ISSN 1644-0692
www.acta.media.pl

Acta Sci. Pol. Hortorum Cultus, 15(6) 2016, 69-86

IDENTIFICATION OF NON-PATHOGENIC FUNGI OF RICE AND THE EVALUATION OF THEIR EFFECT ON BIOLOGICAL CONTROL OF *Pyricularia grisea*, THE CAUSAL AGENT OF RICE BLAST DISEASE *in vitro*

Mohammad Reza Safari Motlagh[1], Pooneh Usefipoor[2]

[1] Rasht Branch, Islamic Azad University, Rasht, Guilan province, Iran

[2] Damghan Branch, Islamic Azad University, Damghan, Semnan province, Iran

Abstract. Rice blast disease, caused by *Pyricularia grisea*, is an important rice disease in Iran and throughout the world. Out of 150 infected samples collected from paddy fields of Guilan province, 57 fungal isolates were isolated for which PDA and W.A media were used. Morphological characteristics were used for identification of these fungi. It was found out that the isolated fungi were belonged to *Pyricularia grisea*, *Ulocladium alternariae*, *Ulocladium* cf. *alternariae*, *Ulocladium* cf. *consortiale*, *Ulocladium* sp., *Curvularia pallescens*, *Preussia* sp., *Epicoccum* sp. and *Trichoderma harzianum*. According to pathogenicity tests, all isolates of *P. grisea* were proved to be pathogenic on rice and fungal isolates belonging to other fungal genera which did not cause disease on rice were selected for biological control studies and to do so, were used different methods. Results showed that eight isolates of *T. harzianum*, *U.* cf. *consortiale*, *Ulocladium* sp., *Epicoccum* sp., *Preussia* sp., *C. pallescens*, *U. alternariae* and *U.* cf. *alternariae*, had the highest inhibitory effect in mycelial growth of *P. grisea*, respectively. In hyperparasitism test, hyphae of *T. harzianum* did not coil around the mycelium of *P. grisea*. In these experiments, *T. harzianum* and *U.* cf. *consortiale* isolates had the highest inhibitory effect on growth rate of *P. grisea* and was known as the most effective isolates. Based on results, volatile metabolites method was best method in control of *P. grisea in vitro*.

Key words: antagonist, biological control, mycelial growth, nonpathogenic fungi

Abbreviations: PDA: Potato dextrose agar, WA: Water agar

Corresponding author: Mohammad Reza Safari Motlagh, Department of Plant Pathology, Faculty of Agriculture, Rasht Branch, Islamic Azad University, Rasht, Guilan Province PO box 41335--3516, Iran, e-mail: ssafarimotlagh@yahoo.com

INTRODUCTION

Rice is the second most important crop after wheat and playing a crucial role in people's nutrition in the world. It has the second highest cultivation area after wheat in the world [Safari Motlagh et al. 2005]. Rice diseases are of the main challenges of rice production in most rice-growing regions [Ou 1985]. Rice blast causes economically significant crop losses annually. Each year it is estimated to destroy enough rice to feed more than 60 million people. This disease is known to occur in 85 countries worldwide [Ou 1985]. Blast caused by *Pyricularia grisea* is the most important rice disease in most rice-growing countries including Iran. Rice blast has been long occurred in Guilan and Mazandaran provinces in Iran. The symptoms and instances of the disease were reportedly observed around Lahijan in Guilan province for the first time [Javan Nikkhah 2001]. At present, the disease is widespread in Iran and occurs in other rice-growing regions than Guilan and Mazandaran [Javan Nikkhah 2001]. So, extensive research has conducted on its control by different means, e.g. production and application of fungicides, identification of resistance gene resources and breeding resistant cultivars, generation of forecasting systems, and investigation of factors affecting the severity and mitigation of disease like nutrients, moisture, temperature and in more recent years, biological control [Khodaparast and Sahragard 2004]. Biological control is an agronomical controlling method for the management of damages caused by plant pathogens using other living organisms. This method is based on thorough knowledge of pathogenic agents, host and their relationship with the environment [Safari Motlagh 2010].

In a study, 400 isolates of bacteria were isolated from the rice fields of International Rice Research Institute (IRRI) and were screened by their antagonism against blast and sheath blight diseases. Nine bacteria were identified including three isolates from *Pseudomonas fluorescens*, five isolates from *Bacillus* spp. and one isolate from *Enterobacter*. Then, the bacteria were evaluated in field and it was revealed that seeds treatment or spraying at leaf stage reduced blast intensity by 50–73% at leaf stage of IR50 cultivar, by 34–80% in C-22 in low lands and by 47–57% in UPLRI-5 in high lands. Then, two antibiotics AB1 and AB2 were isolated from *Pseudomonas* which hindered the germination of *Pyricularia oryzae* spores at 1 ppm concentration and also, reduced leaf blast intensity by 90–92% [IRRI 1989]. In a study in the Philippines, the samples infected by *Pyricularia grisea* were collected from two regions at seedling and tillering stages and the antibiosis effect of the isolated bacteria against *P. grisea* was examined. In total, no difference was observed in the genus of bacteria in the studied regions. However, the population of *Bacillus* was higher at tillering than at seedling stage and also, it was higher in bigger and/or older spots than in smaller and/or younger spots. In turn, the population of *Pseudomonas* was higher at seedling stage. Also, it was revealed that most antagonistic bacteria were with spots and that healthy leaf samples had the minimum number of antagonistic bacteria [IRRI 1989].

In a study on biological and chemical control of *P. grisea* in Greek, it was revealed that *Trichoderma harzianum* and *Chaetomium globosum* inhibited mycelial growth and conidial germination by 70–80% [Gouramanis 1997]. Ouazzani et al. [1998] showed that different isolates of *Trichoderma* reduced infection intensity of *P. oryzae* by 71–88%. In a study on the antagonistic effect of 20 microorganisms on the development

of leaf blast, it was observed that *Chaetomium globosum*, *Micromonospora* sp. and *Trichoderma harzianum* were the most effective antagonists [Padasht Dehkaee 2001]. Boff et al. [2002] investigated the antagonistic effect of *Ulocladium atrum* on strawberry grey mold disease caused by *Botrytis cinerea*. They sprayed *U. atrum* once at transplanting stage and once at the beginning of flowering. It was revealed that *U. atrum* could be effective in reducing the grey mould of strawberry. Abdel-Fattah et al. [2007] studied the antagonistic mechanisms of *Trichoderma harzianum* on *Bipolaris oryzae*. *T. harzianum* anti-fungal metabolites completely suppressed the linear growth of *B. oryzae*. Observations with light and scanning electronic microscope (SEM) showed no evidence of mycoparasitism of *T. harzianum* in farm. Spraying of spore suspension of *T. harzianum* at 10^8 spores significantly decreased disease severity and incidence on the plant leaves and also, significantly increased grain yield [Abdel-Fattah et al. 2007].

It seems that biological control of plant disease is a good alternative for the application of chemicals against blast. For example, *Serratia marcescens* seems to be an optimum agent for controlling blast because it produces chitinolytic enzymes that can degrade the cell walls of fungi inducing defense response of the plant and certain anti-fungal low molecular weight molecules [Jaiganesh et al. 2007]. In another evaluation, 12 isolates of bacteria as *Bacillus circulans*, *B. subtilis*, *B. megaterium*, *Bacillus* sp. and *Pseudomonas fluorescens* were selected and their antagonistic effects against blast disease was evaluated under field conditions. Results showed that some bacteria reduced the disease significantly as compared to control, but they were less effective than fungicide. *B. circulans* had more effect on controlling blast at leaf and heading stages as compared to other bacteria [Padasht Dehkaee and Izadyar 2007]. In a perusal on biological control of Italian millet blast (*Setaria italica*), it was reported that strains from *Pseudomonas fluorescens* and *Bacillus* had inhibitory effects on mycelial growth in vitro and reduced disease intensity in farm conditions [Karthikeyan and Gnanamanickam 2008]. In a study, biological control effect of some actinomycetes isolates obtained from different habitats of Manipur, India was examined on major rice pathogens including *Curvularia oryzae*, *Pyricularia oryzae*, *Bipolaris oryzae* and *Fusarium oxysporum*. LSCH-10C isolated from Loktak Lake was found to be a promising biocontrol agent [Ningthoujam et al. 2009].

De Figueirêdo et al. [2010] examined the biological control of *Sclerotinia sclerotiorum* using eight isolates of *Trichoderma* spp. and one isolate of *Ulocladium atrum* and found that all isolates of *Trichoderma* excluding *U. atrum* had good antagonstic potential against *S. sclerotiorum*.

In greenhouse and in vitro experiments, Naeimi et al. [2011] studied the effect of 200 *Trichoderma* strains isolated from the soil, plant debris and phyllosphere in paddy fields of Mazandaran province on *Rhizoctonia solani* and reported that according to the in vitro experiments, several strains belonging to *T. harzianum*, *T. virens* and *T. atroviride* controlled the disease agent significantly. Among 55 isolates selected for greenhouse experiments, 7 isolates controlled the disease significantly among which *T. harzianum* AS12-2 was found to be the most effective in controlling sheath blight whose effect was even better than the most common fungicide used in Iran, i.e. propiconazole [Naeimi et al. 2011].

Khosravi et al. [2011], in an evaluation of three commercial products of *Trichoderma* isolates in controlling rice blast in field conditions, concluded that these compounds increased rice yield and decrease disease intensity. In a study, the antifungal effect of *Saccharopolysopora erythraea* was studied on *P. grisea* in greenhouse and in vitro. *S. erythraea* showed a high biocontrol potential in disc-agar, leakage from sump to agar and dual culture by producing antifungal metabolites [Amini et al. 2012]. Khalili et al. [2012] studied the effect of native isolates of *Trichoderma* isolated from rice fields of Guilan and Mazandaran provinces on controlling rice brown spot caused by *B. oryzae* for which they screened 145 isolates of *Trichoderma* species belonging to *T. atroviride*, *T. harzianum* and *T. virens* in greenhouse and in vitro experiments in order to find the best biocontrol species for controlling *B. oryzae*. They reported that two strains belonging to *T. harzianum* significantly controlled the disease and two strains belonging to *T. atroviride* improved the seedling growth [Khalili et al. 2012]. In another study, it was revealed that isolates from *Pseudomonas* spp. and *Bacillus* sp. were more efficient than *Streptomyces* sp. and *Seratia* sp. in controlling blast disease under greenhouse conditions [Rostami et al. 2012]. Hajano et al. [2012] evaluated biocontrol of *P. grisea* by antagonistic fungi and concluded that *Paecilomyces lilacinus* and species of *Trichoderma* such as *T. harzianum* and *T. polysporum* had the highest inhibitory effect on mycelial growth.

In a study on the antagonistic effect of seven isolates including *Fusarium verticillioides*, *Alternaria tenuissima*, *Trichoderma harzianum*, *Trichoderma virens*, *Alternaria citri*, *Alternaria infectoria* and *Preussia* sp. on the mycelial growth of *Bipolaris* spp. in laboratorial and greenhouse conditions, it was revealed that *T. harzianum* was the most effective antagonist in suppressing the mycelial growth of *Bipolaris* spp. under laboratorial conditions and *Preussia* sp. and *T. harzianum* were the most effective isolates on reducing the intensity of brown spot disease under greenhouse conditions [Mohammadian 2013].

In a study, the antagonistic effect of *Epicoccum* sp. against *Magnaporthe oryzae*, *Rhizoctonia solani*, *Sarocladium oryzae*, *Monographella albescens* and *Cochliobolus miyabeanus* was studied in dual culture and greenhouse. *Epicoccum* sp. suppressed the colony growth of *M. oryzae* by 42.5%. It suppressed the intensity of *M. oryzae* by 95.68% in greenhouse. So, *Epicoccum* sp. exhibited antagonistic potential and improving the resistance against leaf blast [Sena et al. 2013]. In a study, the biological control of rice blast by indigenous isolates of *Trichoderma* was studied in Mazandaran province and it was found that over 90% of *Trichoderma* isolates had inhibitory effect on the growth of *P. grisea* colony in dual culture method and in greenhouse experiments, *Trichoderma* isolates RP1-6 and RP4-2 with 100 and 99% inhibitory effect, respectively, were found to be the most efficient isolates [Javadi et al. 2014].

Given the fact that world population is growing and food resources do not suffice to meet their food demand, the only solution is to increase crop production. So, it is crucially important to counteract agents damaging crops, especially plant pathogenic agents. Since the current methods for control this disease, such as chemical or traditional methods, are ineffective, a new approach seems to be required. Today, the dangers of the application of chemical pesticides are evident. Therefore, attempts to introduce alternative methods for chemicals have led to considerable progresses so that a lot of

biological products have been introduced into the market and microbial pesticides will replace for chemicals in the near future. Although biological control of plant pathogens works relatively slowly, their effect is long-lasting and they are cheap and environment-friendly. Thus, they can be a good alternative for the application of chemicals. The purpose of the present study was to find fungus or fungi in natural microbiota of rice with the ability to suppress *P. grisea* with no negative impact on rice plants.

MATERIALS AND METHODS

Collection and culture of fungal isolates. Leaves with symptoms of the disease rice were collected in Guilan province of Iran, cut to appropriate sizes and transferred to the laboratory. Samples were surface sterilized with 0.5% sodium hypochlorite solution, washed by sterile distilled water and placed on potato dextrose agar in petri dishes. Then, petri dishes were incubated at 28°C in darkness or light on a 12 hours light/dark photoperiod for 6–15 days. Conidia were single-sporulated and then, monoconidial isolates of the recovered fungi were maintained on half-strength PDA slants in test tubes as stock cultures [Safari Motlagh 2010].

Study and identification of fungi. Morphological studies were carried out on WA medium. Cuts of colonies or each of filter papers were placed onto PDA medium for 2–3 days. Then, section of colonies was transferred to WA medium for 7–30 days in incubator at 27°C and 12h photoperiod. Afterward, morphological observations were taken based on colony, conidium and conidiophore morphology and other characters morphological [Cain 1961, Simmons 1967, Ellis 1971, Sivanesan 1987, Gams and Bissett 1998, Sutton et al. 1998, Arenal et al. 2004, 2007].

Pathogenicity test. The pathogenicity test of the isolated fungi was done in desiccator under completely controlled conditions for which some farm soil was poured into Erlenmeyer flask and was sterilized in autoclave (twice, each time for 30 minutes) and then, some of this soil was put in sterile petri dishes. Afterwards, an amount of seeds of cv. Hashemi was disinfected in sodium hypochlorite solution 30% for one hour and then, 10 seeds were laid in soil in petri dishes. This was done in two desiccators, one as treatment and one as control. Two petri dishes were put in each desiccator. The petri dishes were added with distilled water so that they were waterlogged during the experiment. The spores were inoculated 16–18 days later when the seedlings in petri dishes were at two-leaf stage for which distilled water was first sprayed on all seedlings in control and treatment desiccator by hand sprayers (under sterile hood) and then, the spore suspension required for the inoculation was prepared [Safari Motlagh et al. 2005]. In all experiments, a suspension containing 4×10^4 spores per ml distilled water was used which were counted by hemocytometer. In addition, Tween® 20 with the ratio of 1% was used for improving surface absorption. It should be mentioned that desiccators were kept in incubator at 26°C, > 90% moisture, and 12/12 day/night light periods [Safari Motlagh et al. 2005].

Biological control studies. Inhibition of *P. grisea* growth by culture filtrate. The isolates of the studied fungi were cultured in 250-ml Erlenmeyer flasks containing PDB culture medium and they were shook at 26°C at 70 rpm for 10 days. Then, they were extracted by biological filters and vacuum pump. Next, the extract was added to PDA culture medium. In control, the extract added to PDA culture medium lacked antagonistic fungus. A mycelial disc from 3-day culture of *P. grisea* was placed at the center of treatment and control petri dishes and then these petri dishes were transferred into incubator at 26°C. After 10 days, radial growth of *P. grisea* was calculated in control and treatment. The reduce of radial growth was calculated by:

$$\text{Percentage of inhibition of radial mycelial growth} = \frac{C-T}{C} \times 100,$$

where, *C* is the radial growth of *P. grisea* in control petri dishes and *T* is its radial growth in the presence of other fungi [Dennis and Webster 1971 a, Sivakumar et al. 2000].

Dual culture of the studied antagonistic fungi and *P. grisea* by slide culture method (hyperparasitism test). A laboratorial slide was placed inside a 12 cm petri dish on two L-shaped glass bars and was sterilized. Then, some of molten 2% water agar culture medium was poured on the slide as so a thin layer of agar was formed. Small mycelial discs of the desired antagonistic fungus and *P. grisea* were placed on slide with 2 cm spacing. A few milliliters of sterilized distilled water were added to each petri dish to avoid their drying. Petri dishes were kept at 26°C. As soon as the mycelia of the fungi were reached to each other, the slides were studied under optical microscope [Sivakumar et al. 2000].

The effect of volatile metabolites on inhibition of *P. grisea* growth. A mycelial disc with the diameter of 5 mm from the 3-day culture margin of *P. grisea* was placed in the center of a petri dish containing PDA medium. Forty-eight hours later, a disc with the diameter of 5 mm from the 3-day culture of the studied fungi was placed in the center of another petri dish containing PDA. Then, the caps of these petri dishes were removed under sterile hood and the dish containing *P. grisea* was placed upside-down on the petri dish containing the studied fungi. In control, the studied fungi were replaced by a disc from PDA medium. Inhibition percentage was calculated 10 days later [Dennis and Webster 1971 b, Sivakumar et al. 2000].

Dual culture method. A mycelial disc with the diameter of 5 mm taken from margins of 5–7-day culture of *P. grisea* was placed under sterile hood in an 8 cm petri dish containing PDA with 2 cm spacing from the wall of petri dish. Then, the petri dish was placed in incubator at 26°C for 48 hours so that the fungus started its growth. Then, a mycelial disc with the diameter of 5 mm taken from the margins of 5–7-day fungus was placed at a distance of 3 cm from the pathogenic fungus. The petri dishes were placed at 26°C and the measurements were recorded 7–10 days later [Sivakumar et al. 2000]. In disease controls, a mycelial disc from the margins of 5–7-day culture of *P. grisea* was placed in the center of an 8-cm petri dish under sterile conditions. The control petri dishes were also placed in incubator at 26°C. At the end of incubation,

the radial growth of *P. grisea* was measured in control and treatment. The reduce of radial growth as compared to control was calculated [Sivakumar et al. 2000].

Data analysis. The study was based on a Randomized Complete Design with eight treatments and three replications. Data analysis was done using SAS software. In order to compare average values, least significant difference (LSD) method was used.

RESULTS AND DISCUSSION

All fungal isolates were pathogenically studied and the pathogenic nature of all *P. grisea* isolates was proved on rice. Among other fungal isolates related to fungi other than *P. grisea*, 20 isolates that were not pathogenic or were only so slightly pathogenic on rice were identified and selected for biological control studies.

These fungal were divided into 8 groups, as follows:

Characteristics of first group: Colonies brown to dark brown. Mycelium 3–5 µm diam, pale yellow brown, septate, smooth or sometimes minutely echinulate. Conidiophores erect or ascending, simple or branched, translucent yellow to golden brown 5 µm diam, up to 60 µm long, with 1–8 perforate geniculations. Conidia obovoid to long ellipsoidal, dilute to golden or olive brown, usually smooth to indistinctly depressed pustulose, rarely definitely verrucose, 18–30 × 10–15 µm, with 1–5 transverse and 1–6 longitudinal or oblique septa; base initially conical or rounded, often with a distinct apiculus; apex narrowly to broadly rounded; predominantly solitary but occasionally in short chains following apical production of conidiophores (fig. 1). The characteristics of this group corresponded with *Ulocladium* cf. *consortiale* (Thum) E.G. Simmons [Simmons 1967].

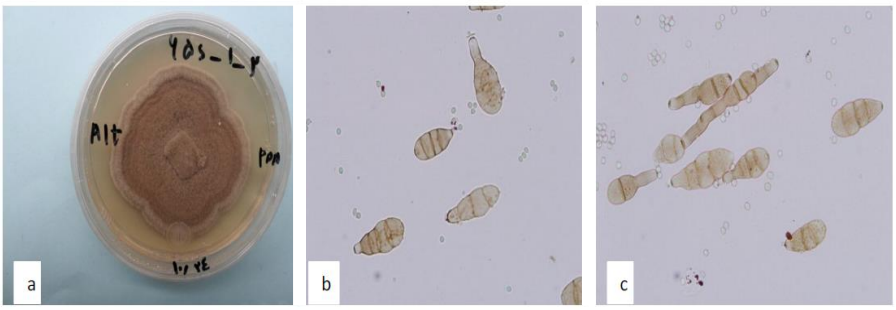

Fig. 1. *Ulocladium* cf. *consortiale*: a) Colony on PDA, b) Conidia (×1200), c) Conidia and conidiophores (×1200)

Characteristics of second group: Colonies brown to dark brown. Mycelium 4–6 µm diam, pale yellow brown, septate, smooth. Conidiophores golden brown, mostly 4–20 × 4–5 µm, simple with an apical pore or with 1–3 close, uniperforate geniculations. Conidia obovoid to broadly ellipsoidal, golden brown to olivaceous, smooth or depressed pustulose, 21–30 × 13–20 µm with 3–5 transverse septa and 1–2 longitudinal or oblique

septa in any or all of the transverse divisions; base broadly conical to rounded, sometimes minutely apiculate; apex broadly rounded (fig. 2). The characteristics of this group corresponded with *Ulocladium alternariae* E.G. Simmons [Simmons 1967].

Characteristics of third group: Colonies brown to dark brown. Mycelium 4–7 µm diam, pale yellow brown, septate, smooth. Conidiophores golden brown, mostly 3–20 × 4–6 µm, simple with an apical pore or with 1–3 close, uniperforate geniculations. Conidia obovoid to broadly ellipsoidal, golden brown to olivaceous, smooth or depressed pustulose, 22–31 × 13–17 µm with 3–5 transverse septa and 1–2 longitudinal or oblique septa in any or all of the transverse divisions; base broadly conical to rounded, sometimes minutely apiculate; apex broadly rounded (fig. 3). The characteristics of this group corresponded with *Ulocladium* cf. *alternariae* E.G. Simmons [Simmons 1967].

Fig. 2. *Ulocladium alternariae*: a) Colony on PDA, b) Conidia (×1200), c) Conidia and conidiophores (×1200)

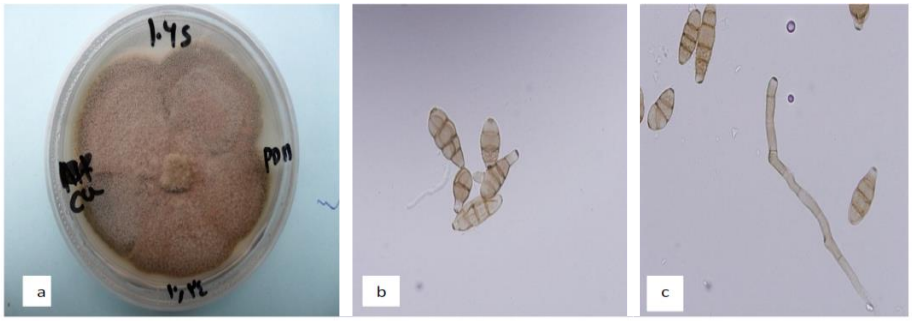

Fig. 3. *Ulocladium* cf. *alternariae*: a) Colony on PDA, b) Conidia (×1200), c) Conidia and conidiophores (×1200)

Characteristics of fourth group: The isolates grow rapidly and produce woolly to cottony or felty colonies on potato dextrose agar at 25°C. From the front, the colonies are yellow to orange, orange to red or pink initially and become greenish brown to black by aging. Mycelium mostly immersed. Conidiophores macronematous or semi-macronematous, in culture often solitary or in dense clusters, unbranched or occasionally branched, short, straight or flexuous, colourless to pale brown, smooth or verrucose.

Conidia solitary, subspherical or pyriform, dark golden brown, often with a pale protuberant basal stalk cell, muriform but with the sepia obscured in mature conidia by the rough opaque wall. The isolates produce a diffusable pigment which turns the color of the inoculated medium to yellow, orange, red or brown. Young conidia are round, non-septate and pale in color. Mature conidia (15–25 μm in diameter), on the other hand, are rough, verrucose to warty, and brown to black in color. Besides, mature conidia contain multiple transverse and vertical septa and have a funnel-shaped base and attachment scar that is formed from aggregated conidiophores on the sporodochium (fig. 4). The characteristics of this group corresponded with *Epicoccum* sp. Link ex Schleet; Link [Ellis 1971, Sutton et al. 1998].

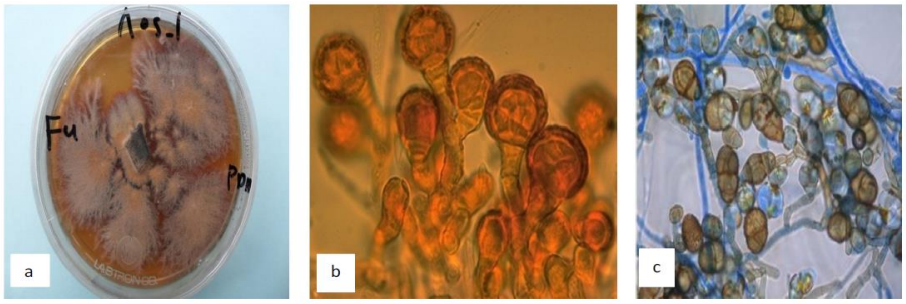

Fig. 4. *Epicoccum* sp.: a) Colony on PDA, b) Conidia (×1200), c) Conidia and conidiophores (×460)

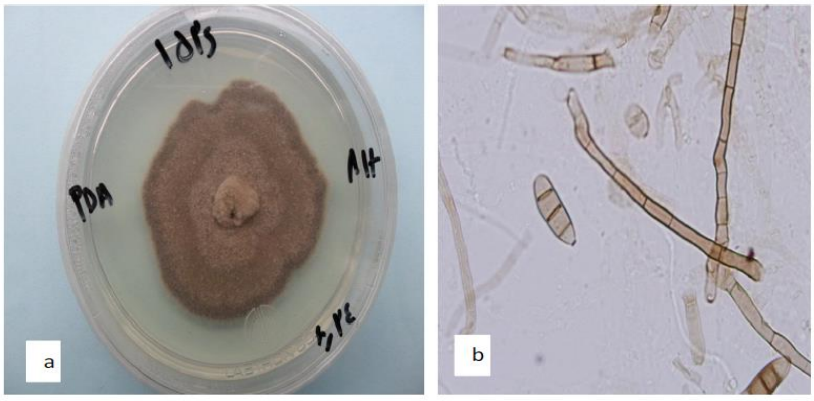

Fig. 5. *Ulocladium* sp.: a) Colony on PDA, b) Conidia and conidiophores (×1200)

Characteristics of fifth group: Colonies effuse, brown, olivaceous brown, dark blackish brown or black. Mycelium partly superficial, partly immersed. Conidiophores macronematous, mononematous, mostly 3–25 × 4–8 µm unbranched or branched, straight or flexuous, often geniculate, pale to mid brown, smooth or verruculose. Conidia solitary in most species but secondary conidia on short secondary conidiophores give rise to chains in some, 15–26 × 6–14 µm simple, mostly broadly ellipsoidal or obovoid, sometimes clavate, frequently with a minute projecting hilum, pale to dark blackish brown, smooth or verrucose, with transverse and usually also longitudinal or oblique septa (fig. 5). The characteristics of this group corresponded with *Ulocladium* sp. Preuss [Ellis 1971].

Characteristics of sixth group: colonies effuse, brown, grey or dark brown, hairy, cottony or velvety. Mycelium immersed in natural substrata. Conidiophores macronematous, mononematous, straight or flexuous, often geniculate, sometimes nodose, brown, usually smooth. Conidia simple, often curved, clavate, ellipsoidal, broadly fusiform, obovoid or pyriform, pale or dark brown, often with some cells, usually the end ones, paler than the others, with dark bands at the septa, smooth or verrucose, with hilum scarcely or not at all protuberant, remaining smooth-walled, predominantly 3-septate, middle septum not median. All conidial cells usually pale or very pale brown. Conidia usually straight or only slightly curved, 17–32 × 7–12 (fig. 6). The characteristics of this group corresponded with *Curvularia pallescens* Boedijn [Ellis 1971, Sivanesan 1987].

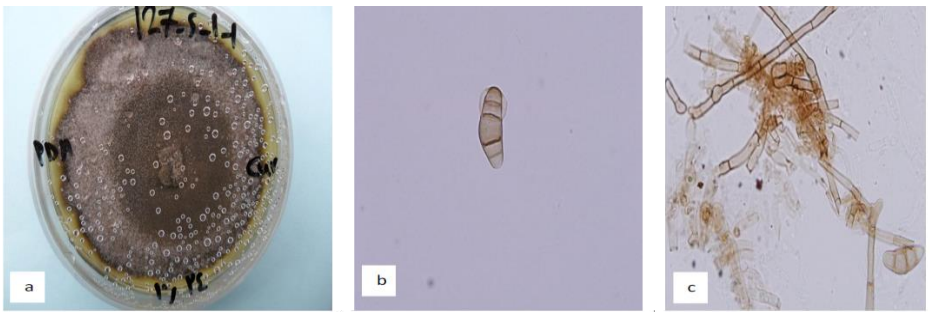

Fig. 6. *Curvularia pallescens*: a) Colony on PDA, b) Conidia (×1200), c) Conidia and conidiophores (×1200)

Characteristics of seventh group: Colonies on PDA medium attaining 80 mm diameter in 14 d at 23°C. Texture cottony, adpressed and partially submerged, light brown to pink. Ascomata scattered to aggregated, developed superficially or partially immersed in culture media when young. Pseudothecia globose to spherical, smooth, almost glabrous, usually not ostiolate, light brown to dark brown. Ascomata ornamentation consisting on septate and flexuose hyphae, 5–10 × 2–2.5 µm. Asci 80–110 × 10–13 µm, eight spored, cylindrical to clavate, broadly rounded above and gradually to abruptly tapering into a robust stipe of 10 × 5 µm. Pseudoparaphyses 10–15 µm, filiform, septate

and longer than the asci, mixed with them and bifurcate. Ascospores 32–47 × 6–10 µm, two-celled, cells easily separable at the central septum, cylindrical, hyaline to olivaceous. When young and finally becoming olivaceous brown to dark brown when mature; transversely septate, constrictions at septa broad and shallow, middle cells of equal length and broader than terminal cells, provided with rounded apices; germ slit diagonal, oblique or parallel and straight to sinuous; gelatinous sheath hyaline and narrow, less than 4 µm wide (fig. 7). The characteristics of this group corresponded with *Preussia* sp. Fuckel [Cain 1961, Arenal et al. 2004, 2007].

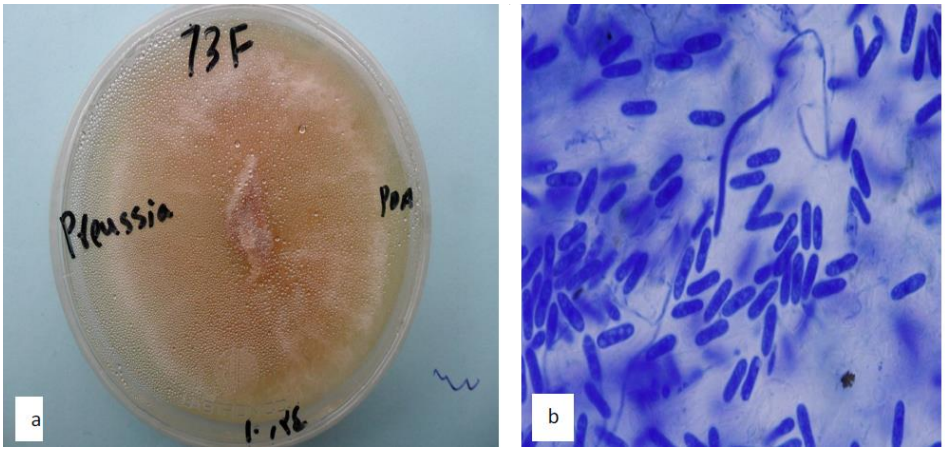

Fig. 7. *Preussia* sp.: a) Colony on PDA, b) Ascospores (×1200)

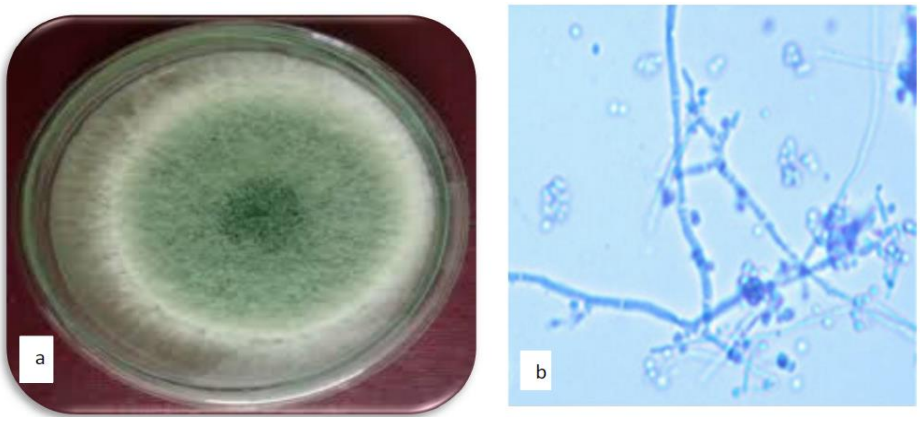

Fig. 8. *Trichoderma harzianum*: a) Colony on PDA, b) Conidia and conidiophores (×1200)

Characteristics of eighth group: colonies growing rapidly (most isolates 7–9 cm). Conidiation predominantly effuse, appearing granular or powdery due to dense conidiation; rapidly turning yellowish-green to dark green, or producing tufts or pustules fringed by sterile white mycelium. Reverse colourless to dull yellowish, buff or drab. Odour indistinct or faintly earthy. Conidiophores as in the section, tending to be regularly verticillate forming a pyramidal structure. Phialides ampulliform to lageniform, usually 3–4-verticillate, occasionally paired, mostly 3.5–7.5 × 2.5–3.8 μm, terminal phialides up to 10 μm long. Conidia subglobose to obovoid, mostly (2.5–) 2.7–3.5 × 2.1–2.6 (–3.0) μm, smooth-walled, subhyaline to pale green (fig. 8). The characteristics of this group corresponded with *Trichoderma harzianum* Rifai [Gams and Bissett 1998].

In evaluation of inhibition of *P. girsea* growth by culture filtrate, it was found that *T. harzianum* had the highest inhibitory effect of 62.25% on the growth of *P. grisea* colony. The isolates of *Ulocladium* cf. *consortiale*, *Ulocladium* sp., *Epicoccum* sp., *Preussia* sp., *Curvularia pallescens*, *Ulocladium alternariae* and *Ulocladium* cf. *alternariae* had the next highest efficiencies in reducing the growth of colony of *P. grisea* (tab. 1). Analysis of variance of the growth inhibition showed significant differences among the studied fungi at the 1% probability level.

Table 1. Comparison of means of mycelial growth inhibition of *P. grisea* by the use of studied fungi

Treatment	Growth inhibition (%)
T. harzianum	62.25 a
U. cf. *consortiale*	53.16 b
Ulocladium sp.	52.71 b
Epicoccum sp.	51.31 b
Preussia sp.	49.21 c
C. pallescens	49.35 c
U. alternariae	48.45 c
U. cf. *alternariae*	45.53 cd

In evaluation of culture of the studied antagonistic fungi and *P. grisea* by slide culture method, it was found that the hyphae of *T. harzianum* did not coil around the mycelium of the fungal agent of rice blast disease. The hyphae isolates of *Preussia* sp., *C. pallescens* and *Epicoccum* sp. penetrated into the mycelium of *P. girsea*, but were not able to deform them. The hyphae of various *Ulocladium* species applied in the present study penetrated into the mycelium of *P. grisea* once they reached them and then, they tore the fungal mycelium and deformed them.

In evaluation of inhibitory effect of volatile metabolites on *P. grisea* growth, *T. harzianum* had the highest inhibitory effect of 82.31% on mycelial growth of *P. grisea*. The next highest inhibitory effect on reducing mycelial growth of blast fungus was exerted by the isolates of *Ulocladium* cf. *consortiale*, *Ulocladium* sp., *Epicoccum* sp.,

Preussia sp., *Curvularia pallescens*, *Ulocladium alternariae* and *Ulocladium* cf. *alternariae*, respectively (fig. 9).

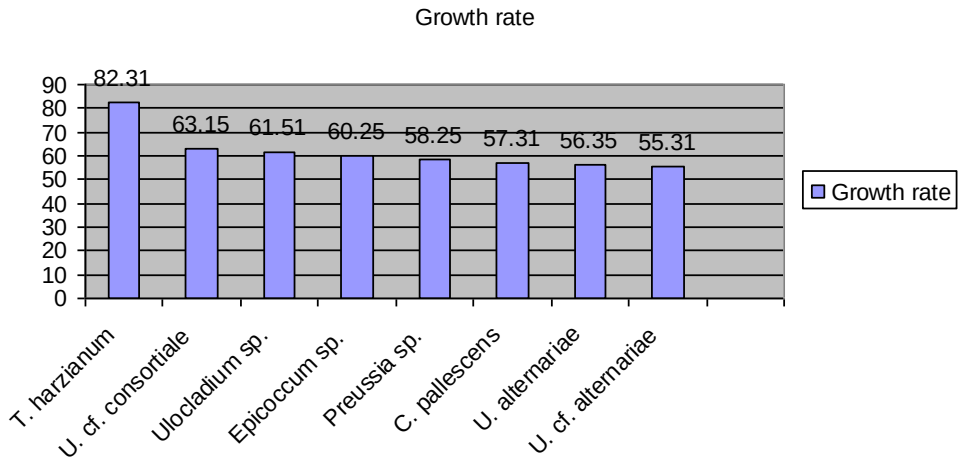

Fig. 9. Effect of volatile metabolites of fungal isolates on the growth rate of *P. grisea*

According to the analysis of variance of inhibition percentage in this method, the treatments showed significant differences at the 1% probability level. It was revealed that fungi had significant differences in inhibition percentage (tab. 2).

Table 2. Analysis of variance of inhibition percentage of mycelial growth

Sources of variations	df	Squares of means
Treatment	7	80.64**
Error	16	4.16
Coefficient of variations		5.03

** – significance at the 1% probability level

According to means comparison of inhibition percentage by LSD method, control by *T. harzianum* (treatment 1) had the highest inhibition percentage with significant differences with other treatments. Also, the lowest inhibition percentage was related to treatment 2, i.e. inhibition by *U.* cf. *alternariae*; however, it had no significant difference with treatments 1, 5 and 6. Therefore, the best result was obtained by controlling with *T. harzianum* with higher efficiency than other fungi (tab. 3).

Table 3. Means comparison of inhibition percentage of mycelial growth by LSD in volatile metabolites method

Treatment	Growth inhibition (%)
1 = *U. alternariae*	56.35 ±3.6cd
2 = *U.* cf. *alternariae*	55.31±4.7d
3 = *U.* cf. *consortiale*	63.15 ±6.3b
4 = *Ulocladium* sp.	61.51 ±4.1b
5 = *C. pallescens*	57.31 ±2.26cd
6 = *Preussia* sp.	58.25 ±1.56cd
7 = *T. harzianum*	82.31 ±4.89a
8 = *Epicoccum* sp.	60.25 ±5.68bc
LSD (5%)	8.531

Treatments having at least one similar letter do not show a significant difference at P = 0.05 level

In dual culture method, *T. harzianum*, *U.* cf. *consortiale*, *Ulocladium* sp., *Epicoccum* sp., *Preussia* sp., *C. pallescens*, *U. alternariae* and *U.* cf. *alternariae* isolates had highest percentage of mycelial growth inhibition, respectively (fig. 10).

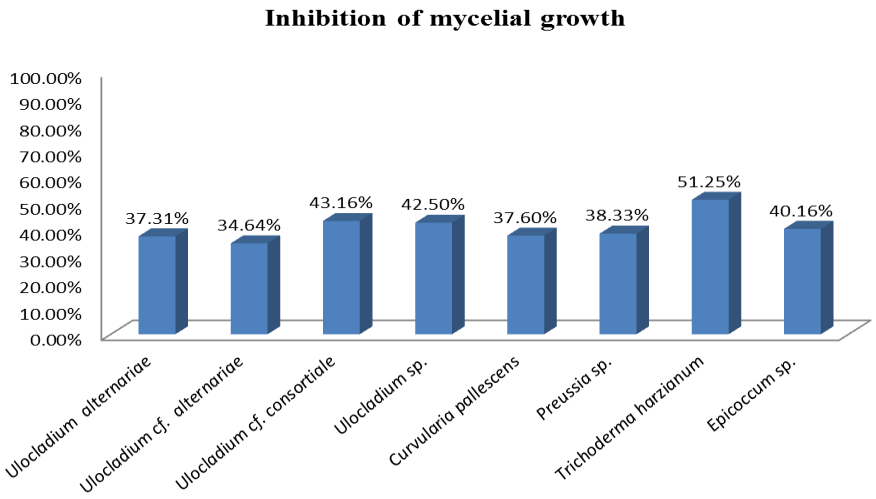

Fig. 10. Comparison of inhibition of mycelial growth of *P. grisea* isolates by studied fungi

In the present study, 150 fungal isolates were isolated from rice plants in paddy fields of Guilan province among which the identified fungi included *Pyricularia grisea*, *Ulocladium alternariae*, *Ulocladium* cf. *alternariae*, *Ulocladium* cf. *consortiale*, *Ulocladium* sp., *Curvularia pallescens*, *Preussia* sp., *Trichoderma harzianum* and *Epicoccum* sp. After elementary identification at genus level, 57 isolates were used for pathogenic studies and their pathogenicity of all isolates of *P. grisea* (33 isolates) was proved

on rice plants. Out of the isolates used for dual culture (two methods), the isolates of *T. harzianum*, *U.* cf. *consortiale*, *Ulocladium* sp., *Epicoccum* sp., *Preussia* sp., *C. pallescens*, *U. alternariae* and *U.* cf. *alternariae* inhibited the mycelial growth of *P. grisea* more than other isolates, respectively.

The evaluation of inhibitory effect of culture filtrate on *P. grisea* growth revealed that *T. harzianum* was the most effective isolate inhibiting the growth of *P. grisea* isolates by 62.25% which is consistent with Javadi et al. [2014] that stated that *T. harzianum* significantly hindered the mycelial growth of *P. grisea* in vitro.

The evaluation of the inhibitory effect of volatile metabolites on the growth of *P. grisea*, also, showed *T. harzianum* to be the most effective isolate in biological control of *P. grisea* which is consistent with Javadi et al. [2014].

The isolates applied in these methods were equally efficient in biological control of rice blast fungus although they were all more useful in volatile metabolites method.

In dual culture, *T. harzianum* was found to be the most efficient isolate in suppression of *P. grisea* growth which is in agreement with Padasht Dehkaee [2001] in which it was reported that the isolates of *T. harzianum* significantly controlled the mycelial growth of *P. grisea*.

The biological control of *Sclerotinia sclerotiorum* by 8 isolates of *Trichoderma* and one isolate of *Ulocladium atrum* was studied. All isolates of *Trichoderma* excluding *U. atrum* had good antagonistic potential against *S. sclerotiorum* and the isolate 3601 had the highest impact [De Figueirêdo et al. 2010] which is consistent with the findings of the study in terms of the comparison of the effect of efficiency of *Trichoderma* and *Ulocladium* isolates.

In another study, the antagonistic influence of *Epicoccum* sp. against rice pathogens including *Magnaporthe oryzae*, *Rhizoctonia solani*, *Sarocladium oryzae*, *Monographella albescens* and *Cochliobolus miyabeanus* was studied in dual culture and greenhouse. In dual culture, *Epicoccum* sp. inhibited the colonial growth of *M. oryzae* by 42.5%. In greenhouse, it inhibited the disease intensity of *M. oryzae* by 95.68%. According to this study, *Epicoccum* sp. exhibited antagonistic ability and higher resistance against leaf blast [Sena et al. 2013] which is inconsistent with our findings. We found that *Epicoccum* sp. was the fourth effective antagonist.

In a study on the antagonistic effect of seven isolates including *Fusarium verticillioides*, *Alternaria tenuissima*, *Trichoderma harzianum*, *Trichoderma virens*, *Alternaria citri*, *Alternaria infectoria* and *Preussia* sp. on the mycelial growth of *Bipolaris* spp. in laboratorial and greenhouse conditions, it was revealed that the fungal isolate *T. harzianum* was the most effective antagonist in suppressing the mycelial growth of *Bipolaris* spp. under laboratorial conditions and *Preussia* sp. and *T. harzianum* were the most effective isolates on reducing the intensity of brown spot disease under greenhouse conditions [Mohammadian 2013] which was consistent with our findings in the present study.

The present study indicated that *T. harzianum* and *U.* cf. *consortiale* were the most effective fungal isolates in controlling rice blast disease agent. So, they can be introduced as antagonistic fungi *in vitro*.

CONCLUSIONS

It was concluded that identification of the fungi in natural rice microbiota and the study of their antagonistic effect on controlling rice blast disease can be an effective approach to the management of this important disease.

ACKNOWLEDGMENTS

This experiment was supported by the Islamic Azad University, Rasht Branch, Iran.

REFERENCES

Abdel-Fattah, G.M., Shabana, Y.M., Ismail, A.E., Rashad, Y.M. (2007). *Trichoderma harzianum*: a biocontrol agent against *Bipolaris oryzae*. Mycopathologia, 164(2), 81–89.

Amini, E., Tajik Ghanbari, M.A., Hasanzadeh, N. (2012). Biological control of rice blast disease by *Streptomyces erythraea in vitro* and greenhouse conditions. Proceeding of 6th National Conference New Ideas in Agriculture, 29–30 February, Khvorasgan, Iran, 234–238.

Arenal, F., Platas, G., Peláez, F. (2004). Variability of spore length in some species of the genus *Preussia* (*Sporormiella*). Mycotaxon, 89, 137–151.

Arenal, F, Platas, G, Pelaez, F (2007). A new endophytic species of *Preussia* (*Sporomiaceae*) inferred from morphological observations and molecular phylogenetic analysis. Fung. Divers., 25,1–17.

Boff, P., Köhl, J., Gerlagh, M., de Kraker, J. (2002). Biocontrol of grey mould by *Ulocladium atrum* applied at different flower and fruit stages of strawberry. BioContr., 47(2), 193–206.

Cain, R.F. (1961). Studies of coprophilous ascomycetes. VII. *Preussia*. Can. J. Bot., 39, 1633–1666.

De Figueirêdo, G.S., De Figueirêdo, L.C., Cavalcanti, F.C.N., Dos Santos, A.C., Da Costa, A.F., De Oliveira, N.T. (2010). Biological and chemical control of *Sclerotinia sclerotiorum* using *Trichoderma* spp. and *Ulocladium atrum* and pathogenicity to bean plants. Braz. Arch. Biol. Tech., 53(1), 1–9.

Dennis, C., Webster, J. (1971 a). Antagonistic properties of species-groups of *Trichoderma*. I. Production of nonvolatile antibiotics. T. Brit. Mycol. Soc., 57(1), 25–39.

Dennis, C., Webster, J. (1971 b). Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. T. Brit. Mycol. Soc., 57(1), 41–48.

Ellis, M.B. (1971). Dematiaceous Hyphomycetes. CMI, Kew, England.

Gams, W., Bissett, J. (1998). Morphology and identification of *Trichoderma*. In: *Trichoderma* and *Gliocladium*. Vol. 1. Basic biology, taxonomy and genetics, Kubicek, C.P., Harman, G.E. (eds)., Taylor and Francis Ltd., London, 3–34.

Gouramanis, G. (1997). Biological and chemical control of rice blast disease (*Pyricularia oryzae*) in Northern Greece. Cah. Options Méditter., 15(3), 61–68.

Hajano, J.U.D., Lodhi, A.M., Pathan, M.A., Khanzada, M.A., Serwar Shah, G. (2012). *In vitro* evaluation of fungicides, plant extracts and biocontrol agents against rice blast pathogen *Magnaporthe oryzae*. Pakistan J. Bot., 44, 1775–1778.

IRRI (1989). Biological control; Blast control, International Rice Research Institute (IRRI), Annual Report for 1988. The Philippines, 231–232.

Jaiganesh, V., Eswaran, A., Balabaskar, P., Kannan, C. (2007). Antagonistic activity of *Serratia marcescens* against *Pyricularia oryzae*. Not. Bot. Hort. Agrobot. Cluj-Na., 35(2), 48–54.

Javadi, L., Naeimi, Sh., Rezaee, S., Khosrav, V. (2014). Biological control of rice blast disease with native *Trichoderma* isolates in Mazandaran province. Biocont. Plant Protect., 2(1), 1–15.

Javan Nikkhah, M. (2001). Study on the genetic diversity of *Magnaporthe grisea*, rice blast pathogen by using molecular, pathogenicity and vegetative compatibility characters in Guilan province, Iran. Ph.D thesis, University of Tehran, Karaj.

Karthikeyan, V., Gnanamanickam, S.S. (2008). Biological control of *Setaria* blast (*Magnaporthe grisea*) with bacterial strains. Crop Prot., 27(2), 263–267.

Khalili, E., Sadravi, M., Naeimi, Sh., Khosravi, V. (2012). Biological control of rice brown spot with native isolates of three *Trichoderma* species. Braz. J. Microb., 43(1), 297–305.

Khodaparast, S.A., Sahragard, A. (2004). Rice diseases. The University of Guilan Press.

Khosravi, V., Naeimi, Sh., Rostami, M., Omrani, M., Bahramei, M. (2011). Evaluation of three commercial biological products on the rice blast disease in Mazandaran. Proceeding of 19th Iranian Plant Protection Congress, 31 July–3 August, Tehran, Iran, 828.

Mohammadian, S. (2013). Biological control of *Bipolaris* spp., the causal agent of brown spot disease of rice by some antagonistic fungi in Guilan province. Master's thesis, Islamic Azad University, Sci. and Res. Branch, Guilan, Rasht.

Naeimi, Sh., Okhovvat, S.M., Javan-Nikkhah, M., Vágvölgyi, C., Khosravi, V., Kredicks, L. (2011). Biological control of *Rhizoctonia solani* AG1-1A, the causal agent of rice sheath blight with *Trichoderma* strains. Phytopathol. Meditterr., 49(3), 287–300.

Ningthoujam, D.S., Sanasam, S., Tamreihao, K., Nimaichand, S. (2009). Antagonistic activities of local actinomycetes isolates against rice fungal pathogen. Afr. J. Microbiol. Res., 3(11), 737–742.

Ou, S.H. (1985). Rice diseases. Commonwealth Mycological Institute, 2nd ed.

Ouazzani Touhami, A., Mouria, A., Douira, A., Benkirane, R., Mlaik, A., El-Yachioui, M. (1998). *In vitro* effect of pH and temperature on the ability of *Trichoderma* spp. to reduce the growth of *Pyricularia oryzae*. Al Awania, 96, 19–24.

Padasht Dehkaei, F. (2001). The final report identifying biological factors controlling rice blast disease. Rice Research Institute of Iran.

Padasht Dehkaei, F., Izadyar, M. (2007). Study on the biological control of rice blast disease in the field conditions. J. Agri. Sci. Nat. Res., 13, 84–92.

Rostami, M., Momeni, A., Khosravi, V., Zare, L., Darvishzadeh, N., Omrani, M., Ghalandari, M. (2012). Identification and investigation of effects of antagonistic bacterial agents on rice blast control. Proceeding of 20th Iranian Plant Protection Congress, 25–29 August. Shiraz, Iran, 221–225.

Safari Motlagh, M.R., Padasht Dehkaee, F., Hedjaroud, G.A. (2005). Rice brown spot disease and evaluation of the response of some rice cultivars to it. J. Sci. Tech. Agri. Nat. Res., 9(2), 171–182.

Safari Motlagh, M.R. (2010). Isolation and characterization of some important fungi from *Echinochloa* spp., the potential agents to control rice weeds. Aust. J. Crop Sci., 4(6), 457–460.

Sena, A.P., Chaibub, A.A., Côrtes, M.V., Silva, G.B., Silva-Lobo, V.L., Prabhu, A.S., Filippi, M.C.C., Araújo, L.G. (2013). Increased enzymatic activity in rice leaf blast suppression by crude extract of *Epicoccum* sp. Trop. Plant Pathol., 38(5), 387–397.

Simmons, E.G. (1967). Typification of *Alternaria*, *Stemphylium*, and *Ulocladium*. Mycologia, 59(1), 67–92.

Sivakumar, D., Wilson Wijeratnam, R.S., Wijesundera, R.L.C., Marikar, F.M.T., Abeyesekere, M. (2000). Antagonistic effect of *Trichoderma harzianum* on postharvest pathogens of Rambutan (*Nephelium lappaceum*). Phytoparasitica, 28(3), 240–247.

Sivanesan, A. (1987). Gramainicolous species of *Bipolaris*, *Curvularia*, *Drechslera*, *Exserohilum* and their telemorphs. CAB International Mycological Institute.

Sutton, D.A., Fothergill, A.W., Rinaldi, M.G. (1998). Guide to clinically significant fungi (1th ed.).Williams & Wilkins Publish., Baltimore.

IDENTYFIKACJA NIEPATOGENICZNYCH GRZYBÓW RYŻU I OCENA ICH WPŁYWU NA ZWALCZANIE BIOLOGICZNE *Pyricularia grisea* POWODUJĄCEGO ZARAZĘ RYŻU *in vitro*

Streszczenie. Zaraza ryżu powodowana przez *Pyricularia grisea* jest ważną chorobą ryżu w Iranie i na całym świecie. Ze 150 zakażonych próbek zebranych z pól ryżowych w prowincji Guilan, wyizolowano 57 izolatów grzybów, dla których zastosowano pożywki PDA i W.A. Do identyfikacji tych grzybów użyto cech morfologicznych. Stwierdzono, że wyizolowane izolaty grzybów należą do *Pyricularia grisea*, *Ulocladium alternariae*, *Ulocladium* cf. *alternariae*, *Ulocladium* cf. *consortiale*, *Ulocladium* sp., *Curvularia pallescens*, *Preussia* sp., *Epicoccum* sp. oraz *Trichoderma harzianum*. Na podstawie testów patogeniczności udowodniono, że wszystkie izolaty *P. grisea* są chorobotwórcze wobec ryżu, a izolaty grzybów należące do innych rodzajów, które nie powodowały choroby ryżu, wyselekcjonowano do biologicznych badań kontrolnych, stosując w tym celu różne metody. Wywnioskowano, że osiem izolatów: *T. harzianum*, *U.* cf. *consortiale*, *Ulocladium* sp., *Epicoccum* sp., *Preussia* sp., *C. pallescens*, *U. alternariae* i *U.* cf. *alternariae* miały najbardziej hamujący wpływ na wzrost grzybni *P. grisea*. Izolaty *T. harzianum* i *U.* cf. *consortiale* miały najsilniejsze działanie inhibicyjne wobec wskaźnika wzrostu *P. grisea* i uznano je za najbardziej skuteczne izolaty. Na podstawie wyników stwierdzono, że metoda badania frakcji lotnej metabolitów jest najlepszą metodą zwalczania *P. grisea in vitro*.

Słowa kluczowe: antagonista, zwalczanie biologiczne, wzrost grzybni, grzyby niepatogeniczne

Accepted for print: 1.06.2016

For citation: Motlagh, M.R.S., Usefipoor, P. (2016). Identification of non-pathogenic fungi of rice and the evaluation of their effect on biological control of *Pyricularia grisea*, the causal agent of rice blast disease *in vitro.* Acta Sci. Pol. Hortorum Cultus, 15(6), 69–86.