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Evaluation of Enzyme Protease Activity and Inhibition Effect on *Pyricularia grisea* with the Leaf Extract of *Commela communis* L.

Chau Thanh Truc, Pham Thi Thu Ha*, Nguyen Thi Ngoc Tram and Do Thi Duyen

Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam.

Abstract

Rice blast is a dangerous disease that causes major damage to rice productivity worldwide. The purpose of this report is to examine the protease activity of *Pyricularia grisea* and the effects of *Commelina communis L.* (common name "day flower") extract on mycelial growth. The result showed that three isolates were collected from wild rice (*Oryza rufipogon*) based on a designation of new international standard and namely as U43-i4-k024-z05-ta532 (isolate 1); U12-i0-k101-z05-ta102 (isolate 2) and U43-i4-k141-z15-ta522 (isolate 3). Particularly, the third isolate showed the highest protease enzyme activity of 30.11 U/ml, followed by isolate 1 (20.27 U/ml) and isolate 2 (12.81 U/ml). The new fungi isolated from wild rice may be important methods to control detrimental diseases infesting cultivated rice. The result showed methanol extract from the day flower inhibitions on *P.grisea*. Besides protease activity, fungi inhibition was also affected. The protease activity was low; the mycelial growth inhibition was high (IC_{50} = 2.35mg/mL at Isolate 2). Plant extracts from the day flower saved money and were environmentally friendly. So the implementation of this substance is very essential.

Keywords: Protease activity, *Pyricularia grisea,* crude extract, antifungal activity, mycelial growth.

***Correspondence:** phamthithuha@tdtu.edu.vn; +84933092584

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INTRODUCTION

Fungi play an important role in the area of microbiology and biochemistry because of their ability to produce useful enzymes (Bhat *et al.* 2013). In fact, fungal enzymes have been employed for the rapid oxidation and decomposition of proteins, carbohydrates, and fats (Portumarthi *et al.* 2017). Proteases are especially known to be enzyme catalysts that hydrolyse peptide bonds in proteins (Shakil *et al.* 2012, Hanan *et al.* 2012). They can be found in all forms of life including plants, animals, and micro-organisms. Proteases are classified into three groups based on their optimum pH for hydrolysis including acid, neutral and alkaline proteases (Khan *et al.* 2011). The presence of extracellular proteases have been reported in several fungal plant pathogens, and proteases produced by pathogenic fungi play an important role in host tissue invasion, providing nitrogenous compounds to fungus during infection (Chen *et al*. 1993). Patil *et al*. (2015) have successfully isolated endophytic fungi from seven different medicinal plants and evaluated for their enzymatic activities such as amylase, protease, cellulose, and lipase.

In recent years, there has been a suggestion that *Pyricularia grisea* Sacc (syn: *Magnaporthe grisea* (Hebert) Barr) is one of the most destructive fungi to the rice production industry in the world. In an attempt to reduce the damage caused by this fungus, the uses of many eco-friendly agrochemicals have recently drawn a lot of attention. For example, the extract of different plants such as *Epicoccum* sp., *Prosophis juliflora*, and *Ziziphus* are known to have the ability to inhibit mycelial growth of *P. grisea* (Kamalakannan *et al.* 2001, Sena *et al.* 2013). *E. aromatica*, *P. guineense* and *G. kola* extracts can also be used as biological fungicides against the development of *P. grisea* (Olufolaji *et al.* 2001). Several other trees were tested and showed *P. grisea* inhibition (Olufolaji *et al*. 2015, Netam *et al.* 2011). In other research, the composition of bamboo leaves containing compounds derived from hexane and ethyl acetate such as fatty acids, oils and phenols, and their derivatives minimize the growth of *P. grisea* (Toan *et al.* 2018).

The day flower, *Commelina communis* L., is distributed widely throughout the world. The whole plants have been used as a febrifuge or a diuretic in Japanese folk medicine. In addition, the plant is also known to contain potent α-glucosidase inhibitors. These extracts and powders are also used as ingredients in the prevention of type 2 diabetes (Shibano *et al.* 2001). The alkaloid extracted from this plant was resistant to the virus A / PR / 8/34 (H1N1) (Fei-Hong Bing *et al.* 2009). However, the inhibitory effect of the leaf extract of *Commela communis* L. on *P. grisea* is still to be determined. Therefore, the purpose of this study is to examine: (1) the protease activity of fungal isolates, and (2) the fungi-inhibitory effects of *Commelina communis* L. extracts which may shed light on the new treatment methods of plant diseases in the agriculture-related field.

MATERIALS AND METHODS

Fungi source

P.grisea is isolated from wild rice (*Oryza rufipogon*) collected from rice fields in south of Vietnam.

Medium preparation

The agar for monoclonal isolation includes glucose (5g), yeast extract (5g), agar (30g), streptomycin (4 mg) and distilled water (1L). The agar agar for long-term storage contains rice flour (15g), agar (15g), sugar (5g), streptomycin (40mg) and distilled water (1L). Micro-organisms produced in the Potato Dextrose Agar (PDA) environment contain potatoes (200g), sugar (20g), agar (18g), streptomycin (40 mg) and distilled water (1L).

Isolation of *Pyricularia grisea* fungus

Isolation of *P.grisea* by methods previously used by Hayashi and Fukuta (2009). Blast samples were collected from wild rice, and single-celled strains were isolated from damaged leaf cells. Single-celled fungal spores were selected on agar and incubated for three days, which were then transferred to rice agar discs for further study.

Enzyme protease activity

Fungal strains cultured on Glucose Yeast Extract Peptone Agar (GYP) (1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 16 g agar and 1 L distilled water) contained 0.4% gelatin at pH 6.0 in a petri dish incubated in a dark room at 28°C to investigate the protease activity. Three plates with 3 replicates containing the fungus were used to measure the activity of the enzyme. Petri dishes were injected with 0.1 ml of 0.1M ammonium sulfate and the apparent area was observed around the active

colonies after six days of fungal growth, indicating the presence of protease enzymes (Prabavathy *et al.* 2013, Sunitha *et al.* 2013). The solution was then filtered and stored in a refrigerator at 4°C for later use. For quantitative testing, the protease activity was calculated by decomposing casein, 1 ml of the filtrate added to 1 ml of 1% w / v casein (pH 7.5) and incubated for 1 hour at 45°C. Next, the reaction was stopped by adding 3 ml of 0.5 M trichloroacetic acid (TCA). Next, the mixture was centrifuged at 5000 rpm for 30 minutes, and the upper portion was measured at 275 nm using the HACH DR / 4000U spectrometer (HACH Company, Loveland, CO)., USA) (Patil *et al.* 2015). Tyrosine of varying concentrations (10 - 100µg/mL) was used to establish the calibration curve. An enzyme activity unit is defined as the amount of enzyme required to release 1µg of tyrosine (Kunitz *et al.* 2015).

Preparation of methanol extract

Fresh leaves of *Commelina communis* L. were collected in Vietnam in September 2016. Dry leaves were extracted with methanol. The extraction of ten grams of leaf powder mixed with 100 mL of distilled water was shaken for 48 hours at room temperature. The extracts were then filtered, concentrated, dried and stored in a refrigerator at 4°C for future use (Kumar *et al.* 2016).

Antifungal activity

The method of extracting and screening mushroom activity was used from Toan *et al.* (2018) *P. grisea* was isolated on potato agar (PDA) at 28°C ± 2°C. The five extractable levels were prepared as 0.1 mg/mL; 0.5 mg/mL; 1 mg/mL; 5 mg/mL and 10 mg/mL with sterile distilled water. Vaccination of 1 mL of the extracted solution into 10 mL of PDA medium was poured onto a sterile Petri dish, and then about 2 mm^2 of the prepared fungi were placed in the middle of Petri dish. Transplanted plates were incubated at 28°C ± 2°C in a warm cabinet. For control samples, the fungus was implanted in distilled water. After three days of incubation, the colony diameter of the control and control plates were measured. The inhibition was calculated by the following equation: I = ((C - T) / C) x 100%, where (I) is the degree of inhibition (%), C is the diameter of the control colonies, and (T) is the diameter of the colonies of the treatments (Kartal *et al.* 2011). The concentration (mg/mL) required inhibiting the growth of 50% mycelium of *P. grisea*. Inhibition and corresponding levels of anti-fungal activity on *P. grisea* are shown in Table 1.

Statistical analysis

Standard errors and significance of enzyme activity were calculated using Mini-tab 16 software. Data comparing $P < 0.01$ are considered to be significantly different. The mean values were compared by the DUNCAN's multiple range test using SPSS statistics.

RESULTS

The three different isolates of *P. grisea* were isolated from wild rice (*Oryza rufipogon*) based on its susceptible response to monogenic

Table 1. Zone of inhibition and relative level of activity of resistance

Zone of inhibition(mm)	Inhibition activity level
>17	+++, strong
12-16	++, moderate
7-11	+, weak
6-0	-, negative

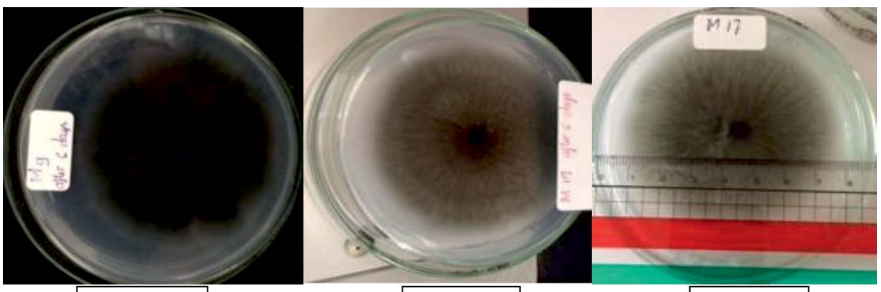

Fig. 1. Production of fungal enzyme of three isolates with GYP agar medium.

rice lines (Hayashi *et al.* 2009). Designation of these isolated fungi species were presented in Table 2. There were two isolates in group "U" that had one pathotype (U43) virulent to monogenic lines (*Pit*, LTH and *Pia*). Besides that, these isolates had pathotype "i4", which was virulent to (*Pi5(t)*). While Isolates 1 and 3 had pathotype "z05", which were virulent to *Piz* and *Piz-t*. Especially one pathotype "i0" belongs to Isolate 2 which means *Pii, Pi3*, and *Pi5* genes are resistant to blast in this group.

Table 2. Isolated designations of three fungus species from wild rice in the South of Vietnam

No.	Region/ Ecosystem	Pathotype	Code
1	Binh Phan, Cho Gao, Tien Giang	U43-i4-k024-z05-ta532	Isolate 1
2	Ngai Tu, Tam Binh, Vinh Long	U12-i0-k101-z05-ta102	Isolate 2
3	Ngai Tu, Tam Binh, Vinh Long	U43-i4-k141-z15-ta522	Isolate 3

Assessment of protease activity

The protease activity of three isolates were evaluated by the degradation of casein using GYP medium for a qualitative test of protease. As shown in Fig. 1, among the three isolates, isolate 3 and isolate 1 showed a large diameter of clear zone (Table 3). It was observed that isolate 3 had the highest density of mycelium on the surface after six days of incubation on the culture GYP medium, when compared to the others.

The objective of the present investigation was to select the fungal isolates with a high level of protease producing ability. The three isolates were checked for the quantitative test of extracellular protease in the GYP medium. Table 4 clearly shows that all isolates secreted protease enzyme at varied levels. The maximum protease activity 30.11 (U/ml) was accomplished after six days by isolate 3. The lowest protease enzyme activity was observed by isolate 2 with enzyme activity (12.810 U/ml).

Table 3. The qualitative evaluation of the fungal protease activity on GYP medium

Isolate name	Diameter of clear zone (cm)
Isolate 1	6.87 b ± 0.12
Isolate 2	6.43 b ± 0.06
Isolate 3	7.67 a ± 0.29

Values with similar letters are not significantly different for each variable, significant at p< 0.01; ± values indicate standard errors of the means.

Table 4. Protease activity from different isolates in production medium

Isolate name	Enzyme activity (Unit/ml)
Isolate 1	20.27 b ± 1.88
Isolate 2	12.81 b ± 1.72
Isolate 3	30.11 a ± 6.20

Values in the columns with similar letters are not significantly different for each variable, significant at p <0.01; ± values indicate standard errors of the means.

Table 5. Inhibitory activity of day flower leaf extract on mycelial growth (mm)

Concen. (mg/mL)	Isolates					
	Isolate 1	Inhibition activity level	Isolate 2	Inhibition activity level	Isolate 3	Inhibition activity level
0.1	9.00 hi	(+)	26.00 b	(+++)	16.33 fg	(++)
0.5	14.67 fg	(++)	22.00 cd	(+++)	21 de	(+++)
1	16.67 ef	(++)	19.00 def	(+++)	8.67 i	(+)
5	9.67 hi	(+)	21.33 cd	(+++)	13.67 gh	(++)
10	16.33 ef	(+++)	29.67 a	(+++)	25.33 bc	(+++)

+++: Strong ++: Moderate; +: Weak. Means were the diameter of the mycelial growth (mm) of *P. grisea*. Means with similar letters are not significantly different at p < 0.01.

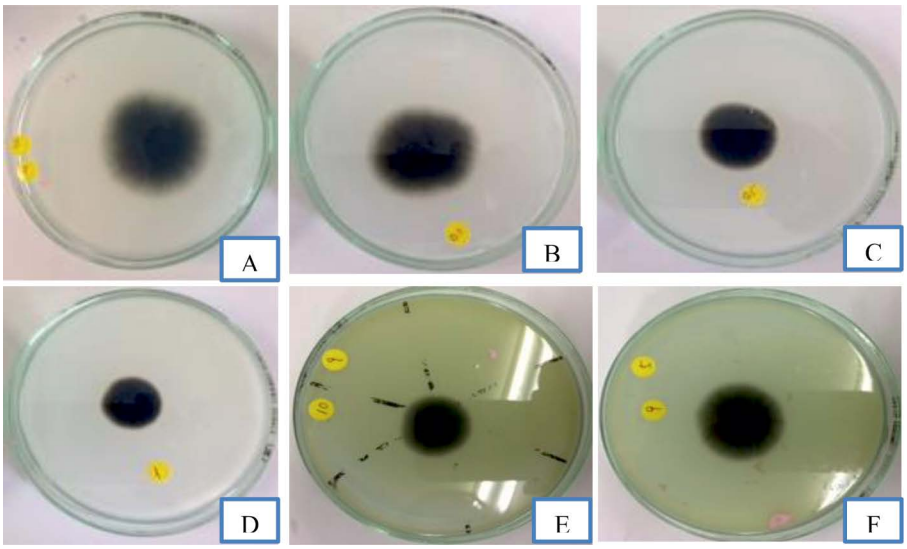

Fig. 2. Mycelia growth of Isolate 1 of *P.grisea* in response to control (A), 0.1 mg/mL (B), 0.5 mg/mL (C), 1 mg/mL (D), 5 mg/mL (E) and 10 mg/mL (F), repetitively of *Commela communis* L. extract on PDA medium.

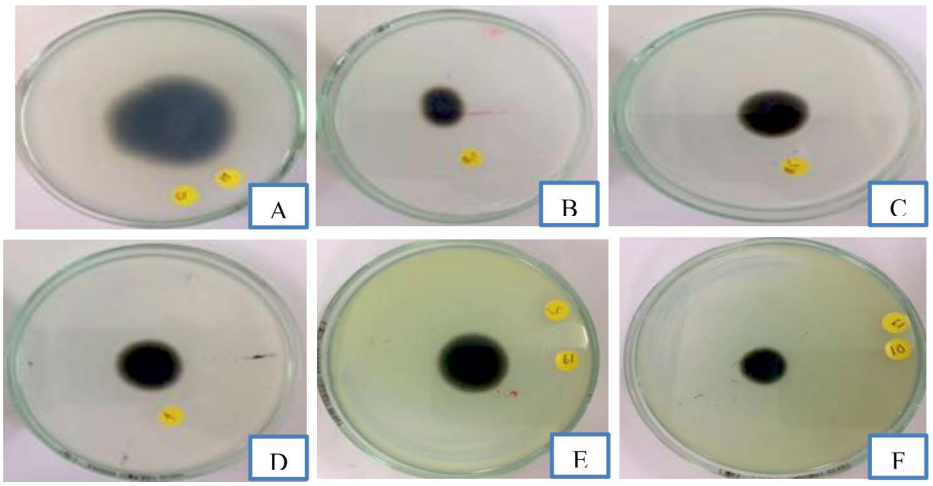

Fig. 3. Mycelia growth of Isolate 2 of *P.grisea* in response to control (A), 0.1 mg/mL (B), 0.5 mg/mL (C), 1 mg/mL (D), 5 mg/mL (E) and 10 mg/mL (F), respectively of *Commela communis* L. extract on PDA medium.

Antifungal activity

The antifungal inhibitory properties of methanol extract from *Commelina communis* L. were tested on *P.grisea* (Table 5; Figs. 2-4). At the highest concentration (10 mg/mL), the extract showed the strongest fungus inhibition. Following were 0.5 mg / mL and 0.1 mg /mL showing moderate inhibition. And finally, the extract at 1-5 mg/mL had the lowest results. Based on the objective of the study, selecting a 10 mg/mL concentration was used to observe the effects on the methanol extract from the day flower inhibition on mycelial growth of *P.grisea*.

For the methanol extract, complete inhibition was found at the lowest IC_{50} value (2.35 mg/mL) by isolate 2 followed by isolate 3 (11.98 mg/mL) (Table 6). The above values show that fungal growth of these isolates were inhibited.

DISCUSSION

In the present study, an investigation was made to isolate the protease enzyme from wild rice collected from different isolates. Fungi are an important component of rice microbiota occurring in natural habitats and change with

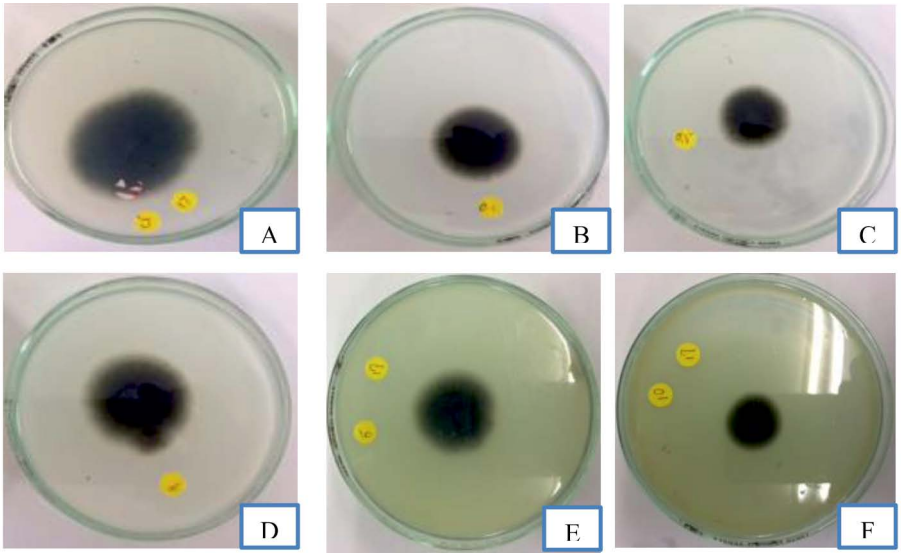

Fig. 4. Mycelia growth of Isolate 2 of P.grisea in response to control (A), 0.1 mg/mL (B), 0.5 mg/mL (C), 1 mg/mL (D), 5 mg/mL (E) and 10 mg/mL (F), respectively of *Commela communis* L. extract on PDA medium.

Table 6. Effect of methanol extract of day flower to different isolates

Isolates	IC_{50} (mg/mL)
Isolate 1	41.34 a
Isolate 2	2.35 c
Isolate 3	11.98

IC_{50} (mg/mL) is the concentration required to inhibit 50% mycelial growth of *P.grisea.* Means with different letters are significantly different at $p < 0.01$.

different environmental conditions. They can be used to characterize differences among strains of various host plants or to assay for production of enzymes with various industrial applications. There is a growing need for new, environmentally friendly antimicrobial agents that may be used safely to control plant pathogens (Capdeville *et al.* 2007). Extracellular protease is important for the hydrolysis of proteins in cell-free environments (Kalisz *et al.* 1998). It reported in a few fungal plant pathogens and it is clear that protease produced by pathogenic fungus plays an important role in the host plant, providing nitrogenous compounds during infection of the fungus (Archetr *et al.* 1997).

Protease is widely distributed among microorganisms including fungi, bacteria, and actinomycetes (Ma 2013). Among fungi genera, *Aspergillus*, *Penicillium*, *Paecilomyces*, *Rhizopus* and *Rhizomucor* are well-known producers of proteases (Devi 2008, Sindhu *et al.* 2009, Krishna 2009). Patil *et al.* (2015) reported that nine different fungi concluding *Cladosporium* sp., *Rhizoctonia* sp., *Aspergillus* sp., *Chaetomium* sp., *Biosporus* sp., *Fusarium* sp., *Curvularia* sp., *Cladosporium* sp., and *Colletotrichum* sp. were isolated from seven medicinal plants. Out of the nine fungi screened, the productivity of protease was the highest in *Biosporus* sp. (11 U/ml). There fungal isolates in this study had protease activity higher than that of *Biosporus* sp. Among the tested isolates, Isolate 3 showed the highest protease enzyme productivity (30.11 U/ml) (Table 4). The present study indicated three isolates might produce a high amount (high activity) of protease enzyme. Although the blast isolates from wild rice are limited, there have been few studies reported on protease enzyme from new sources of *P. grisea* from wild rice (*Oryza rufipogon*) up to the present. The current study opened up the way for wide openings in proteolytic fungal research tending towards the development of resistance in infectious micro-organisms by determining important characterization in agriculture and other industries. Further detailed study on the other enzymes from the available fungus is characterized by examining their sensitivity to specific protease inhibitors to obtain clear results.

Rice blast is one of the most serious diseases affecting rice and is caused by a highly variable fungal pathogen *P. grisea*, which severely affects the rice yield (Khush *et al.* 2009). This disease occurs in much rice growing areas worldwide (Moffat 1994; IRRI 2010). Management of this disease has been focused on the use of synthetic chemicals and resistant cultivates. However, the plant extract was more potent and environmentally friendly compared with synthetic fungicides (Gangawane 1990).

Previous studies showed the aqueous extract of Coffee arabica inhibitions on *P.grisea* (Hubert *et al*. 2015). The Piper caninum Blume crude extract had a very strong effect against *P.oryzae* (Suriani *et al.* 2015). The chemicals identified by GC-MS in the methanol extract were mostly from the bamboo leaf. But they were inactive on the growth of *P.grisa* with all concentrations (Toan *et al.* 2018). In this experiment, *P.grisea* was inhibited by methanol extract (Table 5). With different concentrations of methanol extract, the diameters of growth and spore production could be significantly reduced in isolate 2. While isolate 1 and isolate 3 were dependant on different concentrations that created different inhibition ability (Table 5). IC50 (mg/mL) is concentration required to inhibit 50% mycelial growth of *P.grisea.* Through the IC50 value, isolate 2 showed that only a small amount of extract could be inhibited (IC50 = 2.35mg / mL) and isolate 1 needed the highest amount of extract (IC50 = 41.34 mg / mL). The dayflower leaf extract has shown significant inhibition against *P.grisea*. Although methanol extract from the dayflower inhibited on *P.grisea*. However, it was still a crude extract and had many impurities. The next study will analyze and identify the chemical components in the extract more clearly.

CONCLUSION

The findings of this study highlighted that three isolates from wild rice were collected based on a designation of new international standard. It would be interesting to characterize the enzyme protease activity and established the active inhibition of the dayflower leaf existing source to manage the affection of *P. grisea* in rice.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Archer DB, Peberdy JF. The molecular biology of secreted enzyme production by fungi. *Critical Rev. Biotechnol,* 1997; **17:** 273–306. https://doi.org/10.3109/07388559709146616.
2. Bhat SV, Wathi BR, Rosy M, Govindappa M. Isolation and characterization of Glucose Oxidase (GOD) from *Aspergillus flavus* and *Penicillium* sp. *Int. J. of Curr. Microb. and App. Sci.*, 2013; **2**:153–161.
3. Capdeville G, Souza MTJR, Santos JRP, Paula MS, *et al*. Selection and testing of epiphytic yeasts to control anthacnose in post-harvest of papaya fruit. *Scientia Horticulture,* 2007; **111**: 179–85. https://doi.org/10.1016/j.scienta.2006.10.003.
4. Chen H, Hayn M, Esterbauer H. Three forms of cellobiohydrolase I from *Trichoderma reese*i. *Biochemical Molecular Biology,* 1993; **30**: 901–910.
5. Devi MK. Purification, characterization of alkaline protease enzyme from native isolates *Aspergillus nige*r and its compatibility with commercial detergents. *Indian Journal Science Technology*, 2008; **1**: 1–6.
6. Fei-Hong Bing JL, Li Z, Zhang GB, Liao YF, Li J, Dong CY. Anti-influenza-virus activity of total alkaloids from *Commelina communis* L. *Archives of Virolog*, 2009*;* **154**: 1837. https://doi.org/10.1007/s00705-009-0503-9.
7. Gangawane LV. Fungicide resistance in plant pathogens in India. *Indian Phytopathology*, 1990; **40:** 551–553.

8. Hanan HA. Isolation and screening of extracellular proteases produced by new isolated *Bacillus* sp. *Journal Applied Pharmacy*, 2012; **2**: 071–074.
9. Hayashi N, Fukuta Y. Proposal for a new international system of differentiating races of blast (Pyriculariapryxae Cavara) using LTH monogenic lines in rice (*Oryza sativa* L.) JIRCAS working report No. 63, Tsukuba city, Ibaraki prefecture Japan. *Japan International Research Center for Agricultural Science*, 2009; 11–15.
10. Hubert J, Mabagala R, Mamiro D. Extract against *Pyricularia grisea*, Causal agent of rice blast disease. *American Journal of Plant Sciences*, 2015; **6**: 602–611. https://doi.org/10.4236/ajps.2015.65065.
11. IRRI. *Anaporthe grisea* Rice blast http://www.metapathogen.com.visisted, 2010.
12. Kamalakannan A, Shanmugan V, Suhendran S, Srinivasan R. Antifungal properties of plant extracts against *Pyriclaria oryzae*, rice blast pathogen. *Indian Phytopathol.*, 2001; **54**: 490–492.
13. Khan MA, Ahmad N, Zafar AU, Nasir IA, Qadir MA. Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme. *Africa Journal Biotechnology*, 2011; **10**: 6203–6212.
14. Kalisz MH. Microbial proteinases. *Advance Biochemical Engineering Biotechnology*, 1998; **36**: 17–55.
15. Kartal SN,Terzi E, Kose C, Hofmeyr J, Imamura Y. Efficacy of tar oil recovered during slow pyrolysis of macadamia nut shells. *International Biodeterioration and Biodegradation*, 2011; **65**: 369–373. https://doi.org/10.1016/j.ibiod.2010.08.011.
16. Khush GS, Jena KK. Status and future prospects for research on blast resistance in rice (*Oryza sativa* L.). *In Advances in Genetics*, *Genomics and Control of Rice Blast Disease*, 2009; 1–10. https://doi.org/10.1007/978-1-4020-9500-9_1.
17. Krishna KV. Optimization of growth and production of protease by *Penicillium* species using submerged fermentation. *International Journal of Microbiology Research*, 2009; **1:** 14–18.
18. Kumar NS, Simon N. In vitro anti antibacterial activity and phytochemical analysis of *Gliricidia sepium* L. leaf extracts. *Journal of Pharmacognosy and Phytochemistry*, 2016; **5**: 131–133.
19. Kunitz N. Methods of enzymatic analysis 2nd ed. Velag, Chenie. *Acad. Press N.Y. London*, 2015; 807–814.
20. Ma L. Kinetic studies on batch cultivation of *Trichoderma reesei* and application to enhance cellulase production by fed-batch fermentation. *Journal Biotechnology*, 2013; **166:** 192–197. https://doi.org/10.1016/j.jbiotec.2013.04.023.
21. Moffat AS. Mapping the sequence of disease resistance. *Science*, 1994; **265**: 1804–1805. https://doi.org/10.1126/science.8091208.
22. Netam RS, Bahadur NS, Tiwari U, Tiwari RKS. Efficacy of plant extracts for the control of (*Pyricularia grisea*) blast of rice under field condition of Bastar, Chhattisgarh. *Research Journal of Agriculture*, 2011; **2**: 269–271.
23. Olufolaji DB, Adeosun BO, Onasanya RO. In vitro investigation on antifungal activity of some plant extracts against *Pyricularia oryzae*. *Nigeria Journal Biotechnology*, 2015; **29:** 38–43. https://doi.org/10.4314/njb.v29i1.6.
24. Patil MG, Pagare J, Patil SN, Sidhu AK. Extracellular enzymatic activities of endophytic fungi isolated from various medicinal plants. *International Journal of Current Microbiology and Applied Sciences*, 2015; **4**: 1035–1042.
25. Portumarthi R, Subhakar C, Jetty A. Alkaline protease production by submerged fermentation in stirred tank reactor using Bacillus licheniformis NCIM – 2042: Effect of aeration and agitation regismesm. *Biochemical Engineering Journal*, 2017; **34**: 185–192. https://doi.org/10.1016/j.bej.2006.12.003.
26. Prabavathy D, Nachiyar CV. Screening the bioactivity of ethyl acetate extract of endophytic *Phoma* sp isolated from *Vitex negundo*". *International conference on chemical and Environmental Engineering*, 2013; 15–16.
27. Sena APA, Amanda A, Chaibub VC, Mɹrcio *et al*. Increased enzymatic activity in rice leaf blast suppression by crude extract of *Epicoccum* sp. *Tropical Plant Pathology*, 2013; **38:** 387–397. https://doi.org/10.1590/S1982-56762013005000028.
28. Shakil A, Zunara Z, Azeem H, Hamid M. Isolation and screening of protease producing bacterial species. *Mycopath*, 2012; **1**: 51–54.
29. Shibano M, Kakutani K, Taniguchi M, Yasuda M, Baba K. Antioxiant constituents in the dayflower (*Commelina communis* L.) and their β- glucosidase – inhibitory activity. *Journal of Natural Medicines*, 2008; **62**: 349. https://doi.org/10.1007/s11418-008-0244-1.
30. Sindhu R, Suprabha GN, Shashidhar S. Optimization of process parameters for the production of alkaline protease from *Penicillium godlewskii* SBSS 25 and its application in detergent industry. *African of Journal Microbiology Research*, 2009; **3:** 515–522.
31. Sunitha VH, Aevi DN, Srinvinvas C. Extracellular enzymatic activity of endophytic fungal strains isolated from medicine plants. *Word Journal of Agriculture*, 2013; **9**: 01–09.
32. Suriani NL, Suprata DN, Sudana IM, Temaja IGRM. Antifungal activity of piper canium against *Pyricularia oryzae* Cav. the cause of rice blast disease on rice. *Journal of Biology, Agriculture and Healthcare*, 2015; 2224–3208.
33. Toan NP, Xuan TD, Ha PTT, Anh TTT, Khanh TD. Inhibitory effects of bamboo leaf on the growth of *Pyricularia grisea* fungus. *Agriculture*, 2018; **8:** 92. https://doi.org/10.3390/agriculture8070092.