ISOLATION AND IDENTIGICATION OF SEED BORNE FUNGI FROM KHAO HOM MALI RICE

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Abstract - Seed borne diseases cause significant losses in rice yield. Seed health testing to detect seed borne fungi is an important step in the management of crop diseases. This study was carried out to isolate and identify seed borne fungi associated with stored grain cultivars of Khao Hom Mali rice (Oryza sativa L. cv. KDML 105). The seeds samples were collected from the local barns that located in Lampang province, Thailand. Seed borne fungi were isolated using blotter paper and agar plate methods. A total of 7 fungi isolates were isolated from rice seed samples consisted of LPRU1, LPRU2, LPRU3, LPRU4, LPRU5, LPRU6 and LPRU7. The result from pathogenic fungal test shows that LPRU 3 had the highest effect significantly difference ($P < 0.05$) to incomplete growth of seedling which gave germination and complete percentage include percentage of infection at 82.33, 82.33 and 78%, respectively. This isolate was identified by morphological characterization and gene sequencing analysis. From identification conclude that LPRU3 was Bipolaris oryzae. Isolation of fungal fungi from the rice seeds indicate that they should be treated before sowing to obtain good germination and healthy crop.

Keywords: Seed borne fungi, Rice seeds, Khao Hom mali rice, Isolation, Identification.

I. INTRODUCTION

Rice (Oryza sativa L.) is the most widely consumed staple food for a large part of the world's human population, especially in Asia [1]. Khao Hom Mali rice (O. sativa L. cv. KDML 105) or Jasmine rice or Thai fragrant rice is a long-grain variety of fragrant rice which is grown primarily in Thailand and famous worldwide.

During seedling establishment, rice may be infected by fungal pathogens [2]. Most of the diseases of rice are carried though seed and cause enormous losses to the crop. Seed borne diseases may not only introduce new pathogens or affect quantitatively and qualitatively the crop yield, but also contaminate permanently the soil [3].
Seed borne diseases like brown leaf spot, rice blast, stem rot and leaf blight are caused by the fungal pathogens like Alternaria alternata, A. padwickii, A. longissima, Aspergillus niger, Curvularia oryzae, C. lunata, Drchslera oryzae, Fusarium miniliforme, F. semitectum, F. oxysporum, F. soalni, Pyricularia oryzae, and species of Phoma, Cercospora, Chaetomium, Sclerotium, Pecicillium, Myrothecium and Colletotrichum have been isolated from seeds of different varieties of rice collected from different regions of the country [4].

These seed borne fungi pathogens are disastrous as they may decrease seed germination, cause seed discoloration; produce toxins that may be injurious to man and domestic animals including causes of rice yield reduction in many country [5]. Studies carried out by [1] showed a wide range of seed borne fungi pathogens on the farmers saved rice seeds, which they mainly relay on to propagate the next generation of crop.

II. DETAILS EXPERIMENTAL

2.1 Experimental location
The experiment was conducted at Microbiology Lab, Department of Biology, Faculty of Science, Lampang Rajabhat University, Lampang province, Thailand.

2.2 Sources of experimental materials
Rice seed samples of Khao Hom Mali (Oryza sativa L. cv. KDML 105) were collected from the barns at Sanpateung village (5 samples) and Tonghuachang village (5 samples) that located in Lampang province,

Thailand. Each sample was enclosed in paper bags with proper labeling, taken to the laboratory and kept in the refrigerator at 4 °C until used for the isolation and detection of seed-borne fungi.

2.3 Physical inspection of the rice seeds
The collected seed samples were physically inspected with the unaided eye on the basis of which they were separated into pure seeds, seeds of other crops and inert matter. One kilogram (kg) of each sample was poured into a plastic tray. Inert matter included soil, sand, stones, plant debris, fungal fruiting structures or the effect of fungal pathogens on the physical appearance of the seeds.

2.4 Seeds sterilization
Rice seeds used were surface sterilized using 0.5% sodium hypochlorite (NaOCl) for 5 minutes and later rinsed in several changes of sterile water.

2.5 Preparation of isolation media
The isolation media of potato dextrose agar (PDA) was prepared as described in previous report [6]. The media was autoclaved at 121°C for 15 min before use.

2.6 Isolation of fungi from rice seeds

Three replicates of 90 seeds were surface sterilized as above proceeded. Petri dishes were lined with three layers of filter papers that were soaked in sterile distilled water. Surface sterilized seeds were plated (30 seeds/Petri dishes) and incubated for 7 days at 30°C. Filter papers in the Petri dishes were rehydrated at 24 hrs interval. After 7 days of incubation, fungi which grew out of the seeds were isolated on Potato Dextrose Agar (PDA). Each fungal isolated was further purified by hyphal tip method [7]. Various cultures obtained were maintained on PDA slants and preserved in the refrigerator at 4 °C.

2.7 Pathogenic fungal test

Disinfected seeds of rice with cultures of 7 fungi isolates (LPRU1, LPRU2, LPRU3, LPRU 4, LPRU5, LPRU6 and LPRU7) with 10^6–10^8 cells mL^{-1} suspension for six hours in Petri dishes (5 mL per 30 seeds) and plated on sterile filter paper.

Three pieces of filter paper were properly soaked in sterilized water and were placed at the bottom of Petri dishes. Thirty (30) rice seeds per Petri dish were placed by using a pair of forceps and making sure that seeds are placed equidistantly under aseptic conditions. The lids of each Petri dish were held in place with gummy cello tape. The Petri dishes containing seeds were incubated at room temperature (30 °C) for 7 days under alternating cycles of light and darkness of 12 hours each. After seven days of incubation, growth parameters including seeds germination, seeds infection, completely infection, root and shoot lengths formation were recorded.

2.8 Determination of Seeds Germination/Viability

Germ inability of the seeds was determined by visual observation. Seed germination was assessed by counting the seeds with seed leaf and dividing by the total number of seeds in the Petri plates expressed as a percentage. Thus;

$$\text{Number of seeds Infected} = \frac{\text{Number of seeds infected with fungi}}{\text{Total number of seeds per plate}} \times 100$$

2.9 Determination of Seeds Infected with Fungi

Seeds with fungal infection were determined by counting the infected seeds and dividing by the total number of seeds expressed as a percentage. Thus;

$$\text{Number of seeds Infected} = \frac{\text{Number of seeds infected with fungi}}{\text{Total number of seeds per plate}} \times 100$$

2.10 Identification of fungi

The fungi were identified on the basis of spore morphology and mycelium characteristics using stereoscopic-binocular microscope.

A list of morphological characters of taxonomic importance such as type of spore, septation, color and character of the mycelium were compiled for each fungus. Identification of the fungus was performed using all the characteristics observed and identification reference manuals of Barnett & Hunter [8], and Watanabe [9].

For molecular identification by DNA sequencing using ITSI- 5.8S rDNA – ITS2 [10]. The sequence was searched by basic local alignment (BLAST) on the National Center for Biotechnology Information GenBank database to find the closest matches. Sequence was submitted to GenBank.

2.11 Data Analysis

Data obtained from this study was analyzed using Analysis of Variance (ANOVA) and the Fishers Least Significant Difference was used to separate the means at 5% level of significance.

III. RESULTS AND DISCUSSION

In this study, 10 rice seed samples of Khao Hom Mali were collected from the barns at Sanpateung village (5 samples) and Tonghuachang village (5 samples) that located in Lampang province, Thailand. A total of 7 isolates of fungi were isolated by blotter and agar method. These isolates consisted of LPRU1, LPRU2, LPRU3, LPRU4, LPRU5, LPRU6 and LPRU7. Visual and microscopic observation was used to characterize the selected strains. Details of the hypha and spore characteristic of fungi are noted (Table 1.). The above results gave an idea of morphology and mycelium characteristics of isolated strain which would aid in identification of the isolated fungi strains in the future.

Table 1. Hypha and spore characteristic of isolates fungi

Isolates	Sites	Hypha characteristic	Spore characteristic	
			Color	Type of spore
LPRU1	Tonghuachang	non-septate	black	sporangiospore
LPRU2	Tonghuachang	non-septate	green	conidia
LPRU3	Tonghuachang	septate	grey	conidia
LPRU4	Sanpateung	non-septate	yellow	conidia
LPRU5	Sanpateung	septate	green	ascospore
LPRU6	Sanpateung	septate	black	sporangiospore
LPRU7	Sanpateung	septate	black	ascospore

From isolation, 7 fungi isolates were then tested for seed borne fungal test at room temperature (30 °C) for 7 days under alternating cycles of light and darkness of 12 hours each. The result indicate that

LPRU3 had the highest effect significantly difference ($P < 0.05$) to incomplete growth of seedling which gave germination and complete percentage include percentage of infection at 82.33, 82.33 and 78%, respectively. This isolate were also found to growth of root and shoot length at 33.58 and 46.05 mm, respectively (Table 2.). These results were in coherence with those of many research groups around the world [11] [12] and [13]. Many of the isolated fungi have been reported to be associated with seeds of other crops [14]. Some of them are also known to cause seed rot, decrease seed germination and cause pre and post damping off and seedling death [15]. From this result, isolate LPRU3 was furfure identified by morphological characterization and gene sequencing analysis.

Table 2. Percent of seedlings and root and shoot length of rice showing infection by each of 7 fungi isolates after 7 days and untreated control.

Isolates	Seedlings				
	Germination (%)	Complete (%)	Infection (%)	Root Length (mm)	Shoot Length (mm)
LPRU1	84.67 a*	84.67 a	57.00 d	37.88 bc	57.78 b
LPRU2	86.67 ab	86.67 ab	50.00 d	35.28 bc	55.56 b
LPRU3	82.33 a	82.33 a	78.00 e	33.58 bc	46.05 a
LPRU4	91.33 ab	91.33 ab	78.00 e	30.93 ab	58.63 b
LPRU5	92.33 ab	89.67 ab	33.33 c	38.31 c	60.73 b
LPRU6	87.67 ab	85.67 a	24.33 bc	38.51 c	57.07 b
LPRU	86.67 ab	84.67 a	20.00 b	35.23 bc	60.03 b
Control	86.67 ab	99.00 b	1.00 a	26.26 a	45.51 a

Rapid growth colony of fungal isolate LPRU3 on potato dextrose agar at 30 °C was grey. Colonial texture was woolly to cottony and fluffy (Fig. 1.). Hyphae were septate and hyaline. Conidia were usually curved, fusoid or obclavate, occasionally almost cylindrical, pale to mid golden brown, 5 to 6 septate with hilum. These characters were identified in genus Bipolaris sp. according to Barnett & Hunter [8], and Watanabe [9]. Similarly in the study of [16] found that sporulation was observed in the Bipolaris sp. which were grey and white mixed with cottony growth. On the basis of colony morphology and growth pattern on PDA, 116 isolates were grouped into 8 groups viz., black with suppressed growth, black with cottony growth, black with fluffy growth, grey with suppressed growth, grey with cottony growth, grey with fluffy growth, grey and white mix with cottony growth and white with cottony growth. Majority of the isolates (62.93%) were black followed by grey (33.62%) among the Bipolaris oryzae isolates [17] (Kumar et al., 2016).

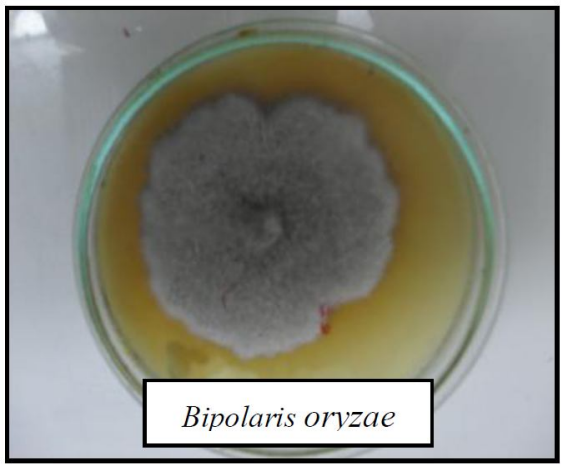

Fig. 1. The Colony morphology on Potato Dextrose Agar of Bipolaris oryzae LPRU3

From the DNA extraction of isolate LPRU3 by using the isoplant DNA extraction kit, the result showed that this isolate had the genomic DNA concentrations at 15.9 ng/μL and had 700 base pairs. The sequences were compared to 18S rRNA gene sequences in the GenBank using the Blast program. From the program, sequence of isolate LPRU3 involved the closest match to that of Bipolaris oryzae with a homology of 99%. Earlier observations reported the occurrence of Bipolaris spp., Fusarium spp. and Curvularia lunata were found more frequently with agar plate method as for Bipolaris spp, they are major plant pathogens, and many are seed borne [18]. Similarly of [19] reported that Bipolaris, Chaetomium, Curvularia, Fusarium, Sarocladium and Trichoderma comprising twelve species among them, the most predominant one was Bipolaris oryzae which was associated with 58.89 per cent seed samples, followed by Alternaria padwickii (52.96%).

CONCLUSIONS

A total of 7 fungi isolates were isolated from rice seed samples which were collected from the barns at Bansanpateung village and Bantonghuachang village that located in Lampang province, Thailand. The pathogenic fungal test of LPRU3 had the highest effect significantly difference($P < 0.05$) to incomplete growth of seedling which gave germination and complete percentage include percentage of infection at 82.33, 82.33 and 78%, respectively. This isolate was identified as Bipolaris oryzae.

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