions of genomic DNA from

100 ng/µL to 1 fg/µL of *B. oryzae* Bo1. The DNA concentration was quantified using a Nanodrop 2000 (Thermo Scientific, Waltham, MA). 1.5 µL of each dilution was used as a template for LAMP assay to assess the amplification potency and sensitivity of the LAMP primers. This experiment was carried out thrice.

Specificity of LAMP assay

For testing the specificity of the developed LAMP protocol, Genomic DNA samples from 22 different phytopathogens obtained from the Culture Collection Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India were used.

B. oryzae Bo1 (positive control) and nuclease-free water (non-template control) were used to ascertain the specificity of the LAMP assay. The results of the assay were analyzed through colorimetric reaction as well as in 2% agarose electrophoresis assay.

Validation of LAMP assay developed for the detection of *B. oryzae*

a. Validation with different isolates of *B. oryzae*

The proposed LAMP test approach was validated using 35 different *B. oryzae* isolates obtained from different locations in Tamil Nadu, India. As previously noted, a modified CTAB technique was used to extract the genomic DNA template. Using nuclease-free water as a non-template control, the LAMP test was performed on the DNA of 35 different *B. oryzae* isolates. The LAMP reaction mixture was incubated at 60°C for 30 min followed by heating it to 80°C for 2 min to halt the reaction. The results were visualized through the colorimetric reaction of HNB dye followed by 2% agarose gel electrophoresis assay and the experiments were carried out thrice.

b. Validation through artificial inoculation of seed samples

Seeds were surface sterilized with a 3% sodium hypochlorite solution for 5 min before being washed three times with sterile water to validate the established LAMP test using artificially inoculated samples. Rice seeds were soaked in the spore suspension of *B. oryzae*

24 h prior to DNA extraction and blot dried to remove excess spore suspension on the surface of seed. Genomic DNA was isolated from rice seeds using the CTAB technique. DNA was extracted from artificially inoculated rice seeds by following the method given by [34] with slight modification. The resulting genomic DNA sample was digested using 10 g/mL of enzyme. To eliminate any RNA contamination, the genomic DNA sample was digested with 10 g/mL RNase and kept at -20°C. The final solution of 2 µL had been employed directly as a template DNA for the LAMP test.

c. Validation through screening of field samples

Discolored seed samples and apparently healthy seed samples as well as symptomatic and asymptomatic leaf samples were collected from rice fields of different districts *viz.*, Coimbatore, Trichy and Villupuram of Tamil Nadu, India. From each district, six leaf samples and five seed samples were collected. Healthy leaves and panicles were used as control. In accordance with [89, 92], genomic DNA was isolated from rice seed and leaf samples using the CTAB technique. The extracted solution (2 µL) was served as the DNA template for the LAMP assessment.

d. Validation using soil DNA

Soil samples were collected from rice fields at the Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India, for investigating the applicability of the assay to detect *B. oryzae* soil borne inoculum. From 1 m² area, four samples each weighing 50 g were collected, dried under shade, finely grounded and sieved. 25 g from each of these four samples were pooled together. Total soil DNA was extracted from the pooled sample in six replications by following Polyethylene glycol and NaCl method with slight modification. One gram of soil sample was mixed with 10 mL of DNA extraction buffer [120 mM Na₂HPO₄ (pH 7.4), 5% SDS (w/v) and 0.02 g PVPP] in centrifuge tubes and incubated for 1 h at 65°C with occasional stirring followed by centrifugation at 8,000 rpm for 10 min [93]. Then, the supernatant was mixed with half volume of PEG and 1 volume of NaCl and incubated at 4°C for overnight. Further, 1 volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 min. The supernatant obtained was precipitated by addition of 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Finally, the pellet was recovered by centrifugation at 12,000 rpm at 4°C and dissolved in 50 µL TE buffer. DNA was quantified using Nano Drop (Nanodrop, Wilmington, DE) and used as template for the LAMP assay. The rice field was monitored for disease incidence for two months after the collection of soil samples.

e. Validation using weed species and seeds of different rice varieties

Following the procedure mentioned in section “Validation through artificial inoculation of seed samples”, template DNA was extracted from the weeds randomly collected from bunds of brown spot infected fields. Using the aforementioned ideal circumstances, the LAMP assay was performed with templates. In

order to evaluate rice seeds of different varieties, 2 µl of total genomic DNA was employed for the assay despite maintaining all other variables fixed.

Conclusion

The LAMP primers designed in this study were specific for the detection of *B. oryzae*. The LAMP-based diagnostic protocol imposed in this dissertation was swift, highly sensitive and precise for *B. oryzae* detection. On top of that, the versatility of the assay to detect the pathogen in soil and weeds grants it a stranglehold over previous equivalent assays established for this pathogen. The aforementioned characteristics and the minimal instruments requisite for LAMP assays, make it desirable for on-site applications. More importantly, the assay may be proficiently implemented to screen rice seed lots at air and sea ports where plant material is swapped on a big scale. From the standpoint of ecological monitoring, this would serve as a checkpoint on the spread of *B. oryzae* all over the world. This assay has the potential to be a future on-site diagnostic tool, since it is quick and sensitive, demands minimal equipment and requires less expert work.

Declarations

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Author contributions

CG devised and planned the experiments. RL carried out the experiments, collected the data, and wrote the initial draft of the manuscript. The manuscript's initial draft was revised by CG. RL updated and refined the draft. All authors contributed to the article and approved the submitted version.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

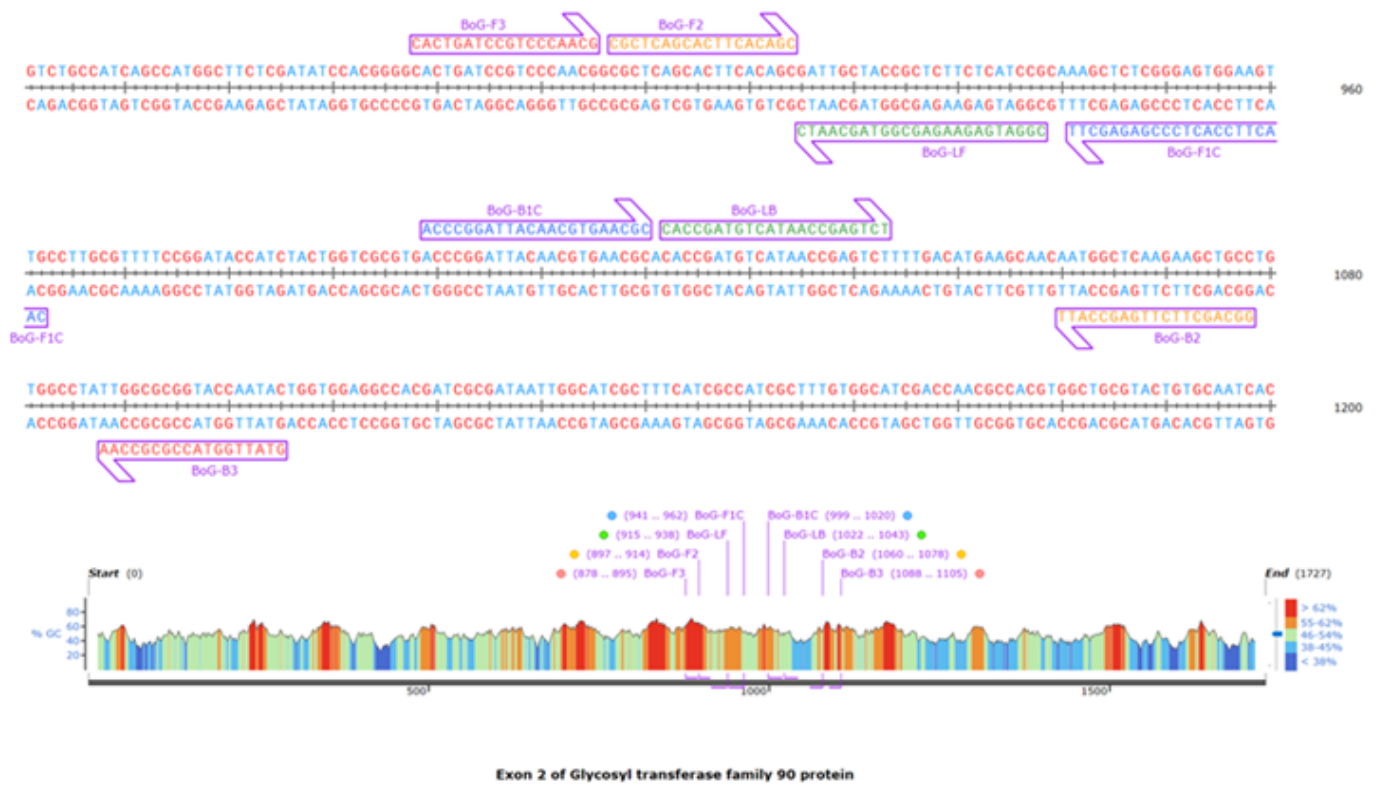


Figure 1

Diagrammatic illustration of *in silico* hybridization of LAMP primers positioned within the exon 2 of the Glycosyl Transferase Family 90 protein domain of the pathogen *Bipolaris oryzae* visualized using SnapGene viewer® 6.1.1. Outer primers: F3 and B3 (Red), loop primers: LF and LB (Green), inner primers: F2 (Orange) and F1c (Blue) and B1c (Blue) and B2 (Orange).

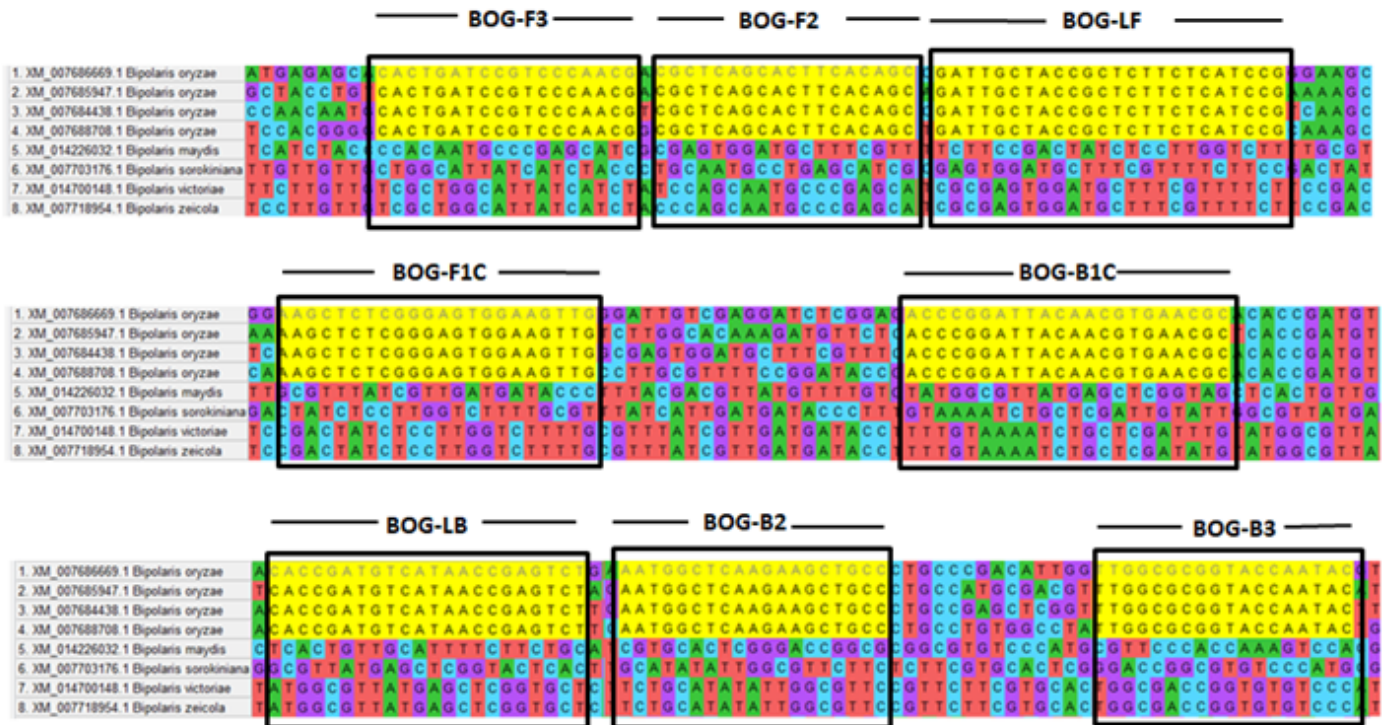


Figure 2

Comparison of the exon 2 of Glycosyl Transferase Family 90 protein region of *B. oryzae* with other *Bipolaris* spp.

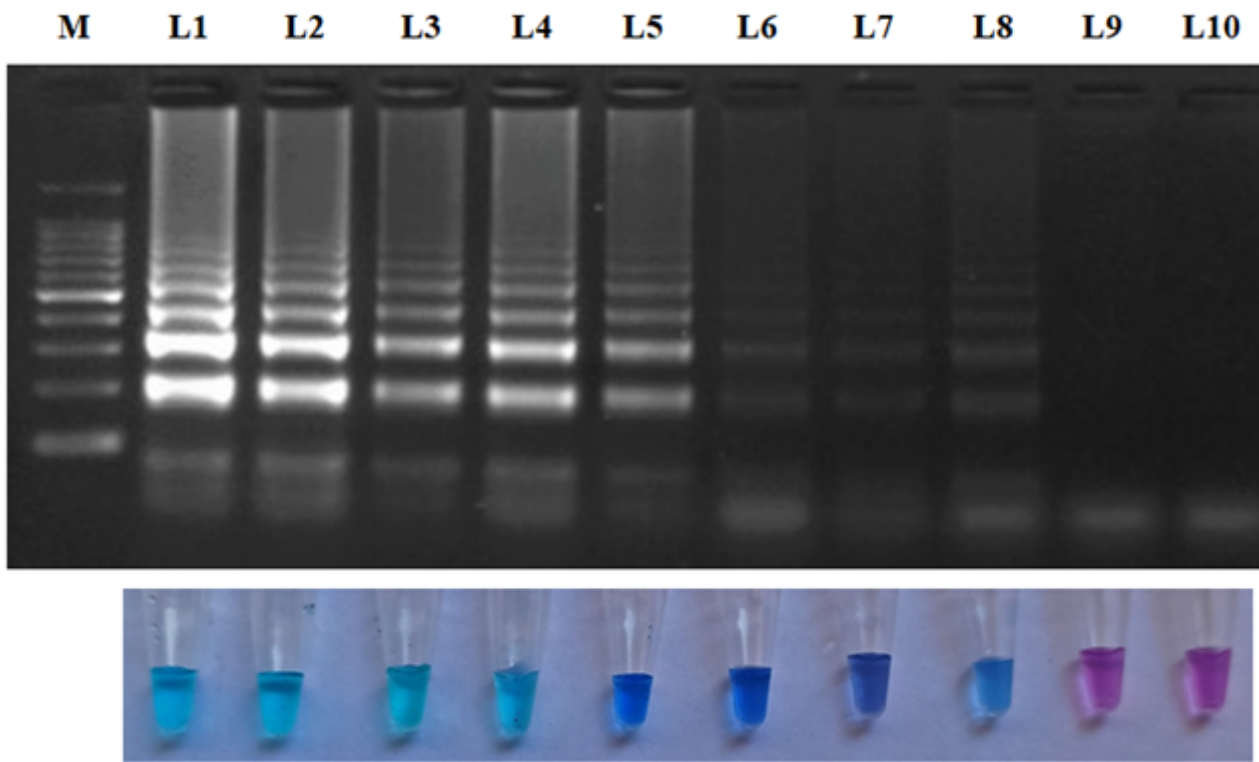


Figure 3

Sensitivity of LAMP assay using ten-fold serially diluted genomic DNA (from 100ng to 1 fg) from *Bipolaris oryzae* isolate MZ995432 as template and nuclease free water as negative control. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection. M- 100bp Ladder; Lane 1: 100 ng; Lane 2: 10 ng; Lane 3: 1 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1 pg; Lane 7: 100 fg; Lane 8: 10fg; Lane 9: 1 fg; Lane 10: Nuclease free water (Control)

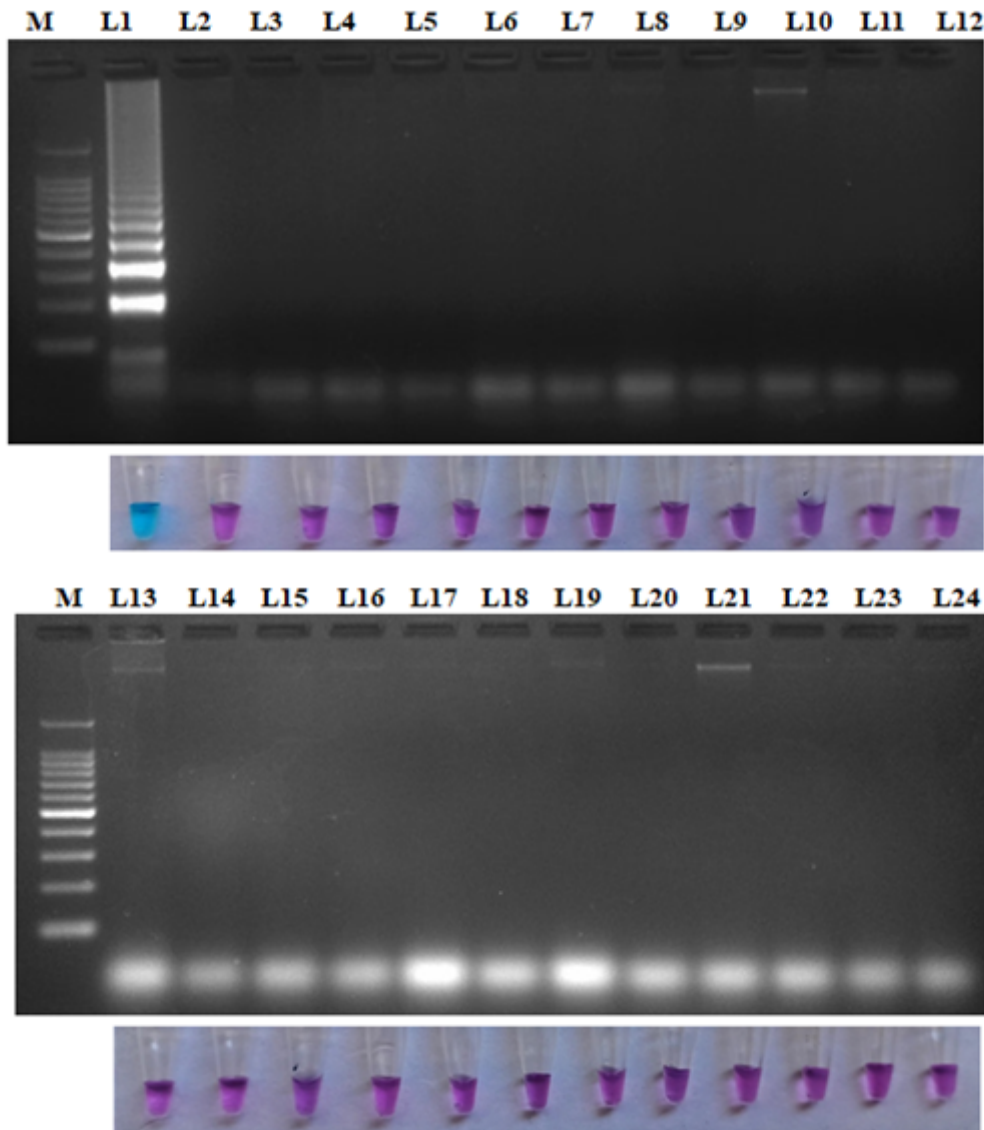


Figure 4

Specificity of LAMP assay using *Bipolaris oryzae* isolate MZ995432 as template and nuclease free water as negative control. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection.

M- 100bp Ladder; Lane 1: *Bipolaris oryzae*; Lane 2: *B. sorokianiana*; Lane 3: *B. cynodontis*; Lane 4: *Sarocladium oryzae*; Lane 5: *S. attenuatum*; Lane 6: *Magnaporthe oryzae*; Lane 7: *Rhizoctonia solani*; Lane 8: *Ustilaginoidea virens*; Lane 9: *Exserohilum rostratum*; Lane 10: *Simplicillium obclavatum*; Lane 11: *Pyricularia grisea*; Lane 12: *Exserohilum turcicum*; Lane 13: *Macrophomina phaseolina*; Lane 14: *Sclerotium rolfsii*; Lane 15: *Sclerotinia sclerotiorum*; Lane 16: *Colletotrichum gloeosporioides*; Lane 17: *C. scovillea*; Lane 18: *Colletotrichum truncatum*; Lane 19: *Fusarium oxysporum* f. sp. *cubense*; Lane 20: *Phytophthora infestans*; Lane 21: *Puccinia arachidis*; Lane 22: *Uncinula nector*; Lane 23: *Pantoea dispersa*; Lane 24: Nuclease free water (Negative control).

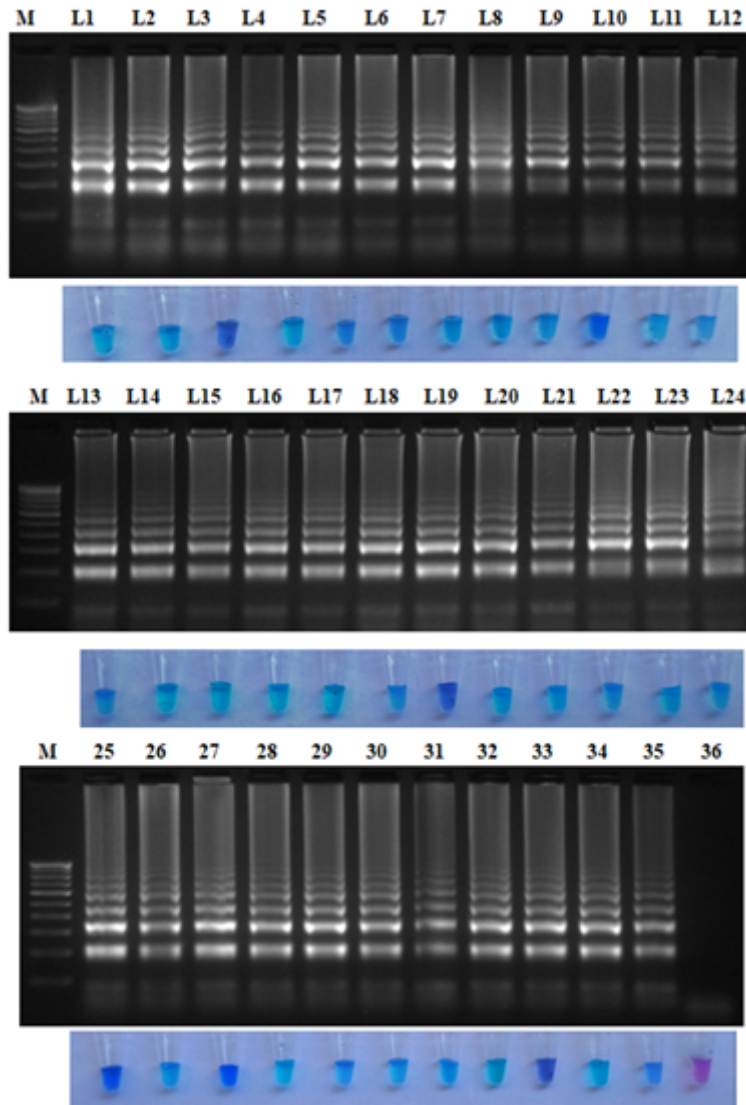


Figure 5

Validation of LAMP assay with different isolates of *S. oryzae*. Bright sky blue colour represents positive reaction while the violet colour indicates the negative reaction. Total DNA isolated from fungi was used as the template. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection.

M- 100bp Ladder; Lane 1-35: *B. oryzae* study isolates 1-35; Lane 36: Nuclease free water (Control).

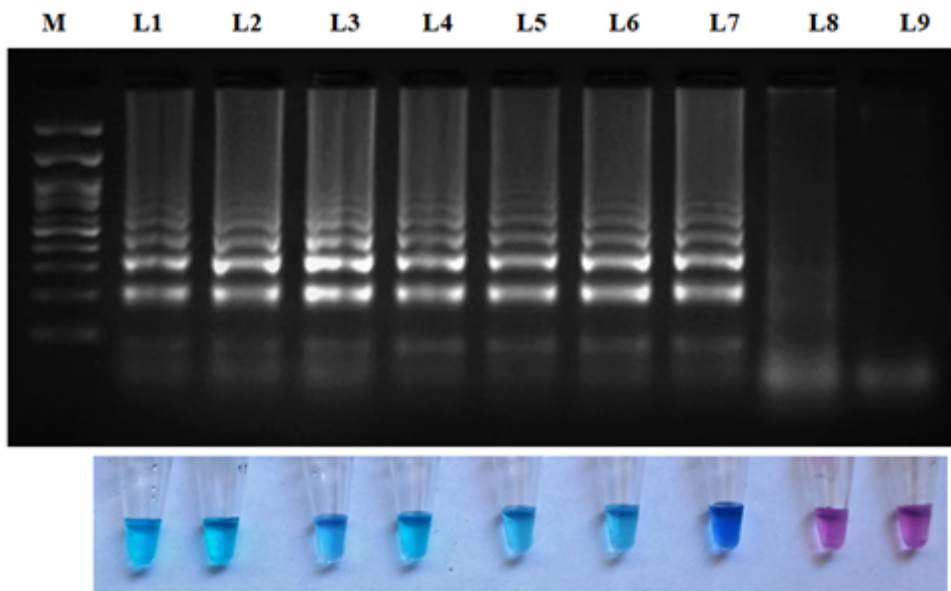


Figure 6

Validation of LAMP assay with artificially inoculated rice seeds. Bright sky blue colour represents positive reaction while the violet colour indicates the negative reaction. Total DNA isolated from fungi was used as the template. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection.

M- 100bp Ladder; Lane 1-35: *B. oryzae* study isolates 1-35; Lane 36: Nuclease free water (Control).

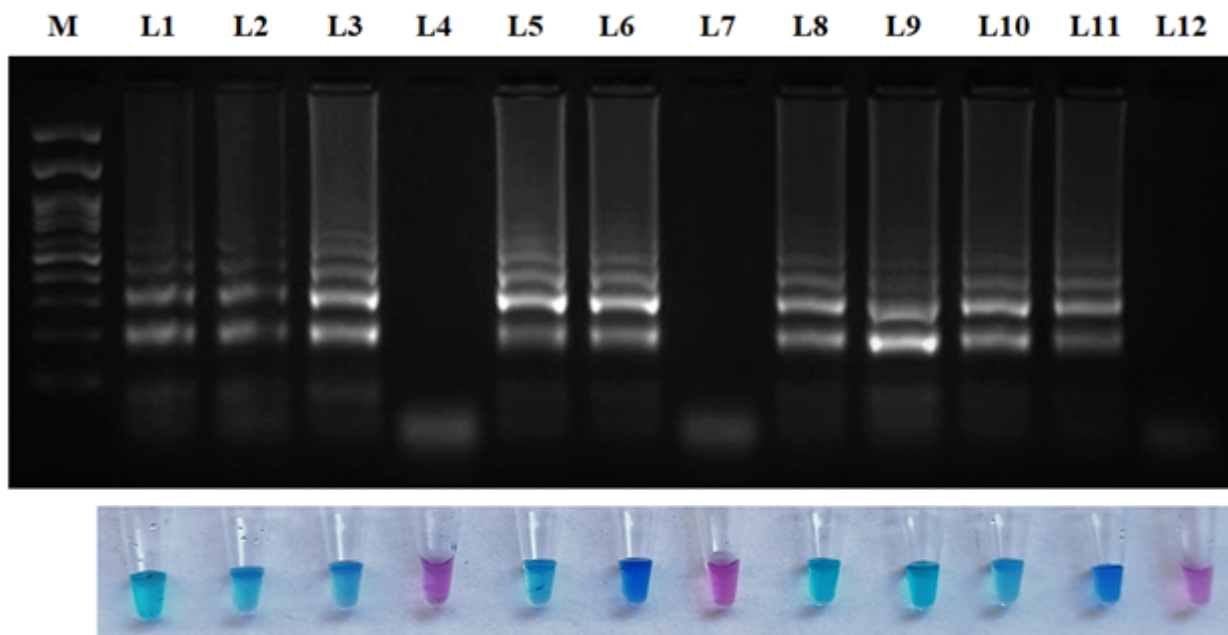


Figure 7

Validation of LAMP assay with *B. oryzae* infected paddy and weed samples. Bright sky blue colour represents positive reaction while the violet colour indicates the negative reaction. Total DNA isolated from fungi was used as the positive control. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection. M- 100bp Ladder; Lane 1: *B. oryzae* isolate MZ995432; Lane 2: *Oryza sativa*; Lane 3: *Echinochloa crusgalli*; Lane 4: *Brachypodium distachyon*; Lane 5: *Typha latifolia*; Lane 6: *Leersia hexandra*; Lane 7: *Phalaris minor*; Lane 8: *Cyanodon dactylon*; Lane 9: *Digitaria sanguinalis*; Lane 10: *Eleusine* spp; Lane11: *Paspalum scrobiculatum*; Lane 12: Nuclease free water.

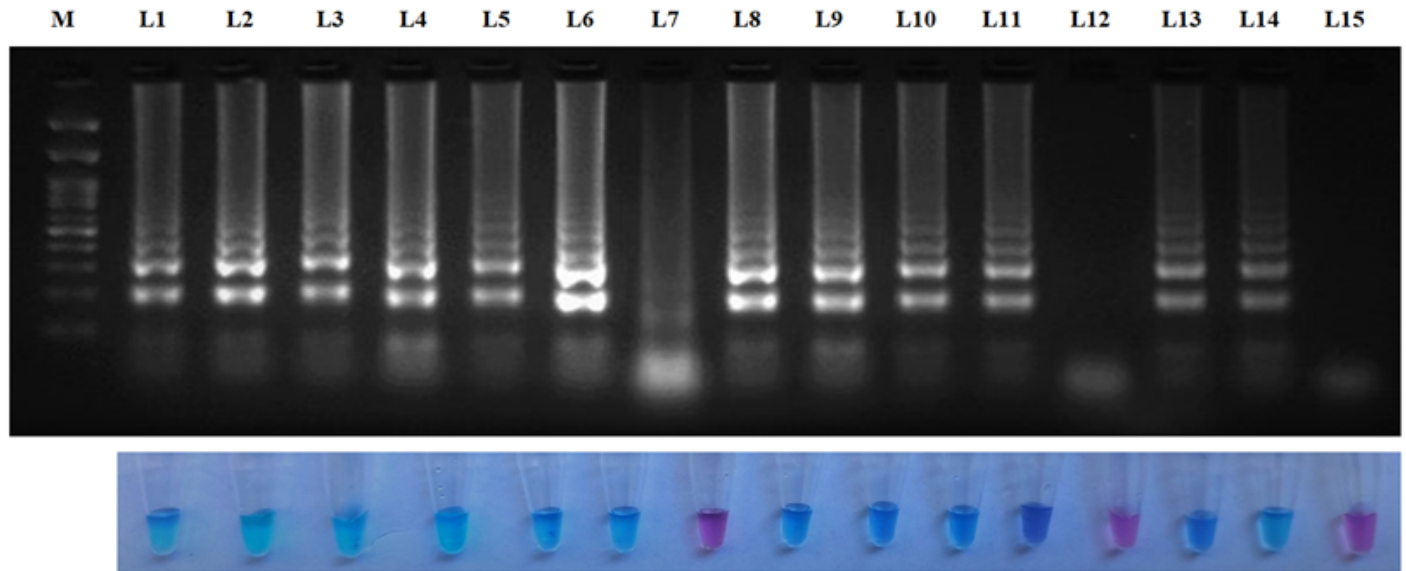


Figure 8

Validation of LAMP assay with naturally infected seeds of different varieties. Bright sky blue colour represents positive reaction while the violet colour indicates the negative reaction. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection. M- 100bp Ladder; Lane 1: *Bipolaris oryzae* isolate MZ995432 (Positive control); Lane 2: Co 39; Lane 3: Co 51; Lane 4: Co 52; Lane 5: Co 53; Lane 6: Co 54; Lane 7: Co 55; Lane 8: ADT 37; Lane 9: ADT 43; Lane 10: TKM 13; Lane11: TN 1; Lane 12: TRY 43; Lane 13: ASD 16; Lane 14: TPS 5; Lane 15: CR 1009; Lane 16: White ponni; Lane 17: RNR 15048; Lane 15: Nuclease free water (Negative Control).

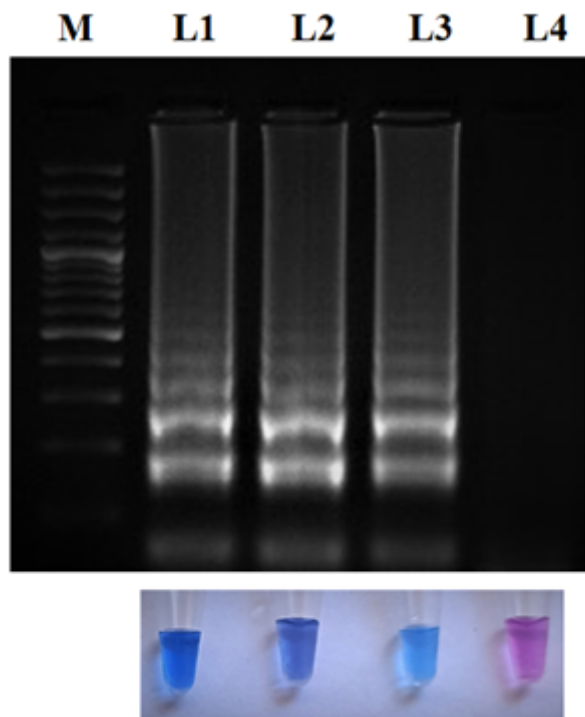


Figure 9

Validation of LAMP assay with soil samples. Bright sky blue colour represents positive reaction while the violet colour indicates the negative reaction. Upper panel in the figure shows agarose gel electrophoresis results whereas lower panel shows reaction tubes for colorimetric detection. M- 100bp Ladder; Lane 1: *Bipolaris oryzae* isolate MZ995432 (Positive control); Lane 2: Soil sample 1; Lane 3: Soil sample 2; Lane 4: Nuclease free water (Negative Control).

Supplementary Files

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