Jurnal Biota Prodi Biologi Universitas Islam Negeri Raden Fatah Palembang

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To cite this article before publication: Istifadah, N., Fitria. S.A. (2024). Endophytic Fungi in Cabbage Roots: Diversity and Antagonistic effects on Rhizoctonia solani. Jurnal Biota. In press
http://jurnal.radenfatah.ac.id/index.php/biota/article/view/20134

Endophytic Fungi in Cabbage Roots: Diversity and Antagonistic effects on *Rhizoctonia solani*

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Abstract

Root endophytic fungi that living inside the plant roots without causing any symptom, basically is part of microorganisms in the rhizosphere or soil. Considering that, the objective of this study was to examine the effect of growth media on the occurrence and variabilities of culturable endophytic fungi in cabbage roots. The growth media examined were soil from pine forest, rhizosphere of cogon grass and elephant grass, inceptisol soil mixed with goat manure, compost or vermicompost (1:1, v/v). Fungal isolates obtained were examined their effect on the growth of cabbage seedlings and their abilities to inhibit the growth of fungal pathogen *Rhizoctonia solani* in vitro. The results showed that the growth media influenced the colonization and variabilities of fungal endophytes isolated from cabbage roots. The media supporting better colonization and variabilities of fungal endophytes was soil mixed with goat manure (1:1, v/v). Among 12 isolates obtained, three isolates (PK-2, PK-4 and PK-5 isolates) tended to improve the growth of cabbage seedlings. There were also three isolates (PK-1, PK-2 and TH-1) inhibited the growth of *R. solani* in vitro by 56.7% -64.7%.

Keywords: *Colonization frequency; Goat manure; Grass rhizosphere; in Vitro*

Introduction

Endophytic fungi are fungi that partially or completely exist in plant tissues without causing symptoms (Hardoim et al., 2015). Endophytic fungi can be found in various plants studied, however, the population and type depend on the host plant and the environment including the habitat where it grows (Chen et al., 2020). Within one plant, the existence and diversity of endophytic fungi is also influenced by the parts or organs of the plant. Compared

to the leaves, the frequency of colonization of root endophytic fungi is relatively more intensive (Qian et al., 2019).

The effect of endophytic fungi on their hosts varies. The presence of endophytic fungi may have no effect on the host plant (neutral) (Hardoim et al., 2015). There are also fungi that are isolated from healthy plant tissues, but they are actually latent pathogens which can be pathogenic if the environment is conducive (Hardoim et al., 2015; Sun & Guo, 2012). However, there are many endophytic fungi that are beneficial for plants because they can promote plant growth (El Mansy et al., 2020; Rigobelo & Baron, 2021) or protect plants from pests and plant pathogens (Busby et al., 2015; Lacava & Azevedo, 2014; Latz et al., 2018).

The abilities of endophytic fungi to inhibit the growth of pathogens *in vitro* as well as plant diseases have been reported (Busby et al., 2015; Latz et al., 2018). Inhibition of pathogens or plant diseases by endophytic fungi can be through several mechanisms either directly or indirectly. The endophytic fungi can directly inhibit the pathogen through production of secondary metabolites that are toxic to the pathogens, parasitism, or through competition for nutrients and space. Endophytic fungi can also suppress the disease indirectly through induction of plant resistance (Latz et al., 2018; Baron et al., 2022).

Root endophytes are essentially part of the rhizosphere or soil microorganism community (Afzal et al., 2019; Qian et al., 2019). Fungal community in bulk soil is usually species rich and more diverse than rhizosphere, but only some of the rhizosphere fungal community that are able to colonize root tissue as endophytes (Urbina et al., 2018). Qian et al. (2019) also reported that endophytic fungal communities in roots have similarities with fungal communities in the rhizosphere rather than endophytic fungi on leaves. Root endophytic fungi can be obtained by baiting method in which the annual plants such as Chinese cabbage on particular soils (Diene et al., 2013). The objective of the study discussed in this paper was to examine influence of growing media such as rhizosphere of several grasses as well as soil that are rich in organic matters on the presence and variability of endophytic fungi isolated from cabbage roots.

The fungi isolated as endophytes may have neutral, detrimental or beneficial effects. Therefore, the effects of endophytic fungal isolates on the cabbage plant and their potential to inhibit one of the cabbage pathogens, *Rhizoctonia solani* were also being evaluated. Fungal pathogen, *R. solani*, causes damping off disease of seedling in various plants including cabbage. In cruciferae plants, the pathogen can also cause stem base rot and crop rot (Erper et al., 2021). In addition to its wide range of the hosts, *R. solani* is also difficult to control due to their rapid growth and its abilities to produce sclerotia that can persist in the soil for many

years (Ajayi-Oyetunde & Bradley, 2018). The information about the potential of the endophytic fungal isolates to promote plant growth and inhibit *R. solani* is important consideration for the development of biological control of the pathogen.

Materials and methods

This study was conducted in several stages. First cabbage seedling was planted in several types of growing media for two months. After that, the endophytic fungi were isolated from the cabbage roots. The effects of endophytic fungal isolates on germination and on the growth of cabbage seedlings were examined. The isolates that did not inhibit the cabbage growth were then selected and examined their abilities in inhibiting the cabbage pathogen, *Rhizoctonia solani*. Detail of each steps are explained in the following sub title.

Planting cabbage in various growing media

The planting media tested in this study were 1) pine forest soils; 2) rhizosphere of cogon grass (*Imperata cylindrica*, L.); 3) rhizosphere of elephant grass *(Pennisetum purpureum)*; 4) soils mixed with goat manure (1:1, v/v); 5) the soil mixed with compost (1 :1, v:v); 6) the soils mixed with vermicompost (1:1, v/v). The soil used was the topsoil (0-10 cm) of soil from Jatinangor area, Sumedang (inceptisol soil). To reduce the possible effects of allelopathy, the sample soil was previously left for two weeks with moisture was maintained.

Cabbage seed was seeded on husk charcoal medium. Before sowing, seeds were pre-soaked using warm water (50 ^{o}C) for an hour. The cabbage seedlings (two weeks old) were transferred into the growing media according to the treatments. After two months since the transplanting, the cabbage roots were taken for isolation of endophytic fungi.

Isolation of endophytic fungi from the cabbage roots, grown in different planting media

The cabbage roots were washed thoroughly and cut into pieces with a size of ± 1 cm. The root pieces was then surface sterilized by soaking in 96% alcohol for one minute, bleach solution (containing 2% chlorine) for three minutes, then rinsing with 96% alcohol for 30 seconds. To ensure that the isolates obtained were not epiphytic fungi, imprint of the sample were made by pressing the sample pieces on *Potato Dextrose Agar* (PDA) medium before plating them on other petridish (Istifadah & Sari, 2017). The root samples (10 pieces) were placed in petridish containing half strength PDA that has been mixed with antibiotic *chloramphenicol* (0.5%). The numbers of replications was determined using Federer's formula, which is $t(r-1) \leq 15$ (Hanafiah, 2012). As the numbers of treatments were six, so each treatment was repeated four times.

Observations were made from 3 days after isolation to one month to ensure that there was enough time for endophytic fungi to colonize the sample root pieces. Frequency of colonization was observed by calculating the percentage of root pieces that have been colonized by endophytic fungal isolates. Isolates from one treatment with different colony characteristics were purified and identified based on their morphological characters (Watanabe, 2010).

Test effects of endophytic fungi on the growth of cabbage seedlings

The endophytic fungal isolates were tested for their effects on germination and growth of cabbage seedlings. The experiment used Completely Randomized Design (CRD) with treatments consisted of the endophyte isolates (12 isolates) and control. Each treatment was repeated three times.

Considering that many fungal isolates did not produce spores, inoculation of the endophytic fungi was carried out based on the method used by Istifadah & Sari (2017). Before planting, cabbage seeds were disinfected by soaking with bleach solution (containing 2% chlorine) for 3 minutes and then rinsed three times with sterile water, then placed for five days on a colony of endophytic fungi. After that, the seeds were transferred to the planting medium which consisted of sterile soil mixed with rice husk charcoal (10%).

The cabbage growth variables observed were plant height, fresh weight and root fresh weight. The observation was conducted at three weeks after planting (WAP). Isolates that caused symptoms or inhibited cabbage growth were considered as pathogenic and they were not used in further tests.

Test of **antagonistic abilities of endophytic fungal isolates**

Isolates of cabbage root endophytic fungi that did not cause disease or inhibit the seedling growth were tested their antagonistic effects against pathogenic fungi *R. solani.* The experiment used CRD with treatments consisted of the fungal isolates (nine isolates) and control. Each treatment was repeated three times.

The antagonistic test was carried out with dual culture method in PDA medium. A plug of fungal endophyte culture (0.8 cm in diameter) was placed 3 cm beside a plug of the pathogen culture. As control/check, the pathogen was cultured without endophytic fungi.

The radial growth of the pathogen towards the endophytic fungi was measured every day. The total radial growth of the pathogen during the observation was determined by calculating area under colony growth curve (AUCGC) using modified Area Under Disease Progress Curve (AUDPC) formula (Istifadah & Herawati, 2018). Level of inhibition was

calculated with following equation: [(AUCGC in control – AUCGC in treatment) / AUCGC in control] × 100%

Data Analysis

The data obtained were statistically analyzed using the SPSS program version 20. The analysis of variance was performed and if there was a significant difference between treatments, further analysis was carried out using the Duncan Multiple Range Test (DMRT) at the level of 5%.

Results and Discussion

The frequency of the fungal endophyte colonization in cabbage roots grown in the tested media were varied depending on the type of planting media. The highest frequency of root pieces colonized by endophytic fungi (57.5%) was found in cabbage roots planted on soil mixed with goat manure. Soil mixed with other organic fertilizers, which were compost made from household waste and vermicompost, only resulted in 5% of colonized root pieces. In the cabbage planted in forest soils and grass rhizosphere, the percentage of colonized roots was only about 7.5-12.5% (Table 1).

Table 1. The effect of planting medium on the frequency of colonization and variability of isolates of root endophytic fungi of cabbage plants.

Types of medium	Percentage of colonized root pieces (%)	Number of isolates
Forest soil	10.0	3
Rhizosphere of cogon grass	12.5	1
Rhizosphere of elephant grass	7.5	1
Soil + goat manure	57.5	5
Soil + compost	5.0	1
Soil + vermicompost	5.0	1

In this study, frequency of fungal endophyte colonisation in the cabbage roots grown in other tested planting media, except from the cabbage grown in goat manure, were quite low. This study was only evaluated the culturable endophytic fungi in which the media used can affect the recovery of the fungi from the roots segments. The use of various media for isolation of endophytic fungi may improve the results. In addition, that may also due to the

difficulties of the fungi from the tested media to colonise the cabbage roots. Brassicaceae plants including cabbage produce secondary metabolites containing sulfur, called glucosinolates. Hydrolysis of glucosinolates results in isothiocyanates which has antifungal effect. Therefore, the endophytic fungi can successfully colonize the cabbage root if they are able to interfere to the production or hydrolysis of glucosinolates in the host plant, or even degrade them directly (Poveda et al., 2022).

In addition to the frequency of colonization, the type of growing medium also influenced variation of endophytic fungal isolates. Relatively more variation of the fungal isolates was found in cabbage roots planted in soil mixed with goat manure. Isolation of the endophytic fungi from cabbage root planted in the tested media resulted in seven types of fungal colonies. One type of colony could be found in several root pieces from cabbage planted in different media (Figure 1). In general, one root piece was colonized by one type of fungal colony. However, some root pieces could be colonized by two types of fungal colony. The fungal colony that was frequently emerged from root pieces was type 2 which was initially white then turned to light brown with smooth shiny surface and grayish color in the middle part. Out of 40 samples of root pieces from cabbage plants grown in each planting media, this fungal colony was found in root pieces of cabbage grown in pine forest soil (3 times), cogon grass rhizosphere (5 times), and soil mixed with vermicompost (5 times). Another isolates that the most frequently found was fungal isolate type 4, which was similar to the fungal colony type 2 but the colony color was dark brown. This type was found in the root pieces of cabbage grown in cogon grass rhizosphere (8 times), root pieces of cabbage grown in elephant grass (7 times) and from root pieces of cabbage grown in soil mixed with goat manure (18 times).

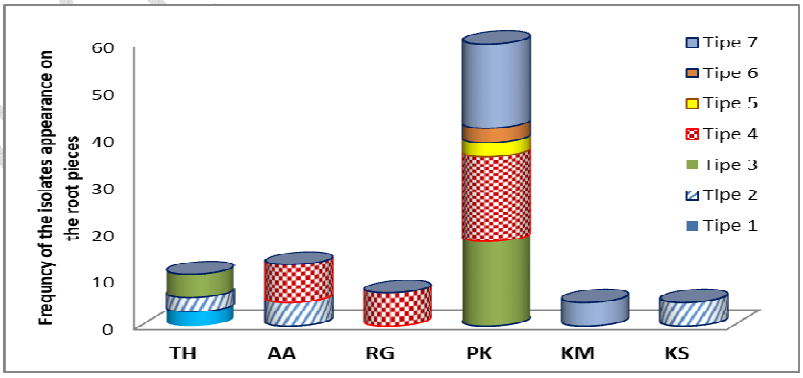

Figure 1. The frequency of the colony type of fungal endophyte isolates emerged from the root pieces. (Note: the numbers of root pieces in each treatment were 40 pieces).

The fungal isolate the most commonly found in this study was fungal isolate with type-4 colony. The isolate was found in cabbage roots grown in soil mixed with goat manure (PK-4 isolate), cogon grass rhizosphere (AA-1 isolate) and elephant grass (RG-1). The fungal isolates has rather smooth and glossy colony surface. The colony was initially white, then turned dark reddish brown, but the middle part was gray (Figure 2A). This fungus did not form spores, even though it has been grown in several media such as Malt Extract Agar (MEA) or V8 Juice Agar. Under microscopic observation, the isolate has septate hyphae with thick, blackish-brown cell walls with many swollen cells (toruloid hyphae) (Figure 2B). Fungi with similar characteristics but with light brown color (type-2 colony) were also isolated from forest soil (TH-2 isolate), soil mixed with goat manure (PK-3 isolate) and soil mixed with vermicompost (KS-1). These isolates were probably dark septate endophyte (DSE) that does not produce spores/conidia. The DSE are endophytic fungi that have dark pigmented (melanization) of hyphae which occasionally form a structure like microsclerotia, especially in root tissue (Berthelot et al., 2019). This type of fungus is widely found as root endophytic fungi in various plants (Berthelot et al., 2019), including Brassicaceae plant (Khastini et al., 2012).

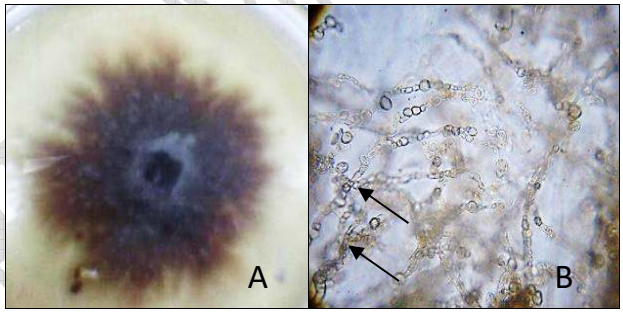

Figure 2. Characteristics of the most frequently found endophytic fungal isolate, A. Fungal colony, B. Fungal hyphae with dark thick cell wall and swollen cell

The isolates obtained in this study have not been identified as many fungal isolates did not form spores/conidia. In addition, although there were some isolates formed powdery-like structures such as PK-1 and PK-2 isolates, the isolates were also very difficult to be identified as the characteristics of conidiophores arrangement was hard to find. Therefore, later identification should be done molecularly on isolates that have the potential to be further studied.

The results of this study showed that the growing media affected the colonization and variability of endophytic fungi in the roots of cabbage plants. These results were consistent with other studies that also reported that soil type played important role in shaping community of root endophytic fungi (Bokati et al., 2016). Soil condition associated with agricultural practices such as in organic farming system or conventional system also affected endophytic communities present in the roots. Agricultural activities such as the use of different types of fertilizers such as organic fertilizers increased the endophytic communities in the roots (Sheoran et al., 2018; Xia et al., 2019). Application of animal manure can provide nutrients, but also enriched the microbial community in the soil (Kracmarova et al., 2020).

Endophytic fungal communities are part of fungal communities that exist in the soil or rhizosphere (Urbina et al., 2018; Qian et al., 2019). However, the host plant will select which microorganisms that can enter and colonize the plant as endophytes (Urbina et al., 2018). In this study, although compost and vermicompost are organic materials rich in microorganisms, only one fungal isolate can be isolated form the cabbage root grown in medium containing such organic materials. In addition, different planting media such as soil mixed with goat manure, cogon grass rhizosphere and elephant grass rhizosphere could result in the same type of fungal endophytes.

The Effect of Root Endophytic Fungi on the Growth of Cabbage Plants

In this study, endophyte inoculation was conducted by placing the cabbage seeds on the fungal endophyte colony for several days to allow the endophytic fungi enter and colonize the seeds and their sprouts. The results showed that out of 12 isolates of endophytic fungi from cabbage roots that were inoculated to cabbage seeds, 7 isolates of the endophytic fungi promoted cabbage seed germination. Cabbage seeds that were placed on colonies of endophytic fungi all germinated (100%) at the third day, mean while at the same time only 20% of the untreated seed that were germinated (Table 2). Manalu et al. (2020) reported that germination of chili seed on colony of DSE fungal isolate were better than the germination of untreated seeds (the check).

The effect of endophytic fungi on the host plant depends on the isolate. Most of the isolates tested were unable to increase the growth of cabbage seedlings. All isolates could not increase the cabbage plant height. Only three isolates of endophytic fungi from the roots of cabbage plants grown on manure-containing soils which were PK-2, PK-4 and PK-5 isolates, tended to support the growth of cabbage shoot 1.3-1.4 times better than controls. Meanwhile, only one isolate which was an endophytic fungal isolate from cabbage roots grown on

elephant grass rhizosphere (RG-1 isolate) supported cabbage root development 7.3 times heavier than the control.

Table 2. Effect of endophytic fungi inoculated to cabbage seeds on the cabbage seedling growth

Endophytic Fungal Isolate	Average height of cabbage plants (cm)	Average of shoot fresh weight (g)	Average of root fresh weight (g)
Control	5.1 a	2.54 ab	0.26 a
TH-1 isolate	7.3 a	2.96 ab	0.42 a
TH-2 Isolate	4.3 a	2.11 ab	0.16 a
TH-3 Isolate	4.1 a	1.54 a	0.19 a
AA-1 Isolate	5.3 a	2.55 ab	0.21 a
RG-1 Isolate	5.7 a	2.88 ab	1.89 b
PK-1 isolate	7.2 a	2.86 ab	0.36 a
PK-2 isolate	7.2 a	3.46 b	0.43 a
PK-3 isolate	5.7 a	2.57 ab	0.36 a
PK-4 isolate	5.1 a	3.59 b	0.36 a
PK-5 isolate	6.7 a	3.29 b	0.32 a
KP-1 isolate	5.4 a	2.56 ab	0.27 a
KS-1 Isolate	4.4 a	2.36 ab	0.21 a

Note: The values in one column followed by the same letter was not significantly different, based on the Duncan Multiple Range Test (DMRT) at the level of 5%. The data were obtained from observation at 3 weeks after planting. Isolate codes: TH: pine forest soils; AA: rhizosphere of cogon grass (*I. cylindrica*, L.); RG: rhizosphere of elephant grass *(P. purpureum)*; PK: soil mixed with goat manure (1:1, v/v); KP: soil mixed with compost (1 :1, v:v); KS: soils mixed with vermicompost (1:1, v/v).

The abilities of some root endophytic fungi to increase the growth of cabbage plants in this study is in line with other studies that also reported the abilities of endophytic fungi from crucifer roots to increase the growth of their host plants (Card et al., 2015). The plant growth improvement by root endophytic fungi on other plants has also been reported. Istifadah et al., (2016) found that 28.7% of endophytic fungal isolates from potato roots and tubers can increase the growth of potato plants. About 50% of endophytic fungal isolates from peanut roots (Istifadah & Sari, 2017) also increased plant growth, especially in the early

vegetative stage. The increase in growth by endophytic fungi can be due to the abilities of endophytic fungi to assist plants in obtaining nutrients or because of their abilities to produce phytohormone such as auxins, gibberellins and cytokinins (Rigobelo & Baron, 2021; Baron et al., 2022).

Among the isolates of root endophytic fungi tested, there were also isolates that inhibited the growth of cabbage plants. The average fresh weight of cabbage plants inoculated with endophytic fungus, TH-3 isolate, was relatively smaller than that of control plants. Although not markedly different, the plant height and root growth of cabbage plants inoculated with these isolates also tended to be smaller than those of controls.

The inhibitory effects of endophytic fungi on their host were also found in other studies. Istifadah et al (2016) reported that among endophytic fungi isolated from potato and tuber root 30.7% were pathogenic (causing symptoms of disease) and 7.7% inhibited the growth of potato plants. The plant growth inhibition by endophytic fungi possibly because they were actually latent pathogens that were inactive when isolated, but could be pathogenic in favourable environment. Hardoim et al., (2015) stated that certain fungi that are actually pathogens, however, under certain conditions can be found as endophytic or in latent conditions.

Effects of endophyte fungi on the growth of fungal pathogen *Rhizoctonia* sp. *in vitro*

Isolates of endophytic fungi that did not inhibit cabbage growth were tested for their abilities to suppress one of cabbage pathogens, *R. solani.* The results showed that the radial growth of *R. solani* in the presence of the endophyte fungal isolates were smaller than the pathogen growth in the check. Six isolates of endophytic fungi tested only inhibited the growth of *R. solani* in vitro by 27.5-40.6%. Three other isolates (PK-1, PK-2 and TH-1 isolates) showed relatively high inhibitory effect (Table 3). The AUCGC value of the pathogen in that treatments were 56.7-64.7% smaller, compared to the control. The highest inhibition of pathogen growth was shown by treatment with PK2 isolates.

Table 3. Effect of root endophytic fungi on the growth of *R. solani* colonies

Fungal endophyte Isolates	AUCGC Value	Level of inhibition*
Control (without isolate)	47.53 e	0,0
TH-1 isolate	20.60 abc	56.7
AA-1 Isolate	28.25 bc	40.6

RG-1 Isolate	28.43 bc	38.9
PK-1 isolate	17.78 ab	62.6
PK-2 isolate	16.77 a	64.7
PK-3 isolate	34.45 cd	27.5
PK-4 isolate	29.50 cd	37.9
PK-5 isolate	32.83 cde	30.9
KP-1 isolate	31.97 cd	32.7

Note: The values in the column followed by the same letter do not differ markedly according to the DMRT at the level of 5%. * Level inhibition is the percentage of AUCGC value in the treatment compared to AUCGC value in the check

The mechanism of antagonism can be inferred from the characteristics of fungal colonies in dual cultures. In dual culture between endophytic fungi PK-2 or TH-1 isolates and pathogenic fungus *R. solani* there were inhibition zone between their colonies (Figure 3). In this case, it was suspected that the inhibition was due to antibiosis mechanism. The endophyte isolates could produce secondary metabolites that diffused into the medium, thus inhibiting the growth of the pathogen. The abilities of endophytic fungi to produce various secondary metabolite compounds have been widely reported (Jha et al., 2023).

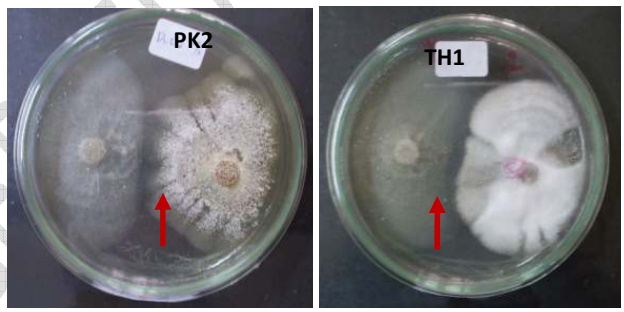

Figure 3. Dual cultures of endophytic fungi isolates PK-2 and TH-1 with *R. solani,* showing inhibition zone (the arrows)

The overall results showed that in addition to their role in supporting plant growth, planting medium also play important role in regulating the root endophytes. The use of goat manure improved colonisation of the root by fungal endophyte. Long term application of animal manure as agricultural practice in organic farming has been reported to increase the root endophyte communities (Sheoran et al., 2018; Kracmarova et al., 2020).

Some endophytic fungal isolate from cabbage root were beneficial as they promoted germination and the growth of cabbage plant as well as inhibited the growth of the pathogenic fungus *R. solani*. The isolate that promoted cabbage growth and best inhibited *R. solani* was PK-2 isolate. This isolate has the potential to be further studied for biological control of diseases in cabbage plants. Root endophytic fungi from Brassica plants have been reported to have antagonistic effects on plant pathogens, suppress plant diseases and also promote the host plant growth (Poveda et al., 2022; Card et al., 2015). The use of endophytes for supporting plant growth and health is very promising as they live inside the plant tissues and hence they are more protected from harsh environment (Baron et al., 2022).

Conclusions

The results of this showed that planting medium affected the presence and variability of endophytic fungi in the roots of cabbage plants. The frequency of colonization and variability of endophytic fungal isolates was highest in the root of cabbage planted in soil containing goat manure. Among 12 isolates of endophytic fungi tested, three isolates (PK-2, PK-4 and PK-5 isolates) enhanced cabbage growth and three isolates (PK-1. PK-2 and TH-1) inhibited the growth of fungal pathogen, *R*. solani *in vitro* by 56.7-64.7%.

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