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Isolation, Classification and Study of the Influence of Glow Discharge Plasma on Dirty Panicle Rice Fungal Pathogens

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Abstract

This study was conducted to isolate and classify fungal dirty panicle disease pathogens from five rice varieties. Observation of rice seeds under stereo microscope showed that the seeds from all five varieties had 100% lesions of dirty panicle disease. When isolating the fungal pathogen with three methods, i.e. (i) ground rice seeds shaken in dH_2O and then spread on potato dextrose agar (PDA), (ii) rice seeds washed with dH_2O twice, then placed on PDA, and (iii) rice seeds shaken in 5% clorox for 5 min, after that washed with dH_2O twice and then placed on PDA, 57 isolates of fungi were obtained. Morphological characterization classified the fungi into 17 genera, i.e. *Alternaria*, *Arthrinium*, *Aspergillus*, *Biporalis*, *Botryotinia*, *Botrytis*, *Corynespora*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Humicola*, *Monilinia*, *Mucor*, *Penicillium*, *Rhizopus*, *Stachybotrys* and *Stemphylium*. Four genera, i.e. *Alternaria*, *Bipolaris*, *Curvularia* and *Fusarium* were reported to cause of dirty panicle disease of rice. The effect of glow discharge plasma on inhibition of fungal pathogen showed that exposed glow discharge plasma for 150s could inhibit mycelium growth of *Curvularia* but did not affect to *Bipolaris* and *Fusarium*.

Keywords : Dirty Panicle Disease; Glow Discharge Plasma; Thai Rice Variety

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1. Introduction

One of staple food crops is rice (*Oryza sativa* L.) that is grown in tropical and sub-tropical climate [1]. In Thailand, about three-quarters of all farm households grow rice. The world's second-largest exporter of rice is Thailand [2]. This country has plans to increase the area for rice production to 9.2 million hectares [3,4]. The Thai Ministry of Agriculture expects rice yield to be 25 million tons in 2016-2017. However, many serious problems affect rice quality and yield losses. In Thailand, one of the most important diseases that affect rice grain, seed quality, seed germination, and seed losses is dirty panicle disease [5] that usually infects rice plants before and after harvest [6]. Many fungi including *Alternaria padwickii*, *Cercospora oryzae*, *Curvularia lunata*, *Fusarium semitectum*, *Helminthosporium oryzae* and *Sarocladium oryzae* were reported to be causal agent of dirty panicle disease [7]. Therefore, finding an effective method to solve this problem should be considerably focused. There are many methods to control this disease including using disease-free seeds, mix seeds with fungicides such as carbendazim or mancozeb, monitor the weather conditions to prepare for disease outbreak.

However, we studied a new method as an alternative way to controlling dirty panicle disease which is glow discharge plasma. Glow discharge plasma is an ionized gas comprising the same concentration of positive and negative electric charges and many neutral species. This kind of plasma is applied to analytical chemistry, micro-electronic industry, and also as lasers, light sources, displays, and many other applications. Plasma is partially ionized gas normally generated by an electrical discharge at near ambient temperatures [8]. Advantages of plasma including low cost, rapid reaction times, high cleaning efficiency, low consumption of gas due to physical effects, and the enclosed and dry nature of process. The One Atmosphere Uniform Glow Discharge Plasma (OAUGDP) performs at atmospheric pressure in air and builds anti-microbial activity species at room temperature. OAUGDP can decrease log number of microorganisms including gram-positive, gram-negative, endospore forming bacteria, yeasts and bacterial virus on surfaces. When exposed to plasma within a range 50-90s, bacterial number decreased in 5 log/sub 10/cfu. Bacterial fragmentation and macromolecular leakage were observed after exposure to plasma for 10-25s [9]. Apart from agricultural products, the method of one atmospheric glow discharge plasma can sterilize *Aspergillus flavus* spores that are coated on glass bead with the ability to inoculate approximately 1.8×10^7 cfu/g within less than 30 min [10]. The present work aims to isolate and identify fungi of rice dirty panicle disease and study the effect of glow discharge plasma on these pathogens.

2. Research Methodology

2.1 Isolation of dirty panicle pathogen

Seeds of five rice varieties, i.e. Pounded Red Rice, RD15, Leum Pua, Riceberry and Black Glutinous Rice were collected from Udon Thani province,

Thailand. After that, they were observed under stereo microscope for disease symptoms and isolation of the pathogen was operated with three methods which were (i) 10 g of ground seed sample of each variety was put into a flask containing 90 ml of dH_2O and shaken at 150 rpm for 1 hr then diluted into the concentrations of 10^{-3}, 10^{-4} and 10^{-5}, after that 0.1 ml of each dilution was spread on potato dextrose agar (PDA) added with 100 ppm streptomycin, with triplicates of each concentration, and incubated at 27°C. (ii) a sampling of 15 seeds of each variety was washed with dH_2O twice and placed on PDA added with 100 ppm streptomycin by placing five seeds per petri dishes with triplicates of each variety and then incubated at 27°C and (iii) a sample of 15 seeds each of all five rice varieties was shaken in 5% clorox for 5 min and then washed with dH_2O twice and then five seeds were placed on PDA added with 100 ppm streptomycin, with triplicates of each variety, and incubated at 27 °C. After incubation for 3-5 days, they were observed for mycelium growth followed by purification of the fungal pathogens.

2.2 Morphological characterization

Morphology of the fungal pathogens was observed 7-10 days after inoculation on potato dextrose agar (PDA). The characteristics consist of mycelium growth and colony color. Therefore, wet mount technique was used for the study of mycelium characteristics, arrangement, size and shape of fungal propagation.

2.3 Effect of glow discharge plasma on dirty panicle pathogens

Fungal pathogens isolated from rice seeds including *Biporalis, Curvularia* and *Fusarium* were inoculated on PDA for 7 - 10 days. Then exposed to the plasma for 0s, 90s, 120s and 150s. The ambient air with a relative humidity of 40% was used as a precursor gas and fed into the chamber via a top electrode. The sample was held to the bottom electrode which was connected to a radio frequency of 10 kHz. An electrical discharge plasma was produced at a power of 80W. The operating pressure during treatment was kept constant at 260 Pa. All treatments were carried out in triplicates for different exposure times [11]. After that, the effect of the plasma on the fungus was examined by using 0.5 mm diameter cork borer to cut the edges of the fungal colonies that exposed to plasma at various times and then inoculated on PDA, with triplicates for different exposure times and incubated at 27 °C. Then mycelium growth was observed and wet mount technique was used to study the difference of mycelium under a light microscope.

3. Results and Discussion

3.1 Isolation of dirty panicle pathogen

Observation of rice seed samples including Pounded Red Rice, RD15, Leum Pua, Riceberry and Black Glutinous Rice under stereo microscope showed that seeds of all five rice varieties had dirty panicle disease symptom. Isolation of fungi from rice seeds was done with three methods, i.e. (i) shaking the ground seeds in dH_2O and then spreading on PDA. (ii)

washing the seeds with dH_2O and placing the seeds on the PDA and (iii) shaking the seeds with 5% clorox and placing the seeds on the PDA. The fifty-seven isolates of fungi were obtained comprising 17 isolates, 13 isolates, 12 isolates, 11 isolates and 4 isolates from Riceberry, Black Glutinous Rice, RD15, Pounded Red Rice and Leum Pua, respectively. Isolation of fungi with three methods showed that method (iii) obtained the most fungal isolates followed by method (i) and (ii), with 23 isolates, 21 isolates and 13 isolates, respectively (Table 1). The method (iii), when we disinfect the seed surface, only the fungi inside the seed will develop. Whereas methods (i) and (ii) may be contaminated by saprophytes, resulting in less isolation. The results are similar to the study of Seelarak and Thummabenjapone [12] that isolated fungi from rice seeds obtaining 10.5% fungi in rice seeds which were not sterilized and 25.5% fungi in surface sterilized seeds.

Table 1 Number of fungi isolated from three methods from seeds of five rice varieties

Method	No. of isolates	Variety				
		Pounded Red Rice	RD 15	Leum Pua	Rice berry	Black Glutinous Rice
(i)	21	5	6	2	7	1
(ii)	13	3	4	0	0	6
(iii)	23	3	2	2	10	6
Total	57	11	12	4	17	13

3.2 Morphological characterization

Classification of 57 isolates of fungi by studying morphology, growth, mycelium, and conidia [13], [14] can classify all fungi into 17 genera, i.e. *Alternaria*, *Arthrinium*, *Aspergillus*, *Biporalis*, *Botryotinia*, *Botrytis*, *Corynespora*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Humicola*, *Monilinia*, *Mucor*, *Penicillium*, *Rhizopus*, *Stachybotrys*, *Stemphylium* and unknown (**Table 2**). Many fungi were reported to be dirty panicle disease pathogens including *Alternaria padwickii*, *Cercospora oryzae*, *Curvularia lunata*, *Fusarium semitectum, Helminthosporium oryzaeqe* and *Sarocladium oryzae*. These fungal pathogens usually infect in the rice fields at panicle forming stage and after harvest [7], [15].

Table 2 Classification of fungi isolates obtained from seeds of five rice varieties

Fungi	No. of isolates					Total
	Pounded Red Rice	RD 15	Leum Pua	Rice berry	Black Glutinous Rice	
Alternaria	-	-	1	-	-	1
Arthrinium					1	1
Aspergillus	-	2	1	-	1	4
Biporalis	2	-	-	1	-	3
Botryotinia	1	-	-	-	-	1
Botrytis	-	-	-	1	-	1
Corynespora	1	-	-	-	-	1
Curvularia	1		1	2	2	6
Cylindrocarpon	1	-	-	-	-	1
Fusarium	-	-	-	1	-	1
Humicola	-	1	-		-	1
Monilinia	-	-	1	1	-	2
Mucor	-	3	-	-	2	5
Penicillium	-	-	-	1	1	2
Rhizopus	1	1				2
Stachybotrys	-	1	-	-	-	1
Stemphylium	1	-	-	-	-	1
Unknown	4	3		10	6	23
Total	12	11	4	17	13	57

3.3 Effect of glow discharge plasma on dirty panicle pathogens

The effect of glow discharge plasma on *Curvularia* revealed that exposure to plasma at 150s was able to inhibit

mycelium growth of *Curvularia*, with a colony diameter of 3.65 cm and 6.10 cm at 3 days and 5 days after inoculation, respectively (**Table 3**). When observing the mycelium growth we found that the treatment not exposed to plasma showed normal mycelium growth, while mycelium exposed to plasma had less growth (**Fig. 1**). Plasma did not affect mycelium growth and conidia germination of *Curvularia* when observed under a light microscope.

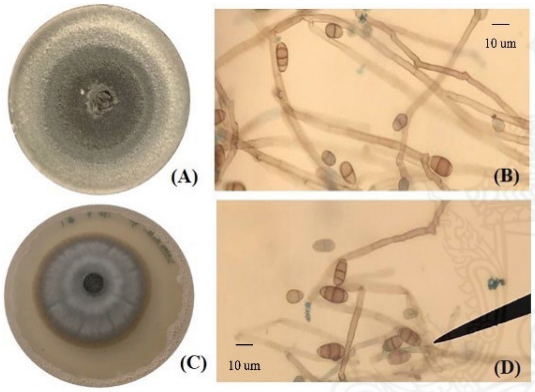

Fig. 1 Colony morphology, mycelium and conidia of *Curvularia*, 5 days after inoculation. (A, B) 0s plasma exposure (C, D) 150s plasma exposure

Results of the effect of glow discharge plasma on *Fusarium* showed that exposure to plasma did not affect the growth of *Fusarium* (**Table 3, Fig. 2**), but there were various crystal shapes in/and around mycelium and conidia when observed under a light microscope. The crystal number varies due to the duration of the plasma exposure. The longer duration of plasma exposure increased crystal number. However, five days after inoculation no crystal was found in the mycelium.

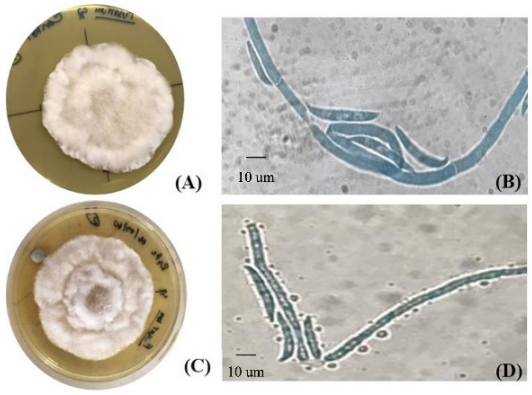

Fig. 2 Colony morphology, mycelium and conidia of *Fusarium*, 5 days after inoculation. (A, B) 0s plasma exposure (C, D) 150s plasma exposure

The effect of glow discharge plasma on *Biporalis* showed that plasma did not affect mycelium growth and conidia germination of *Biporalis* (**Table 3, Fig. 3**).

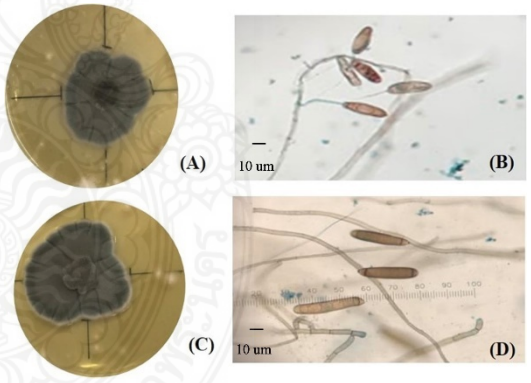

Fig. 3 Colony morphology, mycelium and conidia of *Biporalis*, 5 days after inoculation. (A, B) 0s plasma exposure (C, D) 150s plasma exposure

Study of the effect of glow discharge plasma on fungal seed pathogens showed that the plasma inhibited *Curvularia* mycelial growth, but did not affect the growth of *Bipolaris* and *Fusarium*. This is because of the effect of plasma on

disintegration and becoming free radical O^- and free radical OH^- and oxidation. It can damage cell wall structure and cell membrane and inhibit cell biochemistry. Each fungus has different cell wall composition then the effects of plasma are also different. A regular method used to clean, arrange and improve biomaterial and implant surfaces is glow discharge plasma treatment. Excellence of such treatment is powerfully based on the process parameters [16]. This is similar to the study of the ability of constructed one-atmospheric glow discharge plasma on a reduced aflatoxin contamination from agricultural products including corn, bean, garlic, and shallot. Total plate count of fungal contamination on seed before treatment was 380 cfu/g, 510 cfu/g, 710 cfu/g and $7x10^4$ cfu/g, respectively. After exposure to plasma, total mold was completely reduced on corn and bean. In the case of garlic and shallot, the number of fungi was reduced but remained at 97 cfu/g and $2x10^4$ cfu/g, respectively [10]. Moreover, in the study of the effect of plasma-glow discharge as sterilization of titanium surfaces, *in vitro* osteoblast responses to glow-discharged, commercially pure titanium (Ti) surfaces were investigated. The hypothesis of the glow-discharge treatment would be a practical sterilization system for Ti embedding before implantation. The Ti surfaces were prepared by grinding to 600 grits followed by cleaning. After that, they were divided into two groups, group one being the control and the other group undergoing glow-discharge treatment using oxygen.

Table 3 The effect of glow discharge plasma on mycelium growth of fungal dirty panicle pathogens

Duration of plasma exposure (s)	*Curvularia*		*Fusarium*		*Biporalis*	
	Colony diameter (cm)		Colony diameter (cm)		Colony diameter (cm)	
	3 day *	5 day *	3 day *	5 day *	3 day *	5 day *
0	5.88±0.08 a	8.27±0.06 a	4.00±0.26 a	4.03±0.64 a	2.92±0.34 b	3.42±0.45 b
90	5.08±0.67 b	7.90±0.69 a	3.25±0.22 a	4.57±0.13 a	3.02±0.03 b	3.72±0.08 b
120	5.45±0.17 ab	8.05±0.43 a	4.02±0.49 a	5.23±0.58 a	2.97±0.13 b	3.42±0.44 b
150	3.63±0.03 c	6.10±0.10 b	3.75±0.36 a	5.00±0.46 a	3.43±0.39 b	4.23±016 a
CV (%)	5.01±0.93	7.58±0.97	3.75±0.44	4.91±0.49	3.08±0.31	3.70±0.16

* Means (n=3) in column followed by the same letters are not significantly different based on $p < 0.05$, LSD.

Human embryonic palatal mesenchyme cells, an osteoblast precursor, were used to assess the cell responses to glow-discharged and control Ti surfaces. Ten days after treatment, the results showed that protein production and osteocalcin production on both surfaces exhibited no significant differences. Altogether, this study suggested that the use of glow discharge as an alternative sterilization system for medical and dental embed did not

inhibit osteoblast phenotypic expression [17]. Study of antimicrobial activity in herbal tea was done by using a low-pressure capacitively coupled discharge. *Escherichia coli* and *Staphylococcus aureus* were isolated from herbal tea, after that they were inoculated on NA petri dishes and exposed to the plasma for 0.5 min, 1.0 min, 1.5 min and 2.0 min [11]. From the results of an interdisciplinary cooperation established to evaluate the sterilizing capabilities of the One Atmosphere Uniform Glow Discharge Plasma (OAUGDP), this glow discharge plasma is able to perform at atmospheric pressure in air and other gases and bears antimicrobial active species to surfaces and workpieces at room temperature as decided by viable plate counts. OAUGDP exposures have decreased log numbers of bacteria, *S. aureus* and *E. coli*, *Bacillus stearothermophilus* and *B. subtilis* on seeded solid surfaces, fabrics, filter paper, and powdered culture media at room temperature [18]. Establishment of atmospheric pressure glow discharge was likened to the execution of an equipment used in the first atmospheric pressure glow (APG) experiment, in the matter of sterilization of anew categorized biological indicator such as *Bacillus atrophaeus*, *B. subtilis* var. *niger* and *Geobacillus stearothermophilus*. Stabilization was finished by controlling the experimental conditions at low frequency: 100 kHz and Radio Frequency: 13.56 MHz with water vapor/He dilution. The large volume of meta-stable atomic helium is responsible for the result that aids the generation of hydroxyl radicals [19].

4. Conclusion

In this study we obtained many fungi from seeds. There are four genera which are reported as causes of rice seed diseases including *Alternaria*, *Biporalis*, *Curvularia* and *Fusarium*. Glow discharge plasma effected to inhibit *Curvularia* mycelium growth, but did not affect the growth of *Bipolaris* and *Fusarium*.

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