

Isolation and identification of soil fungi isolates from forest soil for flooded soil recovery

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Abstract. Soil fungi have been evaluated for their ability in increasing and recovering nitrogen, phosphorus and potassium content in flooded soil and in promoting the growth of the host plant. Host plant was cultivated in a mixture of fertile forest soil (nutrient-rich soil) and simulated flooded soil (nutrient-poor soil) in an optimized soil condition for two weeks. The soil sample was harvested every day until two weeks of planting and was tested for nitrogen, phosphorus and potassium concentration. Soil fungi were isolated by using dilution plating technique and was identified by Biolog's Microbial Systems. The concentration of nitrogen, phosphorus, and potassium was found to be increasing after two weeks by two to three times approximately from the initial concentration recorded. Two fungi species were identified with probability more than 90% namely *Aspergillus aculeatus* and *Paecilomyces lilacinus*. Both identified fungi were found to be beneficial in enhancing plant growth and increasing the availability of nutrient content in the soil and thus recovering the nutrient content in the flooded soil.

1. Introduction

Flooding is a natural disturbance which occurs almost everywhere worldwide. Flooding disturbs crop and forages production globally due to the detrimental effects that it provokes on most terrestrial plants [1]. The frequency of flooding events was predicted to be increasing globally by climate change models, making flooding a major environmental threat for plants [2]. Crop damages due to unseasonal and severe flooding events amount to a huge extent in yield losses. Despite the variety of plant tolerant to flooding, there is significant variation in the vulnerability of most crops to wet conditions. Flooding can damage plants by several means. Excessive moisture in soil decreases oxygen levels as plant growth under flooding condition is trying to sustain aerobic respiration of submerged [3]. The inhibition of respiration leads to the build-up of toxic compounds which ultimately suffocate and damage plant [4].

Flooding may also affect nutrient content in the soil. Plants will suffer from nutrient insufficiencies as a result of reduced uptake of nutrients, denitrification, and leaching of mobile nutrients and also ions dilutions in flooded soil [5]. Prolonged waterlogging decreases the concentration of nitrogen, phosphorus and potassium in the soil. Nitrogen appears to have been denitrified and lost from the system [6]. Since phosphorus uptake depends on microbes in most plant, prolonged waterlogging has reduced microbial activity which in turn affects the absorption of phosphorus into the plant roots [7]. Furthermore, soil potassium is less available in soils that remain wet since wet soils are more prone to compaction, which restricts plant root growth and uptake of soil potassium [8].



Application of fungi in increasing soil nutrient content has been widely discussed by many authors. Fungi strongly influence ecosystem structure and functioning and therefore plays a significant role in many ecological services [9]. Soil fungi perform crucial roles within the soil with respect to the cycling of nutrients, disease suppression and water dynamics where all of which help plants become healthier and more vigorous [10]. The use of fungi in recovering nutrient content is currently a favorable method for many agricultural practices over the chemical fertilizers. Since the use of chemical fertilizers and pesticides has created the problem of environmental pollution [11], the use of fungi in agricultural practices seems to be a promising technique to farmers as this technique are an environmentally friendly and are cost-effective. In addition, over application of chemical fertilizer resulting in reduced colonization of plant roots with symbiotic fungi thus reducing the beneficial effect of soil fungi.

In recent years, application of microorganism as biofertilizer has arisen as a significant component of the integrated nutrient supply system and hold a pronounced potential to recover crop productions through environmentally improved nutrient supplies [12]. However, the application in practice, in some way, has not accomplished persistent outcome. The mechanisms and interactions among these microbes still are not well understood, especially in real applications. Hence this study aims to isolate and identify the soil fungi and to evaluate the effect of inoculating fertile soil containing naturally occurring soil fungi and infertile flooded soil with the aim to naturally recover the nutrient in that particular soil.

2. Materials and Method

2.1. Soil collection and preparation

A pot experiment was carried out to determine the effect of naturally occurring soil fungi which were commonly found in the fertile soil when inoculated with infertile flooded soil in recovering the nutrient in the soil. The fertile soil was collected at a secondary forest zone located in Universiti Malaysia Pahang (UMP), Kuantan while the simulated flooded soil was prepared under greenhouse by submerging the collected fertile soil in the water for two weeks [13]. The water was then removed from the soil. The ability of soil fungi in recovering the nutrient in flooded soil was evaluated by the help of onion as a host plant and by analyzing the nutrient content in the soil every day until two weeks of planting.

Five factors were previously selected in a preliminary study namely pH of the soil, soil water content, ratio of fertile to flooded soil, light supply and depth of soil in order to determine the most contributing factor for flooded soil recovery by using the concept of two levels factorial via Design Expert software (Ver 71.6). The analyzed data showed that water content, light supply and depth of soil were the important contributing factors for nutrient recovery in flooded soil. The best condition for flooded soil recovery was determined at pH4, with 28mL water content, 1:5 ratio of fertile forest soil to simulated flooded soil, depth of soil at 5 cm and with the presence of light.

The onion was planted in an optimized soil condition in five pot of 650 g mixed soil with 1:5 ratio of fertile forest soil to the simulated flooded soil each with 28 mL water content, at pH4 and under the presence of light. Onion bulb was planted in 5 cm deep. The soil was harvested for fungi isolation and nutrient testing every day until two weeks of planting and the initial concentration of each nutrient was recorded prior to experimentation.

2.2 Soil sample analysis

The soil was tested for nitrogen, phosphorus and potassium content by using Hach Spectrophotometer. The soil sample was crushed by mortar and pestle first to get the fine structure of the soil. The soil then was diluted with distilled water by two dilution factor for nitrogen and phosphorus testing and three dilution factor for potassium testing. The diluted soil then was filtered by Whatman filter paper to get the clear soil solution before been tested for the presence of nitrogen, phosphorus and potassium. Both nitrogen and phosphorus samples were digested first with DRB 200 reactor before been tested with Hach. Nitrogen was analysed by using persulfate digestion method whereas phosphorus was tested by molybdovanadate method. Meanwhile, potassium was analysed by using tetraphenylborate method. The procedures for each method were referred from DR2800 Hach Spectrophotometer manual.

2.3 Isolation of fungi

The isolation of fungi from soil was carried out in order to identify each fungus up to species level. The serial dilution plating method was used to dilute the soil sample as described by [14] with the purpose of minimizing the fungi in the soil in each dilution. The dilution of soil sample was conducted in two replicate where each replicate was diluted six times and labelled as 10-1 until 10-6. 50 g of soil was added in 100 mL 85% NaCl solution and was thoroughly shake to mix the solution. The solution then was diluted to a series of prepared vials containing 9 mL 85% NaCl solution. 9 mL of the soil-NaCl solution was transferred to the first vial by using a pipette. Subsequently, another 9 mL of the solution from the first vials was transferred to the second vial and the steps continued until the last vial. 0.1 mL of the solution in each vial was pipetted into the prepared PDA plate. The solution was then spread on the plate by using a hockey stick and incubated at room temperature for seven days.

The colony of fungi that appeared in the plate after incubation was then isolated in a new plate. A pure culture of each colony type on each plate was obtained and maintained. The maintenance was done by sub-culturing each of the different colonies onto another new PDA plate. Each colony were cut into 3 mm pieces with a sterilized razor blade, surface sterilized in 70 % ethanol. The colonies were incubated again at room temperature for five days. Each plate of single fungi colony was then sent to UMP Central Laboratory for identification purposes.

2.4 Identification of fungi

Ten pure sample culture was sent to UMP Central Laboratory for identification purpose. The identification was done by Biolog Microbial Identification System as described by Dobranic and Zak [15]. Biolog Microplate tests the ability of a microorganism to assimilate or oxidize compounds from a preselected panel of different carbon sources. The test yields a characteristics pattern of reddish-orange wells and turbidity changes, which constitutes a Metabolic Fingerprint. The Microplate test panel provides a standardized micro method using 95 biochemical tests to identify a broad range of fungi including both filamentous and yeast form.

The identification result by Biolog's Microbial was performed by reading and comparing between plate's phenotypic fingerprint and Biolog's Microbial Identification Systems Database. The result will show no ID if the plate's phenotypic fingerprint produced not matched to the database more than 50%. The no ID result may be due to the isolated fungi was possibly a new species as the profile cannot be found in the database, thus a larger database is needed for further identification by using the molecular technique.

3. Results and discussion

3.1 Nutrient concentration

The average concentration of nitrogen, phosphorus and potassium from the harvested soil sample in five pots was 10.7, 6.5 and 15.2 mg/L respectively. The concentration of all nutrients increased approximately by two to three times from the recorded initial nutrient concentration. The concentration of nutrients shows an increasing trend from day zero until day fourteen. Figure 1 demonstrates the trends of all nutrient from day zero until day fourteen. This finding suggests that application of soil fungi to recover the infertile flooded soil can be implemented for soil nutrient recovery.

A similar observation has also been recorded by Wu et al. [16], who studied the effects of microorganisms containing nitrogen-fixer, phosphorus and potassium solubilizers and arbuscular mycorrhizal fungi on the growth of maize. Inoculation of soil with microorganisms resulted in an increase in phosphorus and potassium uptake to different degrees when compared with the control. Higher nutrient concentration was recorded in pots inoculated with the combination of nitrogen-fixer, phosphorus and potassium solubilizers and arbuscular mycorrhizal fungi.

Meanwhile, according to Rashid et al. [17], co-inoculation of bacteria and fungi with or without organic fertilizer are the most beneficial for restoring the soil fertility and organic matter content rather

than single inoculum. Bacterial and fungal inocula have a potential to restore the fertility of degraded land through various processes. These microorganisms increase the nutrient bioavailability through nitrogen fixation and mobilization of key nutrients (phosphorus, potassium and iron) to the crop plants while remediating soil structure by improving its aggregation and stability.

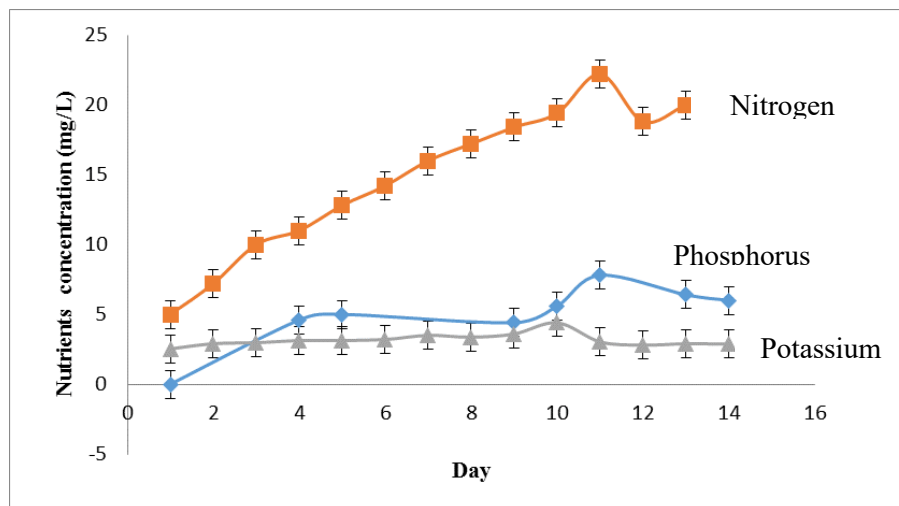


Figure 1. Nutrient profile for nitrogen, phosphorus and potassium

3.2 Identification of fungi

Every single colony of different shapes and colours appeared on the PDA plate after the first incubation was subcultured and maintained on another new PDA plate. Nine single colonies were successfully isolated on a new plate and were presented in Figure 2. The plate was labelled as Colony 1 until Colony 9. Of nine sample plate sent for identification, only five plates have been identified with probability more than 90%. Meanwhile, another five plate shows no ID result. The isolates were identified by Biolog's Microbial identification up until genus level. Only two species were identified, namely *Paecilomyces lilacinus* and *Aspergillus aculeatus*. The result shows that *Aspergillus aculeatus* was the dominant species found in the soil sample.

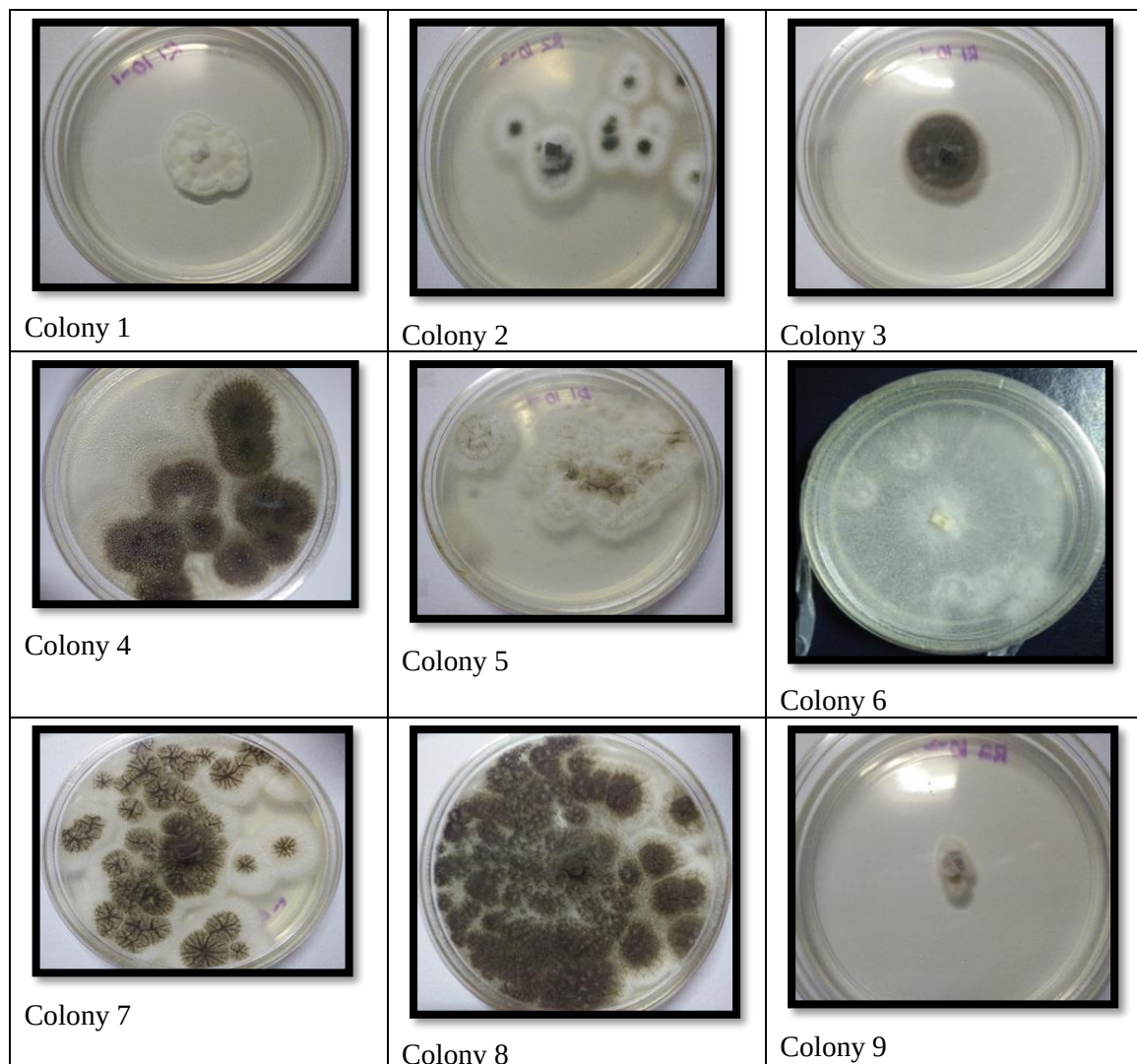


Figure 2. Single isolated colony of fungi in PDA plate.

A. aculeatus was classified under the phylum Ascomycota, Trichomaceae family and under the genus of *Aspergillus*. *A. aculeatus* is a ubiquitous species that is commonly isolated from soil and rotting fruit. Due to its ability to degrade plant cell walls rapidly, *A. aculeatus* isolates have been used to produce a number of important industrial enzymes such as cellulases, hemicellulases, and proteases that are used commercially in the food and feed industries. The biochemical and catalytic properties of several extracellular glycosyl hydrolases from *A. aculeatus* have been studied in detail by numerous researchers. In addition, several *A. aculeatus* polysaccharide degrading enzymes have been studied at the structural level by X-ray crystallography.

Paecilomyces lilacinus was categorized under the phylum Ascomycota, Trichomaceae family and under the genus of *Paecilomyces*. *P. lilacinus* was originally described by the taxonomist in the early 19th under the name *Penicillium lilacinum*. Another taxonomist then transferred the species to *Paecilomyces* in 1974. Until now *P. lilacinus* was declared as synonyms to *P. lilacinum*. *P. lilacinus* is a saprophytic soil fungus and can be found in a wide range of habitats. It has a high frequency of occurrence in the tropics and sub tropic and can be found in most of the agricultural soils.

P. lilacinus has drawn many research attentions due to its promising effect in parasitizing and controlling the population of nematodes. *P. lilacinus* is one the most widely tested biological control

agent for management of plant-parasitic nematodes. The use of *P. lilacinus* as a biological control agent of plant-parasitic nematodes has been discussed and described by many authors. Those include as early as reported by Dube and Smart Jr [18], Kiewnick and Sikora [19] to the latest as described by Wagh and Pramanik [20]. All researchers showed successful nematode control by *P. lilacinus* and thus can act as potential bio control agent of a nematode.

Phosphorus (P) and potassium (K) were both found abundant in soil, however, they exist in the form that is unavailable for the plant to absorb. Plants can only absorb inorganic phosphorus [21], thus the mineralization of organic phosphorus is an important soil process since it results in the release of inorganic phosphorus to the soil solution. Mineralization is facilitated by soil microorganisms, and almost half of the microorganisms present in soil and on plant roots possess the ability to mineralize organic phosphate [22]. Many soil fungi, predominantly of the genera *Aspergillus* and *Penicillium*, have been shown to possess the ability to bring insoluble phosphates in soil into a soluble form by secreting organic acids [23]. These acids lower the pH and bring about the dissolution of bound forms of phosphates. Growth promotion and increased uptake of phosphorus by plants inoculated with P-solubilizing fungi have been reported by many investigators.

Chhonkar and Subba Rao [24] evaluated the phosphate-solubilizing ability of different isolates of fungi associated with legume root nodules under in vitro conditions. Among the fungi tested, isolates of *Penicillium lilacinum*, *Aspergillus* sp., *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans* solubilized insoluble tricalcium phosphate. Several other studies have described the ability of *A. aculeatus* in solubilizing phosphate and potassium in the soil. Narshian and Patel [25] used *A. aculeatus* isolates to test the availability of named fungi in solubilizing rock phosphate and found out that *A. aculeatus* was capable to solubilize all natural form of phosphorus tested. The study of mineralization of organic phosphates via *A. aculeatus* by Narshian and Patel [26] resulting in high acid phosphatase activity in Pikovskaya's broth which indicates that high percentage of phosphorus being released. In addition, *A. niger* has been formerly studied by Cerezine et al. [27] followed by Caravaca et al. [28] as rock phosphate solubilizer. Kang et al. [29] reported that a significant amount of insoluble rock phosphate was solubilized by *Aspergillus* sp as evident by increased soluble phosphorus concentration. This followed by Singh et al. [30] who also tested and prove the ability of two *A. niger* strains as good phosphate solubilizer. Microbial rock potassium solubilization was first discovered by Muentz [31]. Prajapati [32-33] characterized both *A. niger* and *A. terreus* as potassium solubilizing fungi. Inoculation of the isolates with soil treated with insoluble potassium showed a significant increase in the concentration of potassium.

Penicillium pinophilum have been used by Fan et al. [34] in evaluating the ability of isolates to improve the growth of the strawberry plant. *P. pinophilum* formed arbuscular mycorrhizae with the roots of strawberry and the interaction not only improve the plant growth but also enhance the nutrient uptake as well as the rate of photosynthesis of the plant. The inoculation has increased the nitrogen and phosphorus content and also shorten the blossom and ripening period of strawberry.

Nitrogen availability in almost all ecosystems, including agricultural conditions, is a limiting factor that limits primary productivity and nitrogen cycling [35]. A plant needs a very large amount of nitrogen for photosynthesis hence the rate of photosynthesis can be directly linked to nitrogen availability [36]. Plants can absorb nitrogen in the form of inorganic nitrogenous compounds. Fixed nitrogen from the atmosphere in terrestrial systems may be bound in organic matter and must be subsequently mineralized to releases inorganic nitrogen compound that can be used by plants [37]. Nitrogen conversion is largely mediated by fungal decomposer in the soil [38].

Several soil fungi are able to exploit the energy stored in the redox potential of organic nitrogen [39]. The exo enzymes for example proteases and peptidase released by some fungi can break down organic matter and subsequently capture nitrogen-containing compounds, thus providing a direct path from organically bound nitrogen in the soil to the plant [40]. Protease has been reported to be found in several *Aspergillus* species. Olutiola and Nwaogwugwu [41] reported that *A. aculeatus* is able to produce protease with optimum activity expressed at pH 7. Several other *Aspergillus* species that can produce

protease are as described by Ruann Janser and Harumi Sato [42] in *A. oryzae* and by Farnell et al. [43] in *A. fumigatus*.

4. Conclusions

Depending on the type of interaction between fungi and plant, fungi have been proven to have a beneficial effect to plant. Inoculating fertile soil containing microorganisms with flooded soil has significantly increased the nutrient content in the soil. The current study shows an increase of nutrient concentration by two to three times from recorded initial nutrient concentration which suggests that applying fungi in restoring infertile flooded soil can be employed successfully. The application of fungi in agricultural practices should be implemented and replaced the conventional chemical fertilization method. *A. aculeatus* was found to be beneficial in increasing the bioavailability of phosphorus content in the soil by its potential in the solubilizing insoluble form of phosphorus to a form that plant can uptake. Meanwhile, *P. lilacinus* was found to be a very good biological agent for controlling nematodes.

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