Trop J Nat Prod Res, April 2023; 7(4):2790-2795 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Isolation, Molecular Characterization and Application of *Aspergillus niger* and *Penicillium chrysogenum* with Biofertilizer Potentials to Enhance Rice Growth

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ARTICLE INFO

Article history:
Received 01 November 2022
Revised 26 March 2023
Accepted 31 March 2023
Published online 01 May 2023

ABSTRACT

The use of chemical fertilizers has been associated with a persistent decline in soil fertility, which is also detrimental to soil health. Biofertilizers have been reported to be better alternatives to chemical fertilizers. In this study, the mycofertilizer potentials of the species *Aspergillus* and *Penicillium* were investigated. The fungi were isolated from the rice (*Oryza sativa* Linn) rhizosphere and identified using cultural and molecular methods. The fungal isolates were examined for protease synthesis, nitrogen fixation, cellulose breakdown, and phosphate solubilization using standard methods. The mycofertilizer potentials of the isolates were screened for in an *in-situ* experiment that was carried out in the greenhouse using a pot experimental method. Isolates that solubilized phosphate and also produced cellulase and protease were selected for the greenhouse experiment. *Aspergillus niger* and *Penicillium chrysogenum* proved to be the best candidates among the isolates. The results of the greenhouse pot experiment showed that after 30 days of planting, rice (*O. sativa*) in the control group had the best performance, but after 63 days of planting, the rice in the pot inoculated with both *A. niger* and *P. chrysogenum* had the best performance, followed by the plant inoculated with *A. niger*, while the plant in the control group had the least average growth. Plants in the test groups had significant growth compared to the plants in the control group. These isolates could be used in the production of mycofertilizer for the growth of grain crops that are known not to fix nitrogen.

Keywords: Biofertilizer, Fungi, Rice, Rhizosphere, *Aspergillus*, *Penicillium*

Introduction

There are various challenges facing contemporary intensive agricultural practices globally that pose severe risk factors for food security. Chemical pesticides and fertilizers are frequently employed to increase agricultural yields to meet the expanding nutritional demands of the world population, which is anticipated to grow to 9.5 billion by 2050. Two of the most crucial minerals needed for crop yield are phosphorus and nitrogen, and hence they form a major part of chemical fertilizers.[1-3] The challenges of conventional fertilizers include severe environmental pollution and public health hazards.[4] Recent research has focused on developing alternatives to chemical fertilizer application in order to increase plant yield while protecting the soil.[5, 6]

Given its ecologically favourable function in guaranteeing food security and sustainable agro-crop production, the use of advantageous microorganisms as biofertilizers has become a growing topic of research.[7] Since atmospheric nitrogen is typically the main factor limiting plant growth, it has been discovered that microbes can capture it and supply it to the plant directly,[8] as well as help in phosphate solubilization which increases the yield and growth of plants.[9, 10] Plant health and soil fertility are determined by the advantageous plant-microbe interactions in the rhizosphere.[11]

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Citation: David OM., Olawusi, AC., Oluwole OA, Adeola PO, Odeyemi AT. Isolation, Molecular Characterization and Application of *Aspergillus niger* and *Penicillium chrysogenum* with Biofertilizer Potentials to Enhance Rice Growth. Trop J Nat Prod Res. 2023; 7(4):2790-2795 http://www.doi.org/10.26538/tjnpr/v7i4.20

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To regulate the rhizosphere and increase plant productivity, numerous strains of beneficial soil microorganisms have been identified to improve food security and agricultural sustainability.[12]

Fungi are heterotrophic, eukaryotic, mostly multicellular microorganisms that make up the largest biodiversity in the soil.[1] The most common soil fungi are *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp., *Mucor* sp., *Zygorhynchus* sp., and *Chaetomium* sp. *Aspergillus* sp., *Trichoderma* sp., *Verticillium* sp., among the mycorrhiza fungi are perceived to play vital roles as biofertilizers. Fungal biofertilizers (mycofertilizers) have been actively promoted for use in agriculture because of their capacity to boost crop yield and manage plant diseases in an eco-friendly way.[7] In recent years, several fungal biofertilizers have also been produced for application and have been formulated for large-scale crop production.[13] The application of fungal biofertilizers is necessary due to their critical functions in boosting production, encouraging plant growth, enhancing plant health, and improving soil fertility.[14] Additionally, their application to soil strengthens the soil's structure and reduces the proliferation of phytopathogens.[15] Many fungal strains, particularly *Aspergillus niger* and *Penicillium* sp., have been described as phosphate solubilizers; hence, providing plants with mycorrhizal fungi as inoculants that can reduce the amount of phosphate and produce up to 80% of the phosphorus needed by the plants to develop and produce at their best.[16, 17]

Ekiti State is reputable for the cultivated local rice [*Oryza sativa* (Linn)] (Igbemo brown cultivar), although in recent times inorganic fertilizers have to be applied to boost the output of the crop. Consequently, the purpose of this study was to assess the incorporation of fungal isolates as biofertilizers to improve the yield and performance of rice and lessen the negative effects of inorganic fertilizers on the ecosystem and rice consumers.

Materials and Methods

Source of sample collection and study location
Soil samples were collected between June and July, 2019 from rice farmlands in Igbemo-Ekiti and Iworoko-Ekiti, the two towns are known for cultivating and processing local rice in Ekiti State, Nigeria. The soil samples from the rice rhizosphere were put into a sterile polythene bag and transported to the laboratory, where they were processed within two hours of collection. The soil was characterized by wetness, fine texture, and a dark colour. The mycoburden of the soil samples was determined in the Microbiology Laboratory of Ekiti State University, Ado-Ekiti, Nigeria.

Isolation of Fungi from Rice Rhizosphere
Soil samples were serially diluted and plated on Potato Dextrose Agar (PDA) supplemented with chloramphenicol and incubated at 25ºC for 72 h. Colonies observed on the plates after incubation were counted, and thereafter the isolates were subcultured on the freshly prepared agar plates to obtain pure cultures of the isolates. The isolates were subsequently transferred into agar slants, which were kept in the refrigerator at 4ºC as a stock culture for other analyses.

Microscopy
To identify spores and mycelia of the fungal isolates, a 72-hour culture of the fungal isolates was prepared on a sterile, grease-free glass slide. Fungal samples were picked at the growing edge of the plate and placed gently on the centre of the slide containing lactophenol cotton blue. The spores and mycelia morphology of the fungal isolates were examined and identified using the x10 and x40 objectives of a light microscope.

Isolation and screening of fungal isolates for biofertilizer potentials
Screening for phosphate solubilization
To determine the level of phosphate solubilization by the fungi collected from the soil samples' rhizosphere, a modified method described by Elias *et al.*[18] was adopted. The organisms were plated on Pikovskaya's (PKV) agar medium, containing (agar) (gL^{-1}: glucose (10); $MgSO_4 \cdot 7H_2O$ (0.1); KCl (0.2) and $(NH_4)_2SO_4$ (0.5); Yeast extract (0.5); $Ca_3(PO_4)_2$ (5.0); $MnSO_4 . H_2O$ (0.002); $FeSO_4 . 7H_2O$ (0.002); Agar (10).[18] The Pikovskaya agar growth media were inoculated in a circular pattern in triplicate, and a sterile Potato Dextrose Agar was used as a control. The plates were then incubated at temperatures of 25ºC–28ºC for 7 days. Phosphate solubilizing fungi (PSF) were identified by the clear halo zones that formed around their colonies on the Pikovskaya growth media. The solubilization index was obtained by adding the diameter of the colony (mm) with the diameter of the halo (mm), and the resultant value was divided by the colony diameter (mm). The experiment was performed in triplicates, and colonies with better solubilizing potential were used for further studies.

Solubilization index = (Colony diameter + halo zone)/Colony diameter

Screening for cellulase production
Colonies of PSF isolates were cultivated on Casein Yeast Extract agar (containing g/L: casein (5.0); yeast extract (2.5); glucose (1.0); agar (15.0) dissolved in distilled water) amended with 1 % carboxymethyl cellulose.[19, 20] The cultured plates were incubated at 28°C for 7 days, after which the cultured agar was flooded with an aqueous solution of Congo red (1 mg/ml) and allowed to stand for 15 minutes. Clear halo zones around fungal colonies indicate cellulose production by PSB isolates.

Screening for protease production
The proteolytic activity of each of the PSF isolates was determined by the method of Kumar *et al.*[21] PSF was streaked onto casein yeast extract agar amended with 7% skim milk powder. The plates were incubated for 7 days at 28°C. The presence of a clear halo zone around the streak line of incubation was taken as a sign of protease production.

Screening for nitrogen fixation ability
Burk's Nitrogen-free Medium (comprising gL^{-1}: glucose (10.0); KH_2PO_4 (0.41); K_2HPO_4 (0.52); Na_2SO_4 (0.05); $CaCl_2$ (0.2); $MgSO_4.7H_2O$ (0.1); $FeSO_4.7H_2O$ (0.005); $Na_2MoO_4.2H_2O$ (0.0025); and agar (15.0); pH: 7±1) described by Park *et al*.[22] was adopted in this study to screen for the isolates with nitrogen-fixing potential among PSF isolates. The pH of the media was adjusted to 7±1 before autoclaving at 121ºC for 15 min after which the media were inoculated with test fungus and the plates were incubated at 28ºC for 7 days. Only the PSF isolates with the potential for nitrogen fixation were able to grow after 120 hours of incubation.

Molecular characterization of selected fungal isolates
The method described by Choudhary[23] was adopted for the identification of the isolated fungi. The DNA of each of the fungi was extracted by picking from the advanced edge of 5 to 7 days old fungal cultures grown on a PDA medium. The DNA was extracted and the amplification of the DNA was done by using primers ITS4 (R-5'TCCTCCGCTTATTGATATGC3') and ITS5 (F-5'GGAAGTAAAAGTCGTAACAAGG3') after which the and the products were purified and sequenced. The resulting sequences were compared to existing NCBI databases using BLAST analysis software (https://blast.ncbi.nlm.nih.gov) and the phylogenetic tree was constructed using MEGA 7.0 software.

Soil collection and treatment for in situ experiment
Soil samples were collected from the Faculty of Agricultural Sciences Farmland, Ekiti State University, Ado-Ekiti, Nigeria, at a depth of 5–15 cm. The soil showed physicochemical characteristics of silty clay loam and a pH of 6.28. The soil sample was air-dried and later sieved with an 8 mm sieve. The soil was treated at 75℃ for 30 minutes to get rid of potential pathogens. The temperature was not raised any further to avoid mineral binding in the soil and rendering them unavailable to the plant.

Source and treatment of rice seeds
Seeds of the Igbemo brown rice cultivar were collected from an approved source in Igbemo-Ekiti and authenticated at the Plant Science and Biotechnology Department, Ekiti State University, Ado-Ekiti, Nigeria. The rice seeds were suspended in water, and the floated seeds were collected and discarded, while those that sank were thoroughly washed, drained, and air dried immediately. About 25 grains were soaked in fresh water for 24–48 hours, drained, and placed on moistened filter paper in Petri dishes. They were placed in the dark and observed to germinate between 4–6 days. The number of germinated seeds was recorded, and the percentage viability was calculated.

Planting of seeds in the treated soil samples
A wet application method was used to challenge the soil samples before the seeds were planted. Five litres of water were measured into a container, and 5 g of the dislodged fungal mat were mixed thoroughly. This was evenly applied to the soil samples, and the seeds were sown immediately. The research work had four treatment groups, with each group containing four replicas:

Soil sample challenged with *A. niger* AY028 only
Soil sample challenged with *P. chrysogenum* AY005 only
Soil sample challenged with both *A. niger* AY028, and *P. chrysogenum* AY005
Soil sample without any fungal culture (control).

To maintain the water level needed for rice plants, the pots were thereafter placed inside the greenhouse and frequently irrigated with water. The height of the plant was measured at intervals and recorded.
Statistical analysis of data
Statistical analysis was performed using GraphPad Prism 7.4 software for mean comparison using Dunnett's multiple comparisons test and the degree of significance was fixed at 5%.

Results and Discussion

A total of 10 isolates of *Aspergillus* species and *Penicillium* species were obtained from different rice rhizospheres (n=6) from the two locations. The morphology of the isolated fungal species is presented in Table 1. The characteristic features of the isolates, including shape,

pigmentation, and reverse view on the plates, along with their cultural appearance on potato dextrose agar, were observed. Among the soil samples used in this investigation that contained isolates from the rhizosphere of rice plants, six (6) species were culturally identified to belong to the genus *Aspergillus* while four belong to the genus *Penicillium*. The molecular characterization of the isolates confirmed the identities as *Aspergillus niger, Aspergillus flavus,* and *Penicillium chrysogenum*.

Most of the plant growth-enhancing fungi isolated belong to the genus *Aspergillus*. This might be a result of how frequently *Aspergillus* species are found in the roots of crop plants.[24] Plant growth and health are significantly influenced by soil microbes.[25] Fungal species are increasingly finding useful applications in improving soil quality.[26, 27] In this study, we evaluated the plant growth and growth-promoting traits of fungi isolated from the rhizosphere of *Oryza sativa.*

Table 2 shows the ability of the isolates to produce cellulases, proteases, solubilize phosphate, and fix nitrogen. *Penicillium chrysogenum* AY005 was observed to be positive for the four qualitative screening tests, while *A. niger* AY028 was the most effective phosphate solubilizer on Pikovsaya agar medium with a solubilization index of 1.50. Also, *A. flavus* AY026 was negative for nitrogen fixation but positive for phosphate solubilization, cellulase production, and protease production; however, *P. chrysogenum* AY010, was negative for all the tests.

Biofertilizers are live organisms that can fix nitrogen, breakdown cellulose and are also capable of phosphate solubilization.[28] In this study, *A. niger* AY028 and *P. chrysogenum* AY005 were found to be positive for these tests and may be capable of being used as biofertilizers in place of traditional fertilizers. Several studies have investigated and suggested the application of these fungi species for plant growth promotion.[29,30,31,18] Fungi species can enhance growth promotion in plants through beneficial mycorrhizal associations. According to earlier research by Yadav *et al.*[32] the varying potential for phosphate solubilization as determined by the solubilization index on an agar plate in the current investigation may be caused by the different types, quantities, and diffusion rates of unique organic acids produced by fungal isolates. In the report by Iman,[33] *Aspergillus niger* and *P. chrysogenum* produced phosphate solubilization indices (SI) of 2.42 and 3.15 respectively. The values were higher than those reported in this study. Alam *et al.*[34] discovered that solubilization indices for the fungi isolated from the maize rhizosphere ranged from 1.53 to 1.80.

The seeds used for this work are of good quality, with a percentage viability of 96.0%. The effect treating of the rice plant with test fungi with biofertilizer potential was evaluated in a pot experiment and monitored for up to 63 days of cultivation. The observations are shown in Figure 2 for the height of the plant, which was measured at regular intervals. The greenhouse pot experiment showed some significant improvements in the growth of the plants after inoculation with *A. niger* AY028 compared with control. This finding was supported by Mundim *et al.*[35] who reported a strong impact of *A. niger* inoculation on the seedlings when comparing their height and shoot mass with those that were not inoculated. Once the fungus has colonized the seedling rhizosphere, it may have an advantage over the native microbiota, resulting in growth enhancement that lasts throughout the crop cycle.

Araujo *et al*.[36] discovered that inoculating coffee seedlings with *A. niger* increased stem height by 6.5% before transplantation compared to the uninoculated control, demonstrating the potential of *A. niger* for plant growth promotion. At day 30, among the treatment groups, plants in group AN+PC showed the best quality in terms of plant height, followed by those in group PC. The control group had the least height.

Table 1: Morphological and molecular identities of fungal isolates from the rhizosphere of rice soil samples in Igbemo-Ekiti

Macroscopic features	Microscopic features	Identitity using molecular characterization
Greenish to black mycelium and a brown colour on the reverse	Septate hyphae with a smooth, colourless and sometimes brown conidiophores and spores	*Aspergillus niger*
Yellow to green mycelium and a red-brown colour	Aerial hyphae bearing conidiophores	*Aspergillus flavus*
Greenish colour appearing like patches with an orange back view	Hyaline and septate hyphae with radiated conidiophores	*Aspergillus niger*
Greenish mycelium with pale brown pigment on the reverse	Branched and conidiophore with a small hyhae	*Penicillium chrysogenum*
Greenish yellow mycelium forming a circular ring	Presence of brush-shaped conidiophores with dry spores	*Penicillium chrysogenum*
Brownish black mycelium with dark patches with white rings at the tip. Pale yellow on the reverse	Presence of septate hypae with rough conidiophores	*Aspergillus niger*

Table 2: Qualitative screening fungal of isolates for biofertilizer potentials

Isolates	Tests			
	Nitrogen fixation	Phosphate solubilization (index mM)	Cellulase production (mM)	Protease production (mM)
A. flavus AY017	-	+ (1.21)	+ (1.02)	+ (1.43)
A. niger AY011	+	+ (1.40)	+ (0.95)	-
A. niger AY028	+	+ (1.50)	+ (0.89)	+ (1.26)
A. niger AY021	-	+ (1.29)	+ (1.23)	-
F. oxysporum AY018	+	+ (1.21)	+ (2.01)	-
P. chrysogenum AY010	-	-	-	-
P. chrysogenum AY005	+	+ (1.21)	+ (0.32)	+ (1.01)

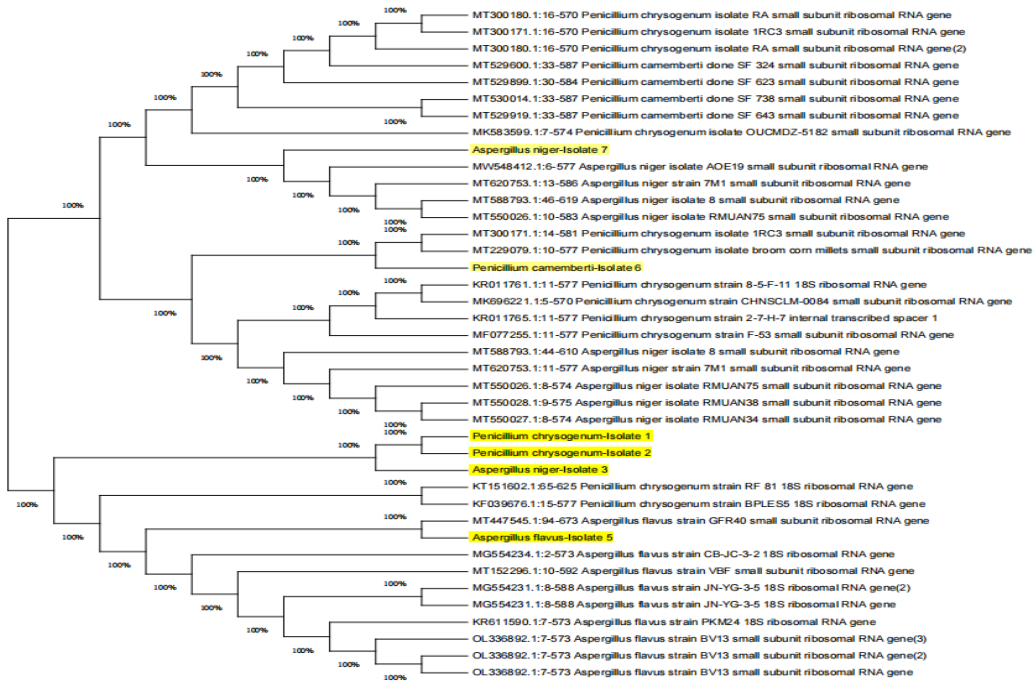

Figure 1: The pylogenetic tree of the fungi isolated from the rhizosphere of rice with biofertilizer attributes. The isolates highlighted are those isolated in this study.

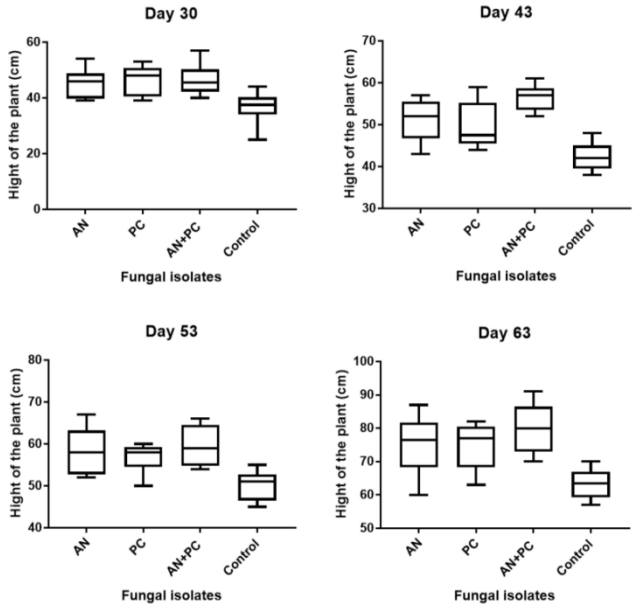

Figure 2: Heights of rice plant inoculated with different potential mycofertilizer fungal culture. AN=Soil sample inoculated with *A. niger* 1a, PC=Soil sample inoculated with *P. chrysogenum* 9a, AN+PC=Soil sample inoculated with both *A. niger* and *P. chrysogenum*, the control group was not inoculated with any fungus

There was an improvement in the height of the plants in only 53 of the 54 treatment groups; however, the performance of the plants in group AN+PC was the best. The height of the plants at the end of the experiment showed the treatment groups to be taller and of better appearance than those in the control group, as shown in Figure 1. On day 30 of the planting experiment, there was a significant difference in the height of the rice plant in groups PC and AN+PC compared to the control. Day 63 revealed a significant difference between the treatment and control groups. The performance of the plant grown in soil samples inoculated with different fungi is shown in Plate 1. On day 30, there was a significant difference in the height of rice in PC and AN+PC compared to the control. At day 53, there was a significant difference between the treatment group and the control group (p 0.05).

There was massive fruiting of the rice plant as well as an increase in growth that was almost twice as much as it had grown. According to the experiment conducted by Chezang and Chhogyel,[37] there was a difference in plant height (cm) between the planting techniques (broadcasting and drum seeding) due to increased crop competition in both direct-seeded procedures. Based on the results so far, *A. niger* AY028 has proven, without further doubt, to obtain the highest average value of plant height than *P. chrysogenum* AY005 after 63 days of planting. Wang *et al*.[38] showed that the biofertilizer potential of A. niger is tied to its high ability to solubilize phosphate, carry out potassium solubilization, express cellulase and hemicellulase in the environment, and inhibit pathogens in the crop rhizosphere and rhizoplane. *A. niger* AY028 dissolved more phosphate than *P. chrysogenum* AY005.According to Subowo,[39] perhaps this was due to the ability of *A. niger* to dissolve more phosphate than *P. chrysogenum.* A. niger and *Penicillium* sp. were found to be capable of dissolving tricalcium phosphate (TCP) in an experiment conducted by Pradhan and Sukla.[40] The samples that were inoculated with *A. niger* AY028 together with *P. chrysogenum* AY005 performed better than the group that was inoculated singly.

Conclusion

In conclusion, the *in vitro* and *in situ* experiments in this study showed that the fungal isolates used in this study could have beneficial impacts on plants. They could improve soil fertility and health, as well as plant growth and yield. This study further demonstrated the possibility of employing fungal candidates as biofertilizers; however, more studies are needed to further verify these claims.

Conflict of Interest

The authors declare no conflict of interest.

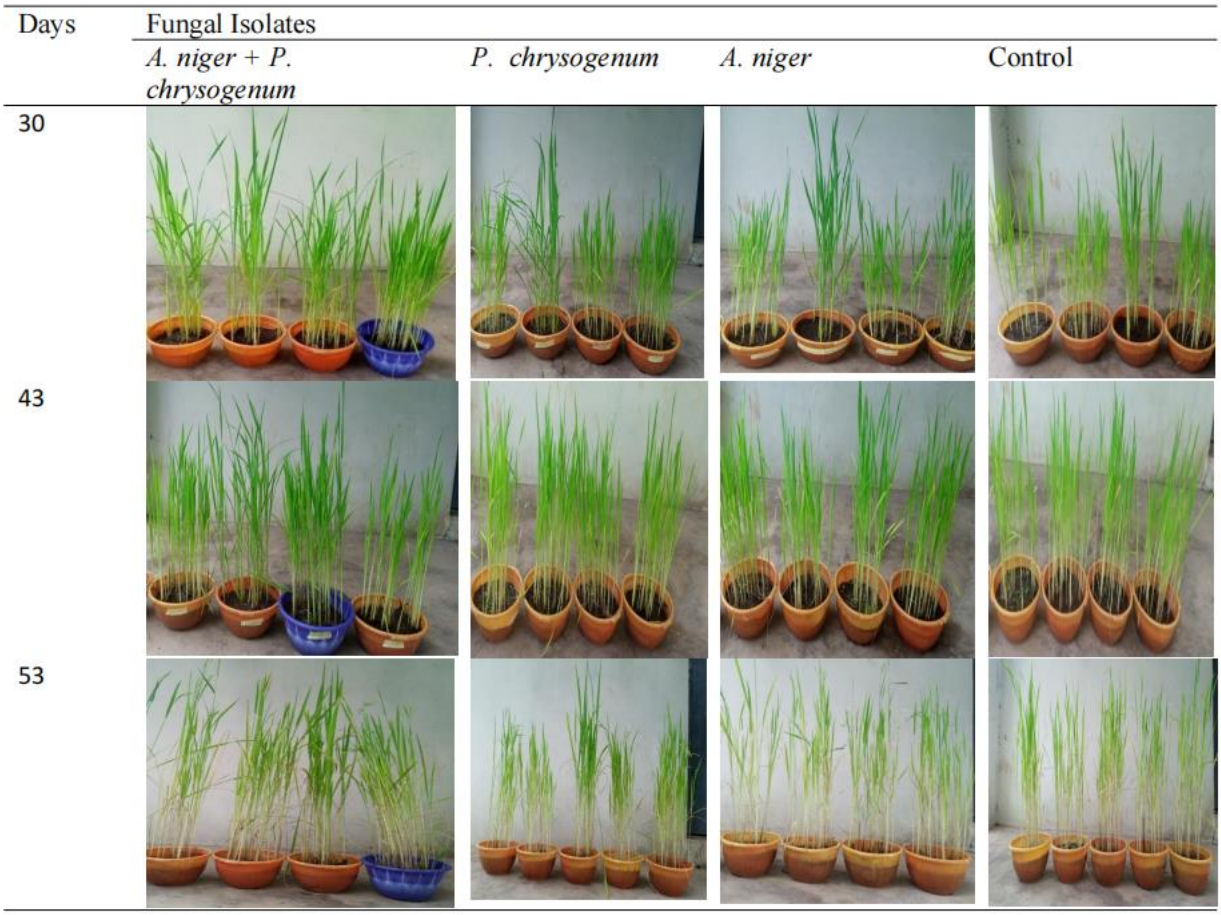

Plate 1: The performance of the rice grow in soil inoculated with different fungal inoculants at different days of growth experiment

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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