Asian Journal of Agriculture and Rural Development
Volume 13, Issue 4 (2023): 277-287.

http://www.aessweb.com/journals/5005

Application of trichoderma and aspergillus as biofertilizers in eco-friendly ratoon rice cultivation

Sutarman[1†]
Andriani Eko Prihatiningrum[2]
Agus Miftahurrohmat[3]

[1,2,3]*Departement of Agrotechnology, Faculty of Science and Technology, Universitas Muhammadiyah Sidoarjo, Indonesia.*

[†] sutarman@umsida.ac.id *(Corresponding author)*

Article History
Received: 5 September 2023
Revised: 12 October 2023
Accepted: 23 November 2023
Published: 14 December 2023

Keywords
Aspergillus
Biofertilizer
Ratoon rice
Trichoderma.

ABSTRACT
The study's goal is to find the best native fungus from rice husk waste so that a solid biofertilizer can be made with high-husk flour as a carrier material and an inert agent. This study was conducted on agricultural land in Seloliman Village, Trawas District, and Mojokerto Regency. Biofertilization and biological agent formulation activities carried out at Muhammadiyah University of Sidoarjo's Microbiology Laboratory aided the research. The experiment was conducted using a factorial randomized block design. The first factor consisted of three treatments: no fungi, *Trichoderma sp.*, and *Aspergillus sp.* The second factor consists of soil treatment and apical treatment. The six treatment combinations were repeated four times (24 samples). The variables measured comprised plant height, number of panicles, weight of grain per plant, weight of 100 grams of grain, and the efficacy of biological agents in improving plant growth and productivity. All data underwent analysis of variety and then an HSD test at the 5% significance level to identify disparities among treatments. The study reveals that isolates Tc-013 and As-022 were identified as *Trichoderma esperellum* and *Aspergillus flavus* or *A. oryzae*, respectively. The application of *Trichoderma* and *Aspergillus* caused a decrease in the intensity of disease symptoms, reaching 64.7% and 37.3%, an increase in plant height and number of panicles, and an increase in the weight of 100 grains of 59.89 and 49.35%, respectively, as compared to the control treatment where the fungus was not applied.

Contribution/Originality: This research demonstrates the potential of a biologically active fungus, which has relatively never been used as a biofertiliser, to increase plant growth and production while maintaining the health of ratoon rice plants, particularly in areas where leaf necrosis pathogens are endemic.

DOI: 10.55493/5005.v13i4.4934
ISSN(P): 2304-1455/ ISSN(E): 2224-4433

How to cite: Sutarman, Prihatiningrum, A. E., & Miftahurrohmat, A. (2023). Application of trichoderma and aspergillus as biofertilizers in eco-friendly ratoon rice cultivation. *Asian Journal of Agriculture and Rural Development*, *13*(4), 277–287. 10.55493/5005.v13i4.4934

1.INTRODUCTION
The need for food for rice increases from year to year in line with the increasing population, while agricultural intensification is one approach to achieving food security that must be achieved and always maintained (Urfels et al., 2023).

Taking into consideration the efficient use of production time and resources, ratooning rice with one land processing and two planting periods is an alternative form of intensification. Ratoon rice cultivation has been developed in several countries and is considered to have higher resource use efficiency and less environmental impact compared to other rice growing systems (Firouzi, Nikkhah, & Aminpanah, 2018; Liu et al., 2019). Ratoon rice also

shortens crop production time (Torres, Natividad, Quintana, & Henry, 2020). Apart from having advantages, this cultivation method also has disadvantages, including that the productivity of the ratoon phase planting is lower than the first phase (Yu et al., 2021).

However, in several tests, ratoon rice cultivation has the potential to contribute to the impact of global warming (Shen, Zhang, & Zhang, 2021). This is due to the fact that during its cultivation, it consistently depends on non-sustainable hydrocarbon-based production resources, particularly in the utilization of chemical fertilizers and pesticides. On the other hand, using production resources wisely, ratoon rice has the potential to overcome the impact of global warming (Yuan, Cassman, Huang, Peng, & Grassini, 2019) and can significantly reduce methane gas emissions and the carbon footprint of rice fields (Lin et al., 2022).

So far, various studies on rice ratoons that have been carried out relate to efforts to improve land use optimization techniques, reduce the potential impact of global warming, and develop strategies for dealing with pest disturbances and increasing soil fertility (Ding et al., 2022; Zheng et al., 2022). As is the case in rice cultivation practices in general, in rice rations, the choice of environmentally specific varieties is a necessity (Xu et al., 2022), which of course requires materials to support soil fertility and plant physiology.

Thus, the ratoon rice system has so far relied on the support of chemical fertilizers and pesticides, bearing in mind that certain diseases and pests are endemic in some areas (Ye et al., 2017; Zaidi, Mukhtar, & Mansoor, 2018). In line with the 2015 Paris Agreement commitment (Zhou et al., 2021), there is a demand for each country to reduce the use of fossil materials, including reducing and replacing the use of petroleum derivatives such as pesticides and fertilizers.

For this reason, alternative fertilizers and pesticides are needed in order to increase plant resistance and protection from biotic and abiotic stresses while being environmentally friendly. The use of biological agents that have the ability to biofertilize and act as biocontrol agents is a prospect for the search for wise alternatives to plant production resources. Trichoderma and Aspergillus are types of fungi that, when applied to plants, have the capacity to substitute for fertilizers and pesticides.

Trichoderma produces enzymatic extracellular compounds that are capable of degrading soil organic matter, which produces nutrients for plants, as well as compounds that act as regulators of plant growth (Amanullah & Khan, 2023; Mezadri et al., 2022; Sutarman, Setiorini, Li'aini, & Rahmat, 2022). With the chitinolytic enzymes it produces and the ability to compete in its niche, this fungus can inhibit and damage pathogenic fungal cells so that it can provide plant protection (Matas-Baca et al., 2022; Tjahjanti, Prihatiningrum, & Miftahurrohmat, 2022). Meanwhile, several species and strains of Aspergillus have many benefits in agriculture because these fungi are able to degrade soil organic matter (Hsieh, Kurzai, & Brock, 2017; Lopes et al., 2021), produce organic acids that can chelate metals from oxides (Klaic et al., 2018), increase the amount of dissolved phosphate in the soil (Klaic, Plotegher, Ribeiro, Zangirolami, & Farinas, 2017), and increase the biological oxidation of elemental sulfur (Majaron et al., 2020). Thus, these two types of fungi have the potential to act as biofertilizer biological agents that have the potential to increase plant growth and health.

So far, not much has been done on the utilization of these two biological agent fungi to help normal paddy rice plants, while ratoon rice research has only relied on the plant's ecophysiological response. This research integrates the function of nutritional support for rice plants through ratoon shoots and crowns and provides protection against various potential disturbances of indigenous diseases in lowland rice crops such as blast (*Pyricularia oryzae*), striped spot (Cercospora spp.), spot (*Helmintosporium*), and stem base rot (*Rhizoctonia* sp. and *Fusarium* sp.).

This research aims to determine the ability of the indigenous fungi *Trichoderma* sp. and *Aspergillus* sp. applied to shoots and shoots of rice cultivation during the rice ratoon period to provide support for the productivity and health protection of lowland rice.

2. METHOD

2.1. Identification of Biological Agents

This experiment used *Trichoderma* Tc-013 and *Aspergillus* As-22, two fungal isolates found by screening a group of indigenous isolates from lowland rice cultivation in Biting Hamlet, Seloliman Village, Trawas District, Mojokerto Regency. These are now in the collection of the Microbiology and Biotechnology Laboratory, Universitas Muhammadiyah Sidoarjo.

The two isolates were propagated in PDA-chloramphenicol media, incubated for 10 days, and observed macroscopically for the shape of the colonies. Furthermore, they were sampled from the culture dish and processed onto the surface of a glass object to observe the shape and dimensions of the hyphae and spores and identify them.

The mycelium from each isolate in a petri dish was taken as much as 50 mg and put in 200 µl dH_2O in a BashingBead™ tube, then deoxyribonucleic acid (DNA) isolation was carried out according to the standard procedure of Quick-DNA Fungal/Bacterial Miniprep Kit™ catalog number D6005. Then the samples were amplified using Forward Primer ITS 1 5'-TCC GTA GGT GAA CCT GCG G-'3 and Reverse Primer ITS 4 5' TCC TCC GCT TAT TGA TAT GC-3'. The cycle used was predenaturation at 95°C for 5 minutes, followed by denaturation at 95°C, annealing at 60°C and elongation at 72°C for 1 minute each. Final elongation (post-elongation) 72 °C, for 5 minutes. The cycle used is 40 cycles.

Sequencing of the polymerase chain reaction (PCR) DNA fragments was carried out using the Sanger sequencing method, with the PCR product sent to a commercial DNA sequencing service (1st Base; Singapore) using an ABI 3730XL sequencer machine.

Then the nucleotide arrangement obtained was compared to the gene bank using the Basic Local Alignment Search Tool (BLAST) program available at the National Center for Biotechnology Information (NCBI) (NCBI, 2022).

Homologous sequences obtained from the NCBI Gene Bank were reconstructed with MEGA 7 software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) using the Neighbor-Joining method to produce a phylogenetic tree.

2.2. Biofertilizer Formulation

Colonies that have grown fill the petri dish for about 7-10 days, ready to be harvested and made as a suspension that was previously crushed using a blender. Suspensions containing isolates of biological agent fungi, each in the amount of one petri dish of the culture mixed with 250 ml of distilled water, were poured and mixed with 2,500 g of husk flour (40 mesh size) as a carrier agent until evenly distributed. After drying for 12-24 hours, a biofertilizer formula is formed. Prior to application, it is important to determine the active spore concentration of the fungus by the implementation of the serial dilution technique. The active population of spores is determined to be 10^6colony forming unit (CFU).gr^{-1}, if the calculation results exceed this amount, dilution will be carried out with the addition of sterile husk flour to reach an average population of 10^6 CFU.gr^{-1}.

2.3. Field Efficacy Test

The rice fields that have been harvested are irrigated for approximately 3 days, and then the rice stalks remaining from harvesting are cut with the aim of growing new rice shoots. The cutting size is about 3-5 cm. Biofertilizer formula is given as a soil treatment, which is considered fertilization, carried out a week after harvesting the first stage of rice plants until the ratoon plants are irrigated, and as an apical treatment after the plants have been watered until the panicles begin to fill. The treatments in this experiment were as follows: (i) without biofertilizer application but using conventional chemical fertilizers, (ii) Trichoderma biofertilizer application, and (iii) Aspergillus biofertilizer application. This experiment was repeated seven times. Each experimental unit is a plot measuring 2x5 m^2. The determination of plot boundaries for each experimental unit is carried out after harvest. As a soil treatment, each biofertilizer is given to the soil around the plant roots at a dose of 200 grams (husk flour formula containing 10^6 CFU.g^{-1} active spores of biological agents) per plant. Apical treatment is applied to the canopy using 200 g of biofertilizer (a husk powder formula containing 10^6 CFU.g^{-1} active spores of biological agents) dissolved in 2,000 ml of neutral water as a suspension which will be sprayed eight times during the growth and filling of the rice grains at intervals of one week. Soil treatment is given before planting or a week after harvest. Next, the plant height, number of panicles per plant, harvest weight per plant, and weight of 100 grains were observed. Since spots caused by *Cercosspora oryzae* and *Helmintosporium oryzae* are common on the land that was used, itt was tested how applying biofertilizer might affect the plants' ability to fight these pathogens. Assessment of plant health is carried out at the beginning of the generative phase, or between 42 and 70 days after planting (DAP). The criteria used to determine the intensity of attack symptoms are as shown in Table 1.

Table 1. Criteria for symptoms of endemic pathogens in rice plants.

Score	Criteria for attack symptom
0	No damage occurred
1	As many as 1-25% of the leaves have striped and spotted spots on the leaves
2	As many as 25-50% of the leaves have striped and dotted spots on the leaves or 25% have wide spots covering each leaf
3	As many as 50-75% of the leaves have striped and dotted spots on the leaves or 50% of the spots expand to cover each leaf or 25% of the leaves die
4	More than 75% of the leaves have striped and dotted spots on the leaves or 75% of the spots have widened to cover each leaf or 50% of the leaves have died

2.4. Data Analysis

Field experiment data were analyzed using analysis of variance (ANOVA) at the 5% level, followed by the honestly significant diffirence (HSD) test at the 5% level to determine differences between treatments.

3. RESULTS AND DISCUSSION

3.1. Identification Results

The results of macroscopic observations of the shape and color of the colonies of the two biological agents, fungi, as well as microscopic observations showing woven hyphae and spores, are shown in Figure 1. The green color on the colony of isolate Tc-013 is typical of *Trichoderma*, with branched hyphae and conidiospores, each with a diameter of 2.56±0.39 µm and 2.68±0.45 µm. Meanwhile, the As-022 colony appeared brown-black with branched hyphae measuring 4.72±0.63 µm and an average spore diameter of 2.74±0.15 µm.

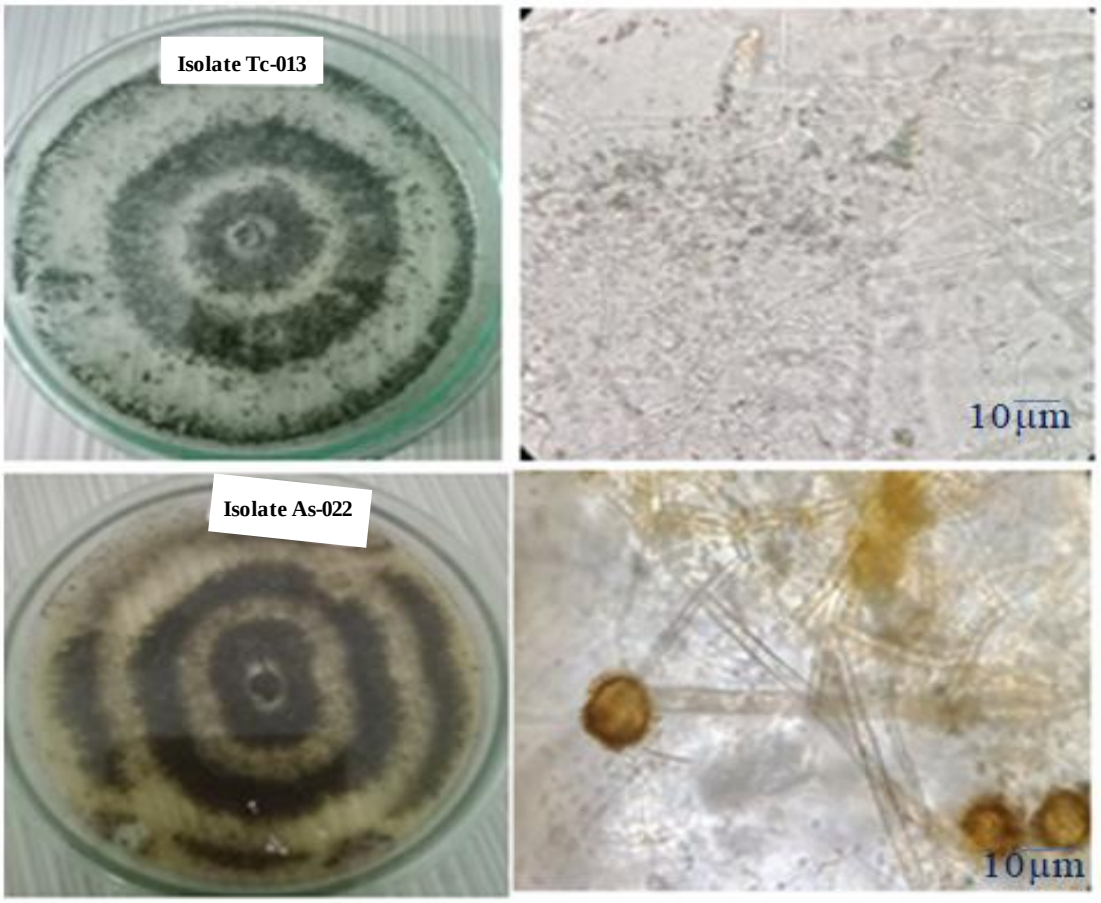

Figure 1. Morphology of Trichoderma sp. Tc-013 (top) and Aspergillus sp. As-022 (bottom) is used as a biological agent.

The nuleotide sequencing results of the two fungal isolates, each of which totaled 579 nucleosides for Tc-013 and 551 nucleosides for As-022, are presented in Figure 2.

ACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCAAT
GTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGG
TGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCA
AACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTG
AGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCAT
TTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGG
ACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGC
CGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGC
GCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTG
ACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCA

GTTCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAG
TTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCA
GCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGA
TCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACT
TTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAG
CGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCAT
CGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGG
CATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGT
GTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAA
GGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTT
TGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAA
TCAATCTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGC
TGAACTTAAGCATATCAATAAGCGGAGG

Figure 2. Nucleoside sequence of DNA sequences of Tc-013 (top) and As-022 isolates (bottom).

In the process of matching nucleotide arrangements to DNA sequences, according to Brock, Döring, and Bidartondo (2009), a species is said to be the same if the ITS homology of the organism's rDNA sequence has a similarity of 97% (Sutarman, 2022). Thus, when matching with collections contained in BLAST, similarities below 97% are ignored, and priority is given to those with 100% similarity.

The BLAST search results from 2022 show that the Tc-013 sequences is 100% identical to the sequence from Trichoderma asperellum (Sequence ID: MH56933331.1) (NCBI, 2022). Meanwhile, isolate As-022 is similar to Aspergilluysoryzae (Sequence ID: MH56933331.1), and Asspergillus flavus (Sequence ID: KX067855.1) with 100% similarity at 557 nucleotide sequence length. Reconstruction results using MEGA software (Kumar et al., 2018) with Neighbor-Joining method obtained a phylogenetic tree as shown in Figure 3.

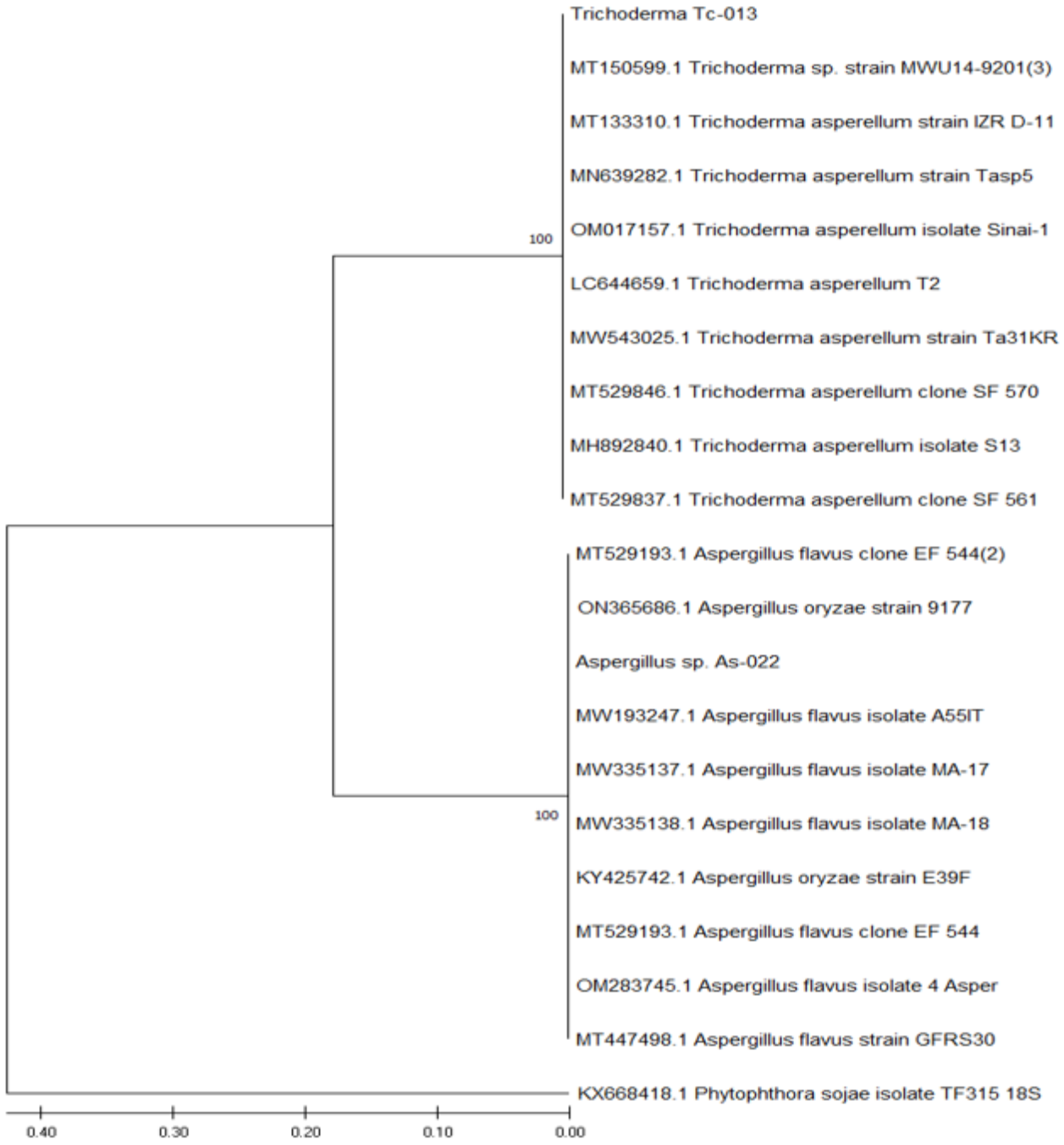

Figure 3. Filogegentik isolate Tc-013 dan As-022.

3.2. Field Test Results

3.2.1. Plant Growth

The response of ratoon rice terms of grain weight and 100 grain weight per plant showed that biofertilizer made the average higher than when no biofertilizer was used Figures 4 and 5. Table 2 shows the idex of necrotic disease symptoms on leaves and how much the symptoms got better when biofertilizer was used compared to when biofertilizer wasn't used.

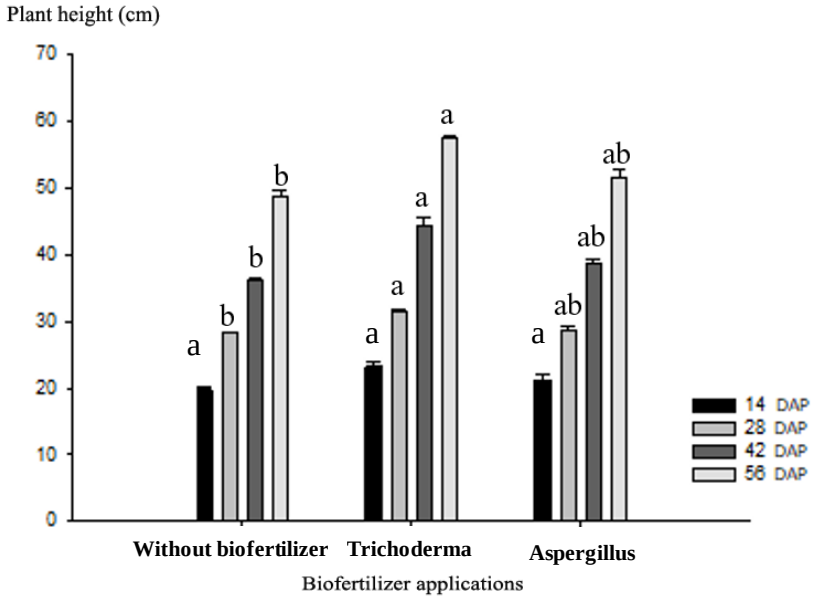

Figure 4. The average effect of biofertilizer application on plant height 14-56 HSP.
Note: Different letters in the column indicating the same observation time indicate differences in the effect of biofertilizer application on the 5% HSD test.

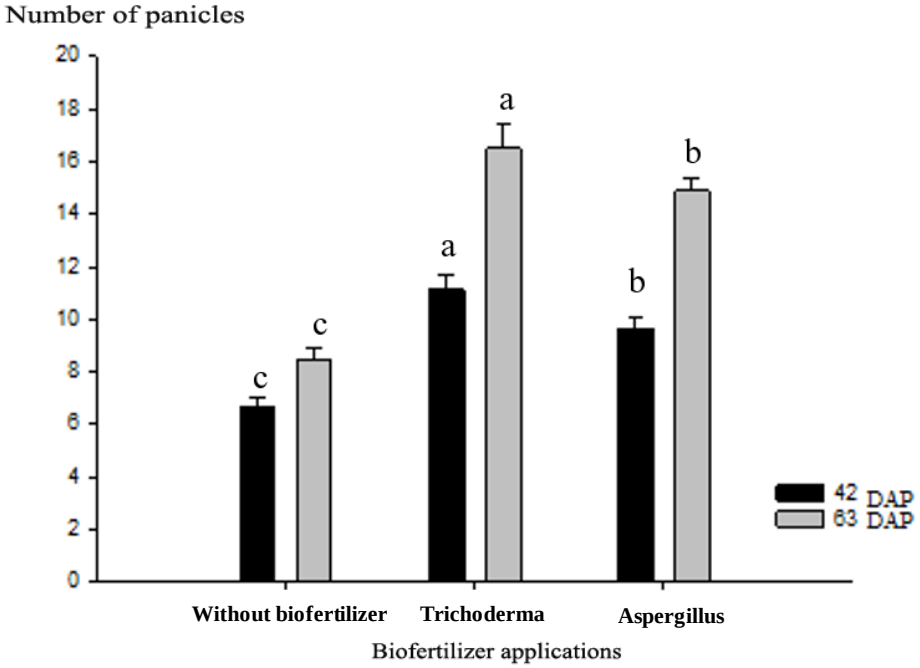

Figure 5. The average effect of biofertilizer application on the number of panicles per plant was 42 and 63 DAP.
Note: Different letters in the column indicating the same observation time indicate differences in the effect of biofertilizer application on the HSD test at 5% level.

Table 2. Effect of biofertilizer application on the intensity of leaf necrotic spot disease symptoms of ratoon rice variety IR 64 and its reduction compared to no biofertilizer at 42 and 70 DAP.

Treatments	42 DAP		70 DAP	
	Intensity of disease symptoms (%)	Reduction in the intensity of disease symptoms (%)	Intensity of disease symptoms (%)	Decreased intensity of disease symptoms (%)
No biofrtilizer	22.77±0.63	-	35.27	-
Trichoderma	8.04±0.77	64.7	14.29	59.5
Aspergillus	14.29±1.17	37.3	23.66	32.9

3.2.2. Plant Production and Biological Age Performance

The response of ratoon rice in terms of grain weight and 100 grain weight per plant showed that the application of biofertilizer made the average higher than when no biofertilizer was used (Figures 6 and 7).

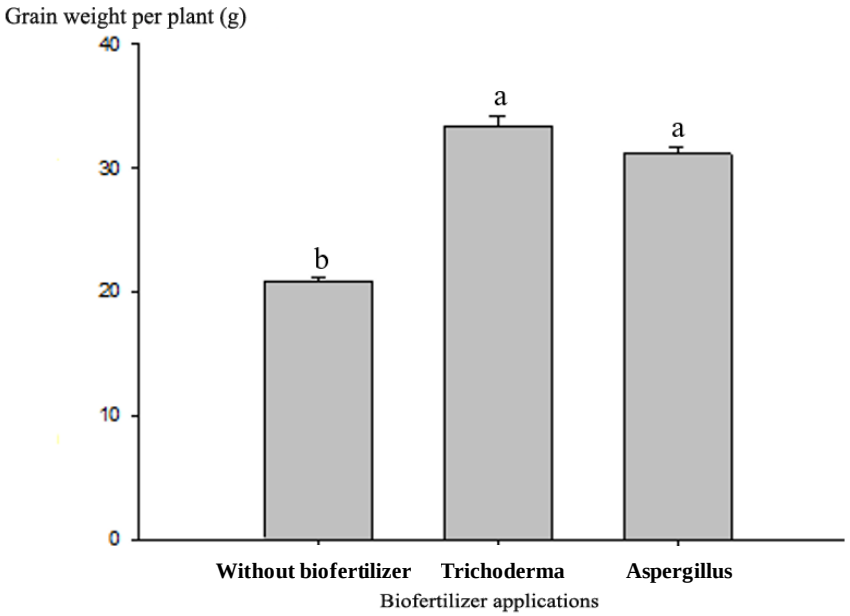

Figure 6. The average effect of biofertilizer application on grain weight per plant.
Note: Different letters in the column indicate differences in the effect of biofertilizer application on the HSD test at 5% level .

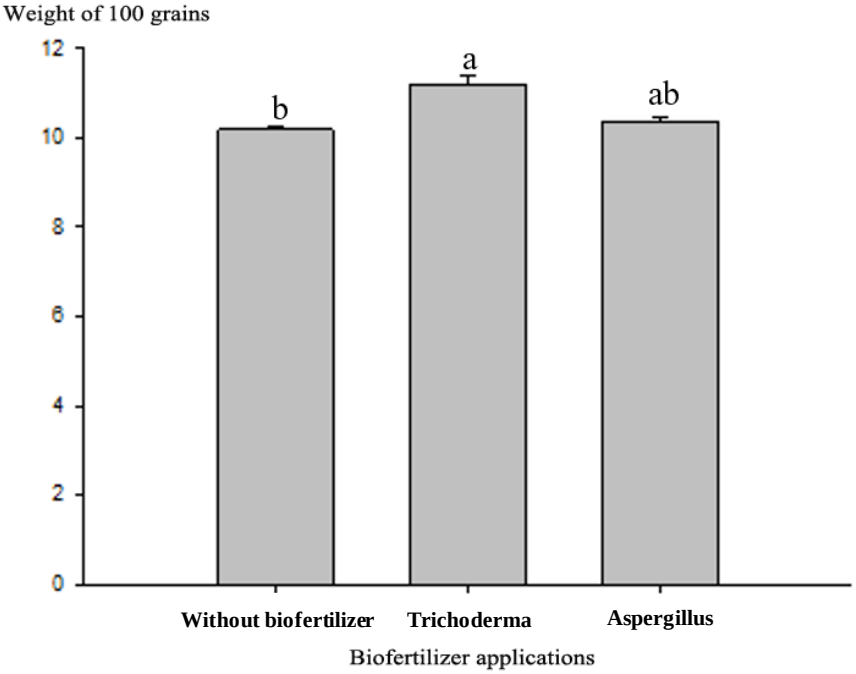

Figure 7. The average effect of biofertilizer application on the weight of 100 grains of grain per plant.
Note: Different letters in the column indicate differences in the effect of biofertilizer application on the HSD test at 5% level.

Application of biofertilizer with Trichoderma and Aspergillus fungi can increase plant height, number of panicles, grain weight per plant, and weight of 100 ratoon rice plants compared to treatments not applied (control) (Table 3).

Table 3. Performance of biofertilizer biological agents on increasing growth response and production of rice varieties IR 64 ratoon model.

Agenhayati biofertilizer	Plant response to performance of biofertilizer biological agents (%)*			
	Biofertilizer biological agent Increase in average plant height 56 DAP	Increase in average number of panicles	Increase in average grain weight per plant	Increase in average weight of 100 grains
Trichoderma	17.94	94.12	9.98	59.89
Aspergillus	5.69	75.00	1.77	49.35

Note: * Increased growth response and plant production compared to control (without biofertilizer).

3.3. Discussion

Isolate Tc-013 has similarities in colony appearance, hyphal branching morphology, and hyphal and spore diameter dimensions to *Trichoderma esperellum* (Sutarman, 2022; Sutarman, Jalaluddin, Li'aini, & Prihatiningrum, 2021). BLAST search results (NCBI, 2022) showed 100% similarity to *T. asperellum* (Sequence ID: MT102403.1). Isolate Tc-013 also has similarities with isolate RM-28, whose data is stored at NCBI with the additional number MK092975 and identified as *T. Asperellum* (Anam, Reddy, & Ahn, 2019), and isolate T1 (accession numbers GenBank LC158827, KU497722, and KU497723) (Baiyee, Ito, & Sunpapao, 2019), andisolate TC01 (GenBank accession numbers MH752042 and MN813963) (Shang, Liu, & Xu, 2020). The shape and dimensions of the conidispores and chlamydospores are also similar to those of the endophytes of *T. asperellum* VM 100 (KY412854) (Leylaie & Zafari, 2018) and *T. asperellum* isolate GDFS1009 (Karuppiah, Sun, Li, Vallikkannu, & Chen, 2019) and isolates Ta1 and Ta2 (Hewedy et al., 2020).

Morphologically, isolate As-022 cannot be differentiated from several isolates found in Indonesia and from various other countries. As-022 has sequences that are similar to many Aspergillus variants, which were shown phylogenetically with primers ITS-1 and ITS-2 with identical levels of up to 100% as *A. oryzae* (KY655350.1) (Devi & Joshi, 2015) and as *A. Flavus* (Alshehri & Palanisamy, 2020). For the final determination of these two naming alternatives, in-depth research is needed regarding their physiological performance and the metabolites they produce.

When Trichoderma and Aspergillus biofertilizers were used, the plants grew taller, had more panicles, and produced more grain. The 100 ratoon rice plants also gained weight (Table 1). This shows that both Tc-013 and As-022 isolates have demonstrated their ability as biological agents that act as biofertilizers.

Various evidence has been shown by the ability of Trichoderma sp. as a biofertilizer, which is able to increase biological activity around the rhizosphere of plants before the soil surface under watery conditions. However, it appears that the role of Trichoderma, which is applied through spraying the plant canopy, makes a significant contribution to helping plant growth and improving the soil structure around plant roots by decomposing organic substances contained in the soil. Many organic substances are available in the rhizosphere. With the application of the fungus *Trichoderma* sp., the organic material will be decomposed and converted into ions that can be absorbed and utilized by plants. In addition, this fungus acts as a mycoparasite against pathogenic fungi and also produces metabolites that act as growth hormones for plants (Vinale et al., 2014) so that it can induce disease resistance (He et al., 2019). Trichoderma degrades organic matter to produce nutrients and increases plant resistance to abiotic environmental stress (Sachdev, Singh, & Singh, 2018).

Aspergillus sp. has the ability to fix nitrogen in the soil, thus helping the plantsmeet their nitrogen needs. Such a function benefits the growth of the plants (Dutta & Das, 2017). *Aspergillus* sp. plays a significant role in decomposition, bioremediation, and biocontrol, being used to synthesize organic acids, enzymes, and secondary metabolites (Kagot, Okoth, De Boevre, & De Saeger, 2019). Numerous studies provide evidence for the effectiveness, quantity, and efficiency of Aspergillus' extracellular hydrolytic enzymes (Brown et al., 2016). Several Aspergillus species' genomes exhibit the genetic expression of cellulase and hemicellulase enzymes' ability to operate proficiently (Cong et al., 2017; De Gouvêa et al., 2018) indicating the organisms' potential as bio-fertilizer agents. This fungus also produces cellulase and hemicellulase (Midorikawa et al., 2018). *Aspergillus* can survive in poor temperature and humidity conditions, has high adaptability to substrate complexity, produces various useful secondary metabolites, and produces various types of enzymes that degrade various polysaccharides and proteins (Flores-Gallegos, Veana-Hernandez, Michel-Michel, Lara-Victoriano, & Rodríguez-Herrera, 2016) and other lignocellulose degrading enzymes (Monclaro et al., 2020), as well as describe the complex structure of lignocellulosic biomass by releasing monomer sugars (Dimarogona, 2016) as an energy source. Therefore, this fungus shows promise for breaking down organic matter and may have practical applications in the development of biological fertilisers.

Aspergillus is generally capable of producing volatile organic compounds (VOCs), including various acid molecules, alcohols, aldehydes, aromatics, ketones, terpenes, thiols, and their derivatives (Wang et al., 2021). Even from the bioconversion process of organic materials, compounds can be produced that are capable of promoting plant growth and acting as a signal of spore germination so as to guarantee positive interactions in their ecology (Lemfack, Nickel, Dunkel, Preissner, & Piechulla, 2014).

The characteristics of the two fungal isolates showed their ability to support plant growth (Table 2), especially through the application of plant canopy spraying. The index of attack symptoms was much lower (Table 1) in the application of biological agents. Meanwhile, the ability to reduce the intensity of attack symptoms in the Trichoderma application was much greater than the Aspergillus application. This is possible because there is a lot of research evidence showing the strength of this fungus as a biocontrol agent. The activity of volatile metabolites produced by *T. eseprellum* is able to inhibit *F. Oxysporum* (Tao et al., 2020) in addition to supporting plant growth (Al-Askar, Saber, Ghoneem, Hafez, & Ibrahim, 2021) considering that T. esperellum is also capable of producing auxin (Wang et al., 2020). In this study, the application of these two biological agents, fungal isolates, increased the grain weight and weight of 100 grain grains, respectively, 75-94.12 and 49.35-58.89% (Table 2). This provides a projection to increase production potential equivalent to planting rice twice. In general, ratoon rice can produce 50% of the first harvest (Oda, Nguyen, & Huynh, 2019). In addition, ratoon rice is a wise choice in order to help reduce pressure on the environment due to conventional rice cultivation while maintaining food security (Jiang et al., 2021; Yang et al., 2022).

4. CONCLUSION

The biological agent fungus isolates Tc-013 and As-022 were each identified based on molecular markers as *Trichoderma esperellum* and *Aspergillus flavu,s* or *A.oryzae*. Application of Trichoderma and Aspergillus formulated as

biofetilizers increased plant height by 17.94 and 5.69%, respectively, 56 days after planting (DAP), increased the number of panicles by 94.12 and 75.00%, respectively, and reduced the intensity of attack symptoms by 64.7% and 37.3% at 42 DAP and 59.5% and 32.9% at 70 DAP. These two biological agents were able to increase the weight of first-plant grain by 9.98 and 1.77%, respectively, and increase the weight of 100 grains by 59.89 and 49.35%, respectively. Biofertilizer with the active ingredients Trichoderma isolate Tc-013 and Aspergillus As-022 has great potential to be applied to wetland plants as ratoon rice to increase growth and provide protection for plant health.

Funding: This research is supported by Directorate General of Higher Education, Research and Technology of the Ministry of Education, Culture, Research and Technology of the Republic of Indonesia thourg Higher Education Basic Research Scheme (Grant number: 019/SP2H/LT-MULTI-PDPK/LL7/2021).
Institutional Review Board Statement: Not applicable.
Transparency: The authors state that the manuscript is honest, truthful, and transparent, that no key aspects of the investigation have been omitted, and that any differences from the study as planned have been clarified. This study followed all writing ethics.
Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.

REFERENCES

Al-Askar, A. A., Saber, W. I., Ghoneem, K. M., Hafez, E. E., & Ibrahim, A. A. (2021). Crude citric acid of Trichoderma asperellum: Tomato growth promotor and suppressor of Fusarium oxysporum f. sp. lycopersici. *Plants*, *10*(2), 222. https://doi.org/10.3390/plants10020222

Alshehri, B., & Palanisamy, M. (2020). Evaluation of molecular identification of Aspergillus species causing fungal keratitis. *Saudi Journal of Biological Sciences*, *27*(2), 751-756. https://doi.org/10.1016/j.sjbs.2019.12.030

Amanullah, F., & Khan, W. (2023). Trichoderma asperellum L. Coupled the effects of biochar to enhance the growth and physiology of contrasting maize cultivars under copper and nickel stresses. *Plants*, *12*(4), 958-958. https://doi.org/10.3390/plants12040958

Anam, G. B., Reddy, M. S., & Ahn, Y.-H. (2019). Characterization of Trichoderma asperellum RM-28 for its sodic/saline-alkali tolerance and plant growth promoting activities to alleviate toxicity of red mud. *Science of the Total Environment*, *662*, 462-469. https://doi.org/10.1016/j.scitotenv.2019.01.279

Baiyee, B., Ito, S.-i., & Sunpapao, A. (2019). Trichoderma asperellum T1 mediated antifungal activity and induced defense response against leaf spot fungi in lettuce (Lactuca sativa L.). *Physiological and Molecular Plant Pathology*, *106*, 96-101. https://doi.org/10.1016/j.pmpp.2018.12.009

Brock, P. M., Döring, H., & Bidartondo, M. I. (2009). How to know unknown fungi: The role of a herbarium. *New Phytologist*, *181*(3), 719-724.

Brown, N. A., Ries, L. N., Reis, T. F., Rajendran, R., Corrêa Dos Santos, R. A., Ramage, G., . . . Goldman, G. H. (2016). RNA-seq reveals hydrophobins that are involved in the adaptation of Aspergillus nidulans to lignocellulose. *Biotechnology for Biofuels*, *9*(1), 1-17. https://doi.org/10.1186/s13068-016-0558-2

Cong, B., Wang, N., Liu, S., Liu, F., Yin, X., & Shen, J. (2017). Isolation, characterization and transcriptome analysis of a novel Antarctic Aspergillus sydowii strain MS-19 as a potential lignocellulosic enzyme source. *BMC Microbiology*, *17*(1), 1-14. https://doi.org/10.1186/s12866-017-1028-0

De Gouvêa, P. F., Bernardi, A. V., Gerolamo, L. E., de Souza Santos, E., Riaño-Pachón, D. M., Uyemura, S. A., & Dinamarco, T. M. (2018). Transcriptome and secretome analysis of Aspergillus fumigatus in the presence of sugarcane bagasse. *BMC Genomics*, *19*(1), 1-18. https://doi.org/10.1186/s12864-018-4627-8

Devi, L. S., & Joshi, S. (2015). Ultrastructures of silver nanoparticles biosynthesized using endophytic fungi. *Journal of Microscopy and Ultrastructure*, *3*(1), 29-37. https://doi.org/10.1016/j.jmau.2014.10.004

Dimarogona, M. (2016). Regulation and heterologous expression of lignocellulosic enzymes in Aspergillus. In New and future developments in microbial biotechnology and bioengineering. In (pp. 171–190): Elsevier. https://doi.org/10.1016/B978-0-444-63505-1.00012-9.

Ding, Z., Hu, R., Styles, D., Wang, X., Tian, Y., Cao, Y., & Hou, J. (2022). Optimized ratoon rice system to sustain cleaner food production in Jianghan Plain, China: A comprehensive emergy assessment. *Environmental Science and Pollution Research*, *29*(17), 24639-24650. https://doi.org/10.1007/s11356-021-17747-1

Dutta, D., & Das, M. D. (2017). Effect of C/N ratio and microelements on nutrient dynamics and cell morphology in submerged fermentation of Aspergillus giganteus MTCC 8408 using Taguchi DOE. *3 Biotech*, *7*(1), 34. https://doi.org/10.1007/s13205-017-0611-2

Firouzi, S., Nikkhah, A., & Aminpanah, H. (2018). Rice single cropping or ratooning agro-system: Which one is more environment-friendly? *Environmental Science and Pollution Research*, *25*, 32246-32256. https://doi.org/10.1007/s11356-018-3076-x

Flores-Gallegos, A., Veana-Hernandez, F., Michel-Michel, M., Lara-Victoriano, F., & Rodríguez-Herrera, R. (2016). Molecular evolution of aspergillus. In New and Future Developments in Microbial Biotechnology and Bioengineering. In (pp. 41-51): Elsevier. http://dx.doi.org/10.1016/B978-0-444-63505-1.00003-8.

He, A.-L., Jia, L., Wang, X.-H., Zhang, Q.-G., Wei, S., & Jie, C. (2019). Soil application of Trichoderma asperellum GDFS1009 granules promotes growth and resistance to Fusarium graminearum in maize. *Journal of Integrative Agriculture*, *18*(3), 599-606. https://doi.org/10.1016/s2095-3119(18)62089-1

Hewedy, O. A., Abdel Lateif, K. S., Seleiman, M. F., Shami, A., Albarakaty, F. M., & M. El-Meihy, R. (2020). Phylogenetic diversity of Trichoderma strains and their antagonistic potential against soil-borne pathogens under stress conditions. *Biology*, *9*(8), 189. https://doi.org/10.3390/biology9080189

Hsieh, S.-H., Kurzai, O., & Brock, M. (2017). Persistence within dendritic cells marks an antifungal evasion and dissemination strategy of Aspergillus terreus. *Scientific Reports*, *7*(1), 10590. https://doi.org/10.1038/s41598-017-10914-w

Jiang, P., Xu, F., Zhang, L., Liu, M., Xiong, H., Guo, X., . . . Zhou, X. (2021). Impact of tillage and crop establishment methods on rice yields in a rice-ratoon rice cropping system in Southwest China. *Scientific Reports*, *11*(1), 18421. https://doi.org/10.1038/s41598-021-98057-x

Kagot, V., Okoth, S., De Boevre, M., & De Saeger, S. (2019). Biocontrol of aspergillus and fusarium mycotoxins in Africa: Benefits and limitations. *Toxins*, *11*(2), 109. https://doi.org/10.3390/toxins11020109

Karuppiah, V., Sun, J., Li, T., Vallikkannu, M., & Chen, J. (2019). Co-cultivation of Trichoderma asperellum GDFS1009 and Bacillus amyloliquefaciens 1841 causes differential gene expression and improvement in the wheat growth and biocontrol activity. *Frontiers in Microbiology*, *10*, 1068. https://doi.org/10.3389/fmicb.2019.01068

Klaic, R., Giroto, A. S., Guimaraes, G. G. F., Plotegher, F., Ribeiro, C., Zangirolami, T. C., & Farinas, C. S. (2018). Nanocomposite of starch-phosphate rock bioactivated for environmentally-friendly fertilization. *Minerals Engineering*, *128*, 230-237. https://doi.org/10.1016/j.mineng.2018.09.002

Klaic, R., Plotegher, F., Ribeiro, C., Zangirolami, T. C., & Farinas, C. S. (2017). A novel combined mechanical-biological approach to improve rock phosphate solubilization. *International Journal of Mineral Processing*, *161*, 50-58. https://doi.org/10.1016/j.minpro.2017.02.009

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547. https://doi.org/10.1093/molbev/msy096

Lemfack, M. C., Nickel, J., Dunkel, M., Preissner, R., & Piechulla, B. (2014). MVOC: A database of microbial volatiles. *Nucleic Acids Research*, *42*(D1), D744-D748. https://doi.org/10.1093/nar/gkx1016

Leylaie, S., & Zafari, D. (2018). Antiproliferative and antimicrobial activities of secondary metabolites and phylogenetic study of endophytic Trichoderma species from Vinca plants. *Frontiers in Microbiology*, *9*, 1484. https://doi.org/10.3389/fmicb.2018.01484

Lin, Z.-M., Li, Z., Weng, P.-Y., Wu, D.-Q., Zou, J.-N., Pang, Z.-Q., & Lin, W.-X. (2022). Field greenhouse gas emission characteristics and carbon footprint of ratoon rice. *The Journal of Applied Ecology*, *33*(5), 1340-1351.

Liu, Q., Su, Y., Zhu, Y., Peng, K., Hong, B., Wang, R., . . . Xiao, L. (2019). Manipulating osa-MIR156f expression by D18 promoter to regulate plant architecture and yield traits both in seasonal and ratooning rice. *Biological Procedures Online*, *21*(1), 1-14. https://doi.org/10.1186/s12575-019-0110-4

Lopes, L. G., Csonka, L. A., Castellane, J. A. S., Oliveira, A. W., Almeida-Júnior, S. D., Furtado, R. A., . . . Moretti, M. L. (2021). Disinfectants in a hemodialysis setting: Antifungal activity against Aspergillus and Fusarium planktonic and biofilm cells and the effect of commercial peracetic acid residual in mice. *Frontiers in Cellular and Infection Microbiology*, *11*, 663741. https://doi.org/10.3389/fcimb.2021.663741

Majaron, V. F., da Silva, M. G., Bortoletto-Santos, R., Klaic, R., Giroto, A., Guimaraes, G. G., . . . Ribeiro, C. (2020). Synergy between castor oil polyurethane/starch polymer coating and local acidification by A. niger for increasing the efficiency of nitrogen fertilization using urea granules. *Industrial Crops and Products*, *154*, 112717. https://doi.org/10.1016/j.indcrop.2020.112717

Matas-Baca, M. Á., García, C. U., Pérez-Álvarez, S., Flores-Córdova, M. A., Escobedo-Bonilla, C. M., Magallanes-Tapia, M. A., & Chávez, E. S. (2022). Morphological and molecular characterization of a new autochthonous Trichoderma sp. isolate and its biocontrol efficacy against Alternaria sp. *Saudi Journal of Biological Sciences*, *29*(4), 2620-2625. https://doi.org/10.1016/j.sjbs.2021.12.052

Mezadri, E. T., Kuhn, K. R., Schmaltz, S., Tres, M. V., Zabot, G. L., Kuhn, R. C., & Mazutti, M. A. (2022). Evaluation of ultrasound waves for the production of chitinase and β-1, 3 glucanase by Trichoderma harzianum through SSF. *3 Biotech*, *12*(5), 122. https://doi.org/10.1007/s13205-022-03179-2

Midorikawa, G. E. O., Correa, C. L., Noronha, E. F., Filho, E. X. F., Togawa, R. C., Costa, M. M. D. C., . . . Miller, R. N. G. (2018). Analysis of the transcriptome in Aspergillus tamarii during enzymatic degradation of sugarcane bagasse. *Frontiers in Bioengineering and Biotechnology*, *6*, 123. https://doi.org/10.3389/fbioe.2018.00123

Monclaro, V. A., Petrovic, D., Alves, G. S., Costa, M., Midorikawa, G. E., Miller, R. N., . . . Varnai, A. (2020). Characterization of two family AA9 LPMOs from Aspergillus tamarii with distinct activities on xyloglucan reveals structural differences linked to cleavage specificity. *Plos One*, *15*(7), e0235642. https://doi.org/10.1371/journal.pone.0235642

NCBI. (2022). *National library of medicine. National Center of Biotechnology Information (NCBI)*. Retrieved from https://www.ncbi.nlm.nih.gov/

Oda, M., Nguyen, H. C., & Huynh, V. T. (2019). Evaluation of cropping method for perennial ratoon rice: Adaptation of SALIBU to triple-cropping in Vietnam. *F1000Research*, *8*, 1825. https://doi.org/10.12688/f1000research.20890.2

Sachdev, S., Singh, A., & Singh, R. P. (2018). Optimization of culture conditions for mass production and bio-formulation of Trichoderma using response surface methodology. *3 Biotech*, *8*(8), 360. https://doi.org/10.1007/s13205-018-1360-6

Shang, J., Liu, B., & Xu, Z. (2020). Efficacy of Trichoderma asperellum TC01 against anthracnose and growth promotion of Camellia sinensis seedlings. *Biological Control*, *143*, 104205. https://doi.org/10.1016/j.biocontrol.2020.104205

Shen, X., Zhang, L., & Zhang, J. (2021). Ratoon rice production in central China: Environmental sustainability and food production. *Science of the Total Environment*, *764*, 142850. https://doi.org/10.1016/j.scitotenv.2020.142850

Sutarman, Setiorini, T., Li'aini, A., & Rahmat, A. (2022). Evaluation of Trichoderma asperellum effect toward anthracnose pathogen activity on red chili (Capsicum annum L.) as ecofriendly pesticide. *International Journal of Environmentgal Science and Development*, *13*(4), 131-137. https://doi.org/10.18178/ijesd.2022.13.4.1383

Sutarman, S. (2022). *Identification of several Aspergillus isolates candidates for bio fertilizer agents using molecular markers*. Paper presented at the IOP Conference Series: Earth and Environmental Science.

Sutarman, S., Jalaluddin, A. K., Li'aini, A. S., & Prihatiningrum, A. E. (2021). Characterizations of Trichoderma sp. and its effect on Ralstonia solanacearum of tobacco seedlings. *Jurnal Hama dan Penyakit Tumbuhan Tropika*, *21*(1), 8-19. https://doi.org/10.23960/j.hptt.1218-19

Tao, L., Zhang, Y., Li, Y., Luo, L., Zhang, Z., & Chen, J. (2020). Antagonistic activity of volatile metabolites from Trichoderma asperellum. *Chinese Journal of Biotechnology*, *36*(6), 1181-1189.

Tjahjanti, P., Prihatiningrum, A., & Miftahurrohmat, A. (2022). Effect of trichoderma formulated with cultivated oyster mushroom waste toward the growth and yield of shallot (Allium ascalonicum L.). *African Journal of Food, Agriculture, Nutrition and Development*, *22*(10), 21743-21760. https://doi.org/10.18697/ajfand.115.19965

Torres, R. O., Natividad, M. A., Quintana, M. R., & Henry, A. (2020). Ratooning as a management strategy for lodged or drought-damaged rice crops. *Crop Science*, *60*(1), 367-380. https://doi.org/10.1002/csc2.20007

Urfels, A., Mausch, K., Harris, D., McDonald, A. J., Kishore, A., van Halsema, G., . . . Singh, V. (2023). Farm size limits agriculture's poverty reduction potential in Eastern India even with irrigation-led intensification. *Agricultural Systems*, *207*, 103618. https://doi.org/10.1016/j.agsy.2023.103618

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Woo, S. L., Nigro, M., Marra, R., . . . Lanzuise, S. (2014). Trichoderma secondary metabolites active on plants and fungal pathogens. *The Open Mycology Journal*, *8*(1), 127–139. https://doi.org/10.2174/1874437001408010127

Wang, S., Mo, H., Xu, D., Hu, H., Hu, L., Shuai, L., & Li, H. (2021). Determination of volatile organic compounds by HS-GC-IMS to detect different stages of Aspergillus flavus infection in Xiang Ling walnut. *Food Science & Nutrition*, *9*(5), 2703-2712. https://doi.org/10.1002/fsn3.2229

Wang, Y.-F., Hou, X.-Y., Deng, J.-J., Yao, Z.-H., Lyu, M.-M., & Zhang, R.-S. (2020). Auxin response factor 1 acts as a positive regulator in the response of poplar to Trichoderma asperellum inoculation in overexpressing plants. *Plants*, *9*(2), 272. https://doi.org/10.3390/plants9020272

Xu, Y., Liang, L., Wang, B., Xiang, J., Gao, M., Fu, Z., . . . Huang, C. (2022). Conversion from double-season rice to ratoon rice paddy fields reduces carbon footprint and enhances net ecosystem economic benefit. *Science of The Total Environment*, *813*, 152550. https://doi.org/10.1016/j.scitotenv.2021.152550

Yang, D., Peng, S., Zheng, C., Xiong, Z., Yang, G., Deng, S., & Wang, F. (2022). Stubble height affects the grain yield of ratoon rice under rainfed conditions. *Agricultural Water Management*, *272*, 107815. https://doi.org/10.1016/j.agwat.2022.107815

Ye, M., Song, Y. Y., Baerson, S. R., Long, J., Wang, J., Pan, Z., . . . Zeng, R. S. (2017). Ratoon rice generated from primed parent plants exhibit enhanced herbivore resistance. *Plant, Cell & Environment*, *40*(5), 779-787. https://doi.org/10.1111/pce.12897

Yu, X., Yuan, S., Tao, X., Huang, J., Yang, G., Deng, Z., . . . Peng, S. (2021). Comparisons between main and ratoon crops in resource use efficiencies, environmental impacts, and economic profits of rice ratooning system in central China. *Science of the Total Environment*, *799*, 149246. https://doi.org/10.1016/j.scitotenv.2021.149246

Yuan, S., Cassman, K. G., Huang, J., Peng, S., & Grassini, P. (2019). Can ratoon cropping improve resource use efficiencies and profitability of rice in central China? *Field Crops Research*, *234*, 66-72. https://doi.org/10.1016/j.fcr.2019.02.004

Zaidi, S. S.-e.-A., Mukhtar, M. S., & Mansoor, S. (2018). Genome editing: Targeting susceptibility genes for plant disease resistance. *Trends in Biotechnology*, *36*(9), 898-906. https://doi.org/10.1016/j.tibtech.2018.04.005

Zheng, C., Wang, Y., Yang, D., Xiao, S., Sun, Y., Huang, J., . . . Wang, F. (2022). Biomass, radiation use efficiency, and nitrogen utilization of ratoon rice respond to nitrogen management in central China. *Frontiers in Plant Science*, *13*, 889542. https://doi.org/10.3389/fpls.2022.889542

Zhou, S., Tong, Q., Pan, X., Cao, M., Wang, H., Gao, J., & Ou, X. (2021). Research on low-carbon energy transformation of China necessary to achieve the Paris agreement goals: A global perspective. *Energy Economics*, *95*, 105137. https://doi.org/10.1016/j.eneco.2021.105137