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Isolation, Morpho-Cultural Identification and Pathogenicity of Fungal Endophytes of Potato (*Solanum tuberosum*) Leaves Collected in Natubleng, Buguias, Benguet

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Abstract

Endophytes are microorganisms that live within plant tissues for part or all of their life cycle. These microorganisms have properties that are valuable in the agriculture and medical sectors. In the field of agriculture, endophytes are studied for their potential as biological control agents, in managing abiotic stress, and as plant growth promoters. Thus, this paper presents fungal endophytes isolated from asymptomatic potato leaves collected from Buguias, Benguet. The four fungal endophytes were isolated and identified based on morpho-cultural characteristics as *Apiospora* sp., *Cladosporium* sp., while the other two were unidentified. None of the tested isolates was pathogenic in a detached leaf assay of Granola potato, as evidenced by the absence of symptoms. The dual culture assay against Fusarium sp. showed varying levels of antagonism ranging from low to moderate (29%-60%), with *Apiospora* sp. exhibiting the highest inhibition. However, the risk of pathogenic behavior cannot be completely dismissed since endophyte-to-plant interactions depend on multiple factors. Hence, further pathogenicity and antagonistic ability tests of the endophytes under greenhouse conditions are recommended. To the best of our knowledge, this is the first report of fungal endophytes from potato leaves in the Philippines.

Introduction

Potato (*Solanum tuberosum*) is a high-value crop in the Philippines, produced mainly in the Cordillera Administrative Region, specifically in Benguet and Mountain Province, at temperatures below 21°C and high elevations of about 1,200 to 2,000 m.a.s.l. Buguias and Mankayan are among the municipalities in Benguet with the highest potato production in terms of area and production volume, along with Atok, and Bakun. Meanwhile, Granola and Igorota varieties are widely grown for their fast-growing characteristics (Gonzales et al., 2016).

Despite the increasing demand for potatoes, their production is continually challenged by various biotic and abiotic factors during crop cultivation and post-harvest. For this reason, farmers extensively apply agrochemicals (Lu, 2010). However, extensive chemical use may prove ineffective against persistent phytopathogens such as *Fusarium* spp. Several species of this genus cause the dry rot of potato tubers worldwide. *Fusarium* spp. have a wide host range affecting economically important crops such as banana, rice, corn, tomato, and sweet potato. Some species develop chlamydospores that serve as survival structures

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during adverse conditions, making *Fusarium* spp. relatively difficult to manage. Aside from being a phytopathogen, Fusarium spp. have mycotoxins that may pose threats to human and animal health (Balendres et al., 2019; Carreon et al., 2021; Xue et al., 2023).

As part of integrated pest management, endophytes are being studied as potential biological control agents. Endophytes are microorganisms that spend either part or their entire lifecycle inside plant tissues. Each plant, including potatoes, is believed to host more than one endophyte. The population and diversity of endophytes depend on various biotic and abiotic factors, such as stress levels, plant organ, adaptive capacity of the species, and farming practices (Pageni et al., 2013; Carbungco et al., 2017; Yasser et al., 2019). Yasser et al. (2019) found that most of the fungal endophytes from potato tissues were isolated from the leaves, highlighting the role of specific plant tissues in supporting endophyte population. Although host reactions depend on both abiotic and biotic factors such as environmental conditions, genotypes of both microorganisms and plants, and the population of microorganisms, the preferred definition of endophytes refers to microorganisms that do not induce disease symptoms on host plants (Hardoim et al., 2015; *Collinge* et al., 2022). Endophytes caught the interest of many researchers worldwide for their potential applications in medicine and agriculture. Some of the reported endophytic fungi isolated from potato tissues around the world include the species of *Aspergillus, Fusarium, Penicillium, Alternaria tenuissima, Chaetomium cervicola, Curvularia lunata, Lasiodiplodia theobromae, Stemphylium vesicarium, Trichoderma harzianum,* and *Ulocladium* sp. (Yasser et al., 2019).

In the Philippines, numerous endophytes from endemic plants, medicinal plants, and economically important crops, such as strawberry, cacao, banana, and sweet potato, were reported. Endophytic fungi commonly isolated from different hosts belonged to the genera *Fusarium, Xylaria, Pestalotiopsis, Aspergillus, Nigrospora, Rhizoctonia, Colletotrichum, Cladosporium,* and *Penicillium* (Carbungco et al., 2017; Guerrero et al., 2019; Ramirez et al., 2020). However, the isolation of potato endophytes in the Philippines has not yet been reported.

With the growing concerns about environmental degradation, health concerns, food safety, and increasing demand for food, there is a need to continually explore ways for sustainable crop production. The use of beneficial organisms in crop production has long been recognized for promoting food safety and security, maintaining ecosystem balance, and reducing farmers' input costs. Apart from the well-known *Trichoderma* spp., other beneficial microorganisms could be explored. Identifying endophytes is a key step in discovering potential beneficial microorganisms for integrated pest management. Hence, to contribute to the expanding knowledge of endophytes isolated from potatoes worldwide, this study presents the isolation and identification of fungal endophytes from asymptomatic potato leaves collected from Buguias, Benguet. It also examines the pathogenicity of the endophytes on potato leaves and their antagonistic potential against *Fusarium* sp. The results serve as a baseline for future research on potato endophytes as biological control agents against phytopathogens such as *Fusarium* spp.

Methodology

Collection of Samples

The sampling site was in Lamot, Natubleng, Buguias, Benguet. Natubleng is one of the barangays in Buguias, elevated at 2,237.7 meters or 7,341.4 feet above mean sea level. Buguias has an average annual temperature ranging from 17 to 29°C and a relative humidity reaching 78.4%, with the coldest from January to February and hottest from April to June (World Weather Online, n.d.). The study was conducted from January to March 2024.

Fifteen potato leaves at the vegetative growth stage were randomly sampled from potato farms in Natubleng, Buguias, Benguet (Pageni et al., 2013). Leaf samples without discolorations or malformations were collected in plastic bags and processed within 48 hours at the Crop Protection and Biotechnology Laboratory, Research and Extension Building, Benguet State University, La Trinidad, Benguet, Philippines. Samples were collected from the 2nd to the 4th order from the youngest leaves, where visibly asymptomatic leaves were observed.

Isolation and Morpho-Cultural Characterization of Fungal Endophyte

Leaf samples were washed with tap water and surface-sterilized following the procedure described by Yasser et al. (2019). A sterilized 6mm one-hole puncher was used to cut leaf discs. The leaf discs were sequentially immersed in 70% ethanol for 1-3 minutes, 4% aqueous solution of sodium hypochlorite for 1-2 minutes, another 1 minute in 70% ethanol, then finally rinsed five times in sterile distilled water.

Four-leaf discs per sample were inoculated on potato dextrose agar (PDA) with 1 ml/L of 10% lactic acid to prevent bacterial growth. Control plates were prepared by leaf prints through dipping each leaf disc for 5 seconds in PDA before inoculation in another plate of PDA. The corresponding plate of Control plates with fungal growth after 10 days was not included (Fernandes et al., 2015; Guerrero et al., 2019). Isolates were incubated at 26-28°C using the Memmert BE 400 Incubator and observed daily for fungal growth. Growing colonies were sub-cultured and maintained in PDA at 4-5°C.

The fungal isolates were identified based on comparison of cultural and morphological characteristics with published and online articles. After obtaining the pure culture of the endophytes, macroscopic and microscopic characteristics were recorded. The colony texture, surface, and reverse color, zonation/ margin, and average diameter at 7 days after incubation on PDA were noted for macroscopic characteristics (n=5). The agar block method was utilized to prepare specimens for microscopic observation, then specimens were mounted on plain lactophenol and iodine glycerol after 7 DAI. The spore size and shape, length, and diameter of hyphae and septations (n=30) were noted for microscopic characteristics (Carbungco et al., 2017).

Pathogenicity Test

The pathogenicity test was performed by detached leaf assay (Karki & Halterman, 2021). Healthy leaves without deformities or discolorations from the 4th to 5th order of the apical shoot leaves of 5-week-old Granola potatoes were collected from the Northern Philippines Root Crops Research and Training Center, La Trinidad, Benguet, Philippines. The leaves were surface-sterilized by sequentially wiping with 70% ethanol and twice with sterile distilled water (SDW) before drying in a laminar flow hood. Clear plastic tubs measuring 15cm x 10cm x 5cm were utilized for the setup. Sterilized paper towels were placed in plastic tubs, topped with plastic screens, and moistened with sterile distilled water (SDW).

The inoculum suspension was prepared and adjusted to 50,000 spores/ml using a hemocytometer before drop inoculating on the abaxial side of the leaves. Each leaflet received 10 µL of the suspension in 3-5 droplets (Karki & Halterman, 2021). Each treatment had 4 replicates with 3 sub-samples, each incubated at room temperature in a Completely Randomized Design. The Control treatment was inoculated with SDW. UI2 was excluded for pathogenicity testing because of its failure to produce spores, making it impossible to standardize the inoculum population. The presence or absence of necrotic or chlorotic symptoms at the inoculation point was recorded within 7 days after inoculation (DAI). The treatments were as follows,

T1- Unidentified Isolate 1
T2- *Apiospora* sp.
T3- *Cladosporium* sp.
T4- Control (SDW)

After 7 days of incubation, leaves from each treatment were randomly sampled for microscopic observation and re-isolation. Two to three centimeters of leaf sections near the point of inoculation were cut and surface-sterilized. Surface sterilization was performed by sequential immersion in 10% sodium hypochlorite for 2-3 minutes, followed by 3 changes of SDW. The growth of the inoculated endophyte was observed within 7 days.

Dual Culture Assay

Seven-day-old isolates of the endophytes and *Fusarium* sp. were used (Ahmad et al., 2020). A stock culture of *Fusarium* sp. isolated from soil collected from an ornamental farm in Tublay, Benguet, Philippines was provided by the Department of Plant Pathology Laboratory, Benguet State University, La Trinidad, Benguet, Philippines. The isolate was identified and maintained in the Department. Five-millimeter plugs from the growing margin of each colony were

placed 4 cm apart on each plate. Each treatment had 5 replicates arranged ina Completely Randomized Design and incubated for 7 days at 26-28°C. The treatments were as follows,

T1- Unidentified Isolate 1
T2- *Apiospora* sp.
T3- *Cladosporium* sp.
T4- Unidentified Isolate 2
T5- *Fusarium* sp. only

Percent growth of inhibition at 5 and 7 DAI was obtained using the formula PIRG R1-R2/R1 X 100, where R1 is the colony radial growth of *Fusarium* sp. on the Control plates, while R2 is the colony radial growth of *Fusarium* sp. grown with endophytes. The degree of antagonism was categorized as very high antagonistic activity (>75%), high antagonistic activity (61–75%), moderate antagonistic activity (51–60%), or low antagonistic activity (< 50%). The data were analyzed using the Statistical Tool for Agricultural Research (STAR) with ANOVA. The normality and homogeneity were checked using the Shapiro-Wilk Test for Normality of Residuals and Barlett's Test for Homogeneity of Variances, respectively (Sadoral & Cumagun, 2021; Taping et al., 2023).

Results and Discussion

Cultural and Morphological Characteristics of Fungal Isolates

Apiospora sp.

After 7-14 days of incubation, 4 fungal isolates with distinct characteristics were isolated (Table 1). Two isolates were identified under the *Apiospora* and *Cladosporium* genera, while the other 2 remained unidentified. *Apiospora* sp. colony has white obverse and creamy white reverse color, loose fluffy aerial mycelia, and is characterized by a rapid growth reaching a maximum of 90mm as early as 4 days after incubation (DAI) in PDA. Spores are usually found in clumps, globose to spherical, and dark in color, with a diameter ranging from 2.97 to 5.4 µm. Hyphae are septate and hyaline, measuring 0.27-2.7 x 5.4-56.7 µm (Figure 1). These characterizations coincide with descriptions of reported endophytic *Apiospora* spp. (Tian et al., 2021; Liao et al., 2023; Zhang et al., 2023).

Apiospora species exist as endophyte, saprobe, or pathogen (Tian et al., 2021; Liao et al., 2023; Zhang et al., 2023). *Apiospora* spp. are widely found in Asia on a relatively narrow host range, mainly on grasses. While endophytic *Apiospora* spp. are mostly associated to Poacea and Cyperaceae, Aktar and Shamsi (2009) isolated and identified Arthrinium from *Datura metel*, a poisonous plant of Solanaceae. The genus *Arthrinium* previously included *Apiospora* until a recent study distinguished *Apiospora* as a distinct genus from Arthrinium (Sorensen et al., 2022).

Cladosporium sp.

Cladosporium sp. is characterized by a greyish green obverse and dark greyish green reverse side growing 30-40mm in diameter. Its spores have either one septum or are aseptate, measuring 2.97-24.3 x 2.7-5.4 µm, while its septate hyphae measure 5.4-59.4 x 2.7-5.4 µm (Figure 2). These descriptions were verified with existing literature (Quimio & Hanlin, 1999; Marin-Felix et al., 2017).

Cladosporium species thrive as either a pathogen, a saprobe, or an endophyte (Marin-Felix et al., 2017). Unlike *Apiospora* spp., *Cladosporium* spp. have a wide range of hosts across varying ecological zones and are among the commonly identified endophytes in the Philippines and worldwide. In the Philippines, reported hosts of endophytic *Cladosporium* spp. include mangrove trees and endemic ginger (Ramirez et al., 2020; Magday et al., 2023).

Unidentified Isolates

Unidentified Isolate 1 (UI1) has a rough texture with a verrucose and umbonate topography measuring 15-20mm at 7 DAI. Its surface has a red-brown to rust-red color with a white cream margin, while the reverse side appears brown-red with a white cream to yellowish margin. The colony features a curly to slightly wavy margin. The hyaline spores are smooth, globose to ellipsoid in shap,e measuring 2.7-8.1 x 1.35- 5.4 µm with an average of 6.9 x 3.9 µm. While it is usually aseptate, spores can have one septum. Its hyphae are branched, slightly wavy, sometimes forming hyphal coils, coenocytic, and hyaline, measuring 0.54 to 2.7 µm and an average of 1.8 µm. Aside from the spores, Unidentified Isolate 1 develops numerous clumped chlamydospores in PDA after 7 days of incubation (Figure 3).

Table 1

Morphological and Cultural Characterization of Fungal Isolates in PDA Incubated for 7 Days at 26-28°C

	Isolates			
	Apiospora sp.	*Cladosporium* sp.	Unidentified Isolate 1	Unidentified Isolate 2
Obverse Color	White	Greyish-green	Rust-red	White
Reverse Color	Cream white	Dark greyish-green	Rusty brown	White
Growth Diameter in PDA (mm)	90	30-40	15-20	45-65
Spore	No septum; 2.97- 5.4 um	0-1 septum; 2.97-24.3 x 2.7-5.4 um	0-1 septum; 2.7-8.1 x 1.35- 5.4 um	Not observed
Hyphae	Septated; 0.27-2.7 x 5.4-56.7 um	Septated; 5.4-59.4 x 2.7-5.4 um	No septum; 0.54-2.7 um	Septated; 0.54-2.7 x 10.8-37.8 um
Reference	Tian et. al. (2021); Liao et. al. (2023); Zhang et. al. (2023)	Quimio and Hanlin (1999); Marin-Felix et. al. (2017)	-	-

Figure 1

Growth of Apiospora sp. in PDA at 7 DAI Showing (A) Obverse View (B)Reverse View and (C) Conidiophores Bearing Clusters of Conidia Under 400x Magnification

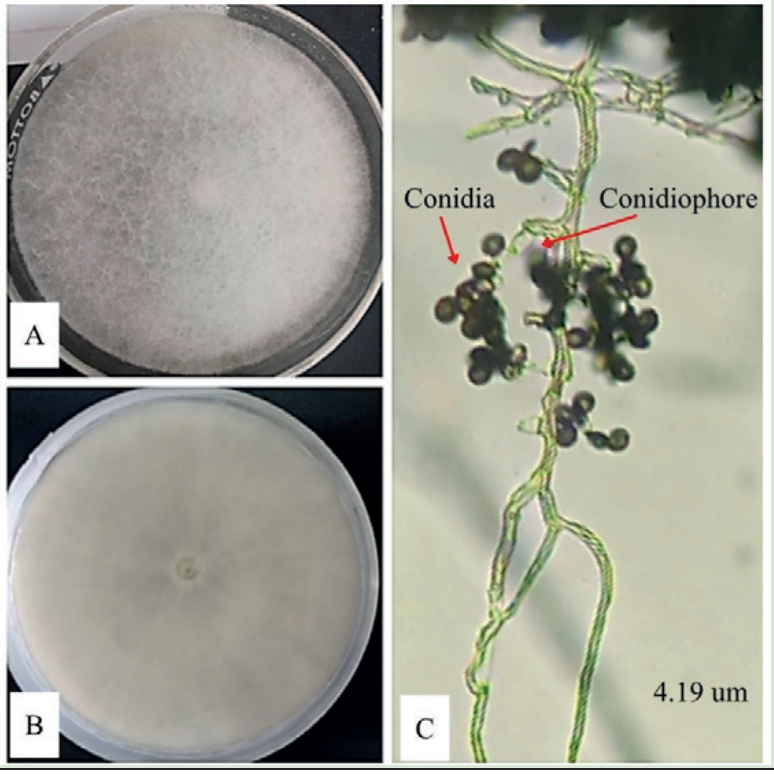


Figure 2

Structures and Colony of Cladosporium sp. in PDA at 7 DAI Showing (A) Obverse View (B) Reverse View, (C) Macro and Microconidia and (D) Conidiophore Bearing Conidia

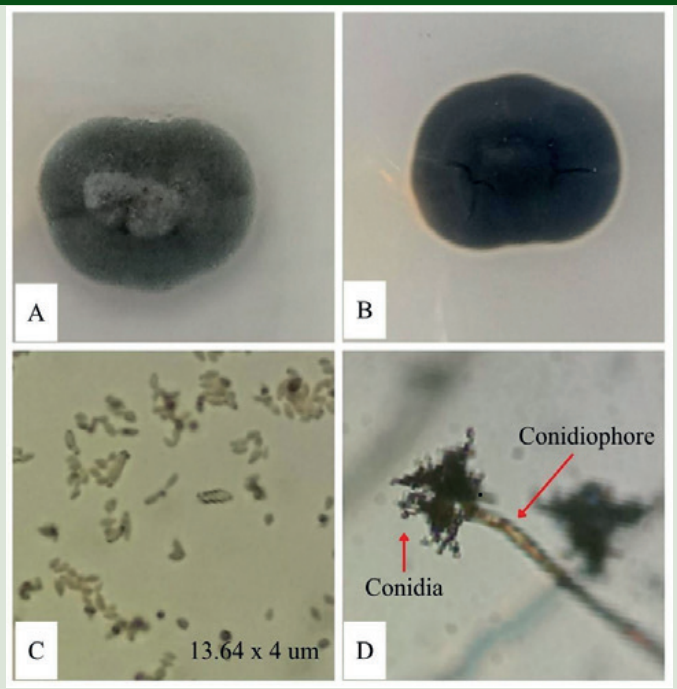
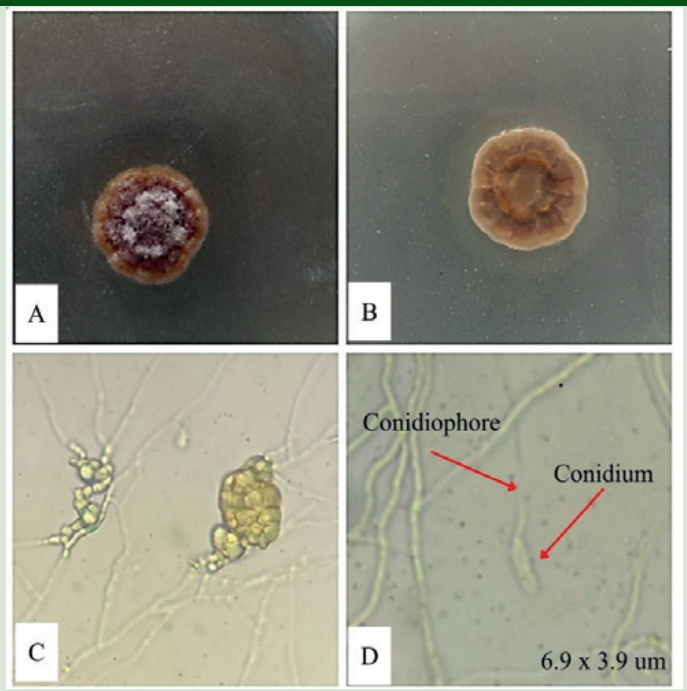


Figure 3

Colony Growth of Unidentified Isolate 1 in PDA at 7 DAI Showing (A) Obverse View, (B) Reverse View, (C) Chlamydospores, and (D) Conidiophore Bearing Conidium



Unidentified Isolate 2 (UI2) is characterized by a white surface color with a slightly granular to flat cottony white texture. It has a filamentous margin and a white to creamy white with a brown to dark brown mass of hyphae, which are observable on the obverse and reverse sides. The masses of hyphae appear as flat, dark specks on both sides. Its colony diameter ranges from 45 to 65mm at 7 DAI. Its hyphae usually form hyphal coils, hyaline and septate, measuring 0.54-2.7 x 10.8-37.8 µm with an average of 2.16 x 24.3 µm (Figure 4). Spores were not observed on either PDA or Malt Extract agar even after prolonged incubation of up to one month at 26-28°C.

Every plant hosts a diverse population of endophytic microorganisms, where the majority of reported fungal endophytes were identified under the phylum Ascomycota. (Torres & dela Cruz, 2015; Rashmi et al., 2019). Potato, having been grown in different regions of the world in relatively different growing practices, has its rich and diverse endophytic community explored from the leaves to the roots and tubers.

Pathogenicity Test

After 7 DAI, no symptoms were observed on the points of inoculation (Figure 5). The UI1, *Apiospora* sp., and *Cladosporium* sp. were recovered from the inoculated leaves, but none of the endophytes were isolated from the uninoculated leaves (Table 2). While some species of *Cladosporium* are pathogens of tomato, which is of the same family as potato, certain endophytic strains are beneficial rather than harmful (Raut et al., 2021; Amatuzzi et al., 2018).

In contrast to the asymptomatic result of this study, the pathogenicity of Arthrinium on Datura metel cannot be determined, as the research did not go that far (Aktar & Shamsi, 2009). This is also true for identified *Apiospora* species of ferns and grasses, which lack pathogenicity tests (Tian et al., 2021; Liao et al., 2023; Zhang et al., 2023). While some species are pathogenic, the same authors demonstrated that Apiospora is associated with healthy plant tissues (Aktar & Shamsi, 2009; Liao et al., 2023; Zhang et al., 2023).

Figure 4

Colony of Unidentified Isolate 2 in PDA Showing: (A) Obverse View (B) Reverse View with Visible Mass of Hyphae at 7 DAI and (C, D) Hyphae Structure at 14 DAI

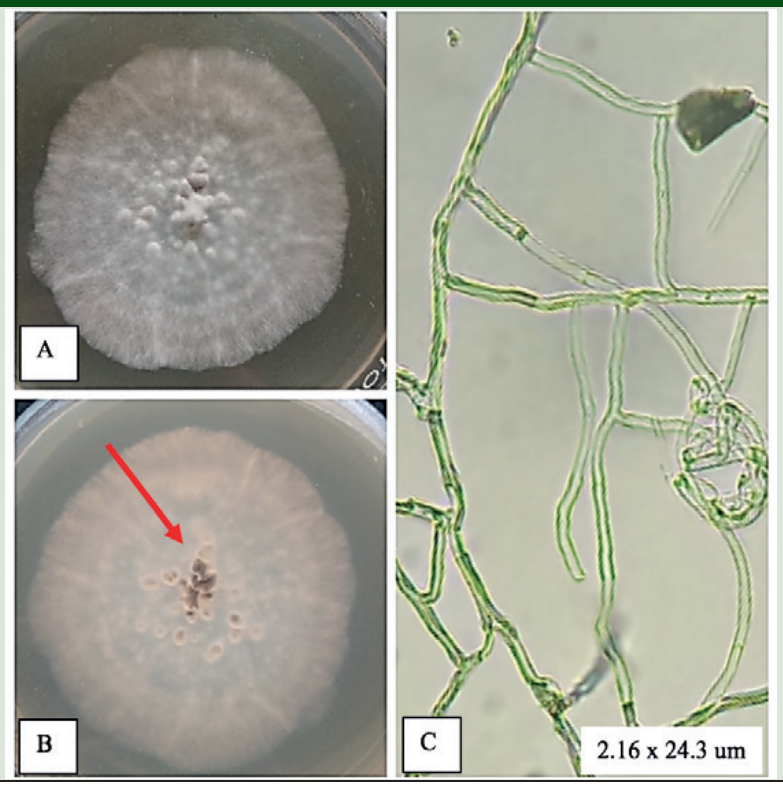


Figure 5

Detach Leaf Assay of Endophytes on Potato Leaves at Room Temperature Showing Inoculation Points of (A) Unidentified Isolate 1, (B) Apiospora sp., (C) Cladosporium sp. Showing Asymptomatic Reaction and (D) SDW at 5 DAI

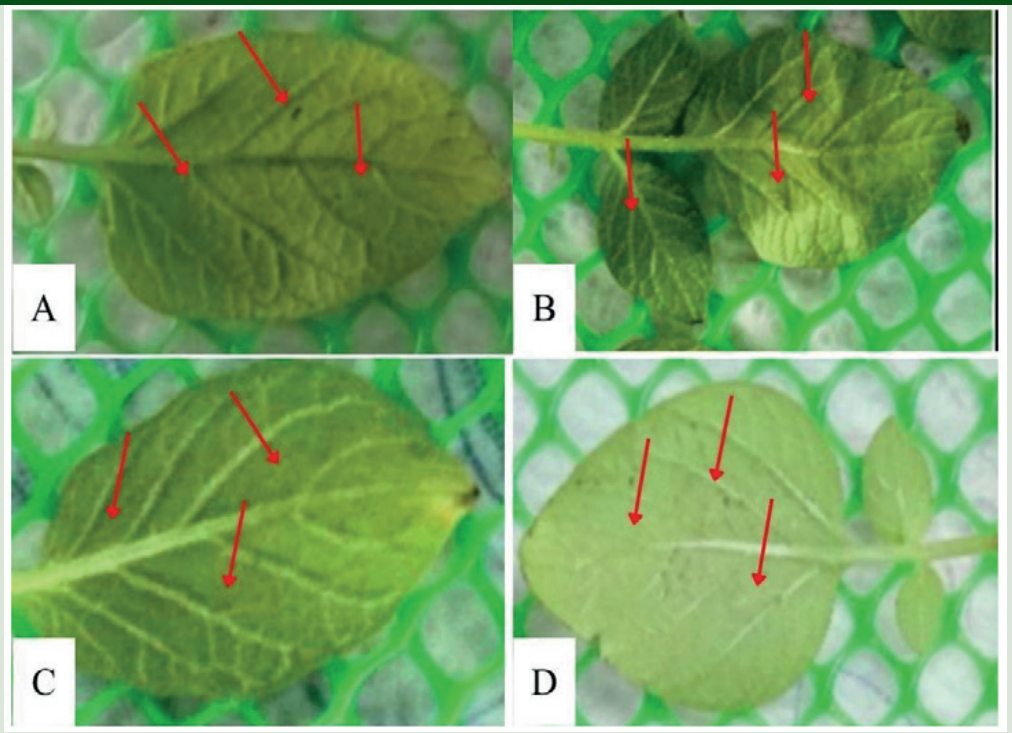


Table 2

Pathogenicity Test Result and Re-isolation of Endophytes Inoculated on Detached Leaves of Potato Incubated at Room Temperature for 7 Days

	Observed Symptom	Re-isolation
T1- UI1	-	+
T2- *Apiospora* sp.	-	+
T3- *Cladosporium* sp.	-	+
T4- Control	-	-

Note: Legend
– no symptom observed, or endophyte not recovered
+ chlorotic or necrotic symptom observed, or endophyte recovered /re-isolated

The pathogenicity result is supported by the studies of Ahmad et al. (2020) and Sadoral and Cumagun (2021), who concluded that endophytes from asymptomatic hosts are not pathogenic when re-inoculated. It is worth noting that species in the same genus do not necessarily share the same lifestyle adaptation. For instance, the genus *Colletotrichum* encompasses a complex of pathogenic species, but an endophytic strain isolated from asymptomatic cacao did not induce disease symptoms on the same host (Sadoral & Cumagun, 2021).

The asymptomatic reaction of plants to endophytes is said to be governed by the balance between host plant defense mechanisms and fungal virulence, called the balanced antagonism. The fungal virulence is kept in check by the plants' defense mechanism while allowing their colonization. An imbalance, such as the increase of the plant defense mechanism, will make it impossible for endophyte survival, while the loss in check of fungal virulence would lead to disease manifestation (De Silva et al., 2019). Similarly, a review of endophytes by Collinge et al. (2022) revealed that the pathogenicity of endophytes is held in control not by a single factor but by the balanced defense mechanism of the host and the presence of the entire microbial community to keep each other in check.

Antagonistic Effect of the Fungal Endophytes Against *Fusarium* sp.

The four isolates of endophyte showed varying levels of ability to inhibit the growth of *Fusarium* sp. (Table 3). *Apiospora* sp. had the highest mean PIGR during the fifth and seventh DAI, followed by Unidentified Isolate 2. *Cladosporium* sp. and UI1 had a comparable percent PIGR during the 5th day, but UI1 had the lowest percent growth inhibition on the 7th day. *Apiospora* sp. had a mean PIGR of 60%, indicating moderate antagonistic activity, while the rest showed low antagonistic activity of <50% (Figure 6).

Table 3

Percent Inhibition Growth Rate at 5 and 7 DAI of Endophytes and Fusarium sp. in a Dual Assay

Fungal Endophytes	PIGR Mean at 5 DAI (%)	PIGR Mean at 7 DAI (%)
Apiospora sp.	44.45[a]	60.00[a]
Cladosporium sp.	15.43[c]	40.87[c]
UI2	24.08[b]	47.83[b]
UI1	12.96[c]	29.56[d]
Coefficient of Variation	24.09%	9.06%

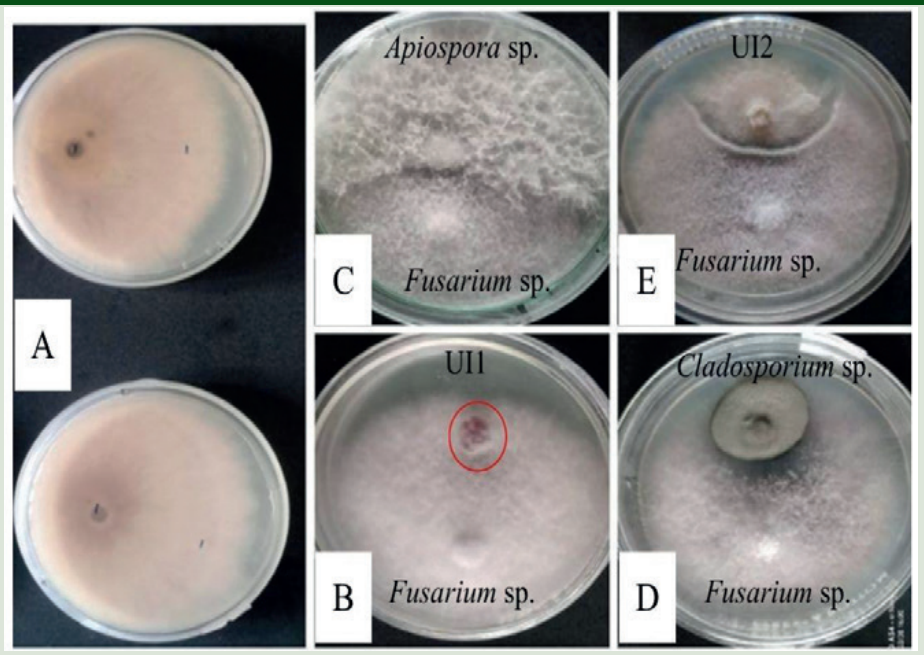
Note: Means with the same letter are not significantly different at p=0.0000 using Analysis of Variance.

The highest PIGR of *Apiospora* sp., as compared to the rest, may be attributed to its fast-growing ability, which is one of the characteristics of a biocontrol agent, to effectively compete for space and nutrients against a pathogen. This mechanism is displayed by fungal endophytes such as *Aspergillus, Albifimbria, Macrophomina,* and *Cylindrobasidium* against potato late blight *Phytophthora infestans* (El-Hasan et al., 2022). However, the general antagonism mechanism of *Apiospora* species to plant pathogens is relatively not understood due to a lack of studies. Meanwhile, the low antagonism of other endophytes could be due to their slow-growing ability, making them unable to compete for space and nutrients (Sadoral & Cumagun, 2021).

Some endophytic species of *Cladosporium* are better known as entomopathogens, but apart from that, they exhibit antagonism against microbial phytopathogens, including *Fusarium graminearum*, possibly through the secretion of hydrolytic enzymes that degrade cell walls

Figure 6

Dual Culture Assay of Fusarium sp. (A) on Control Plate, (B) with Unidentified Isolate 1 (C) with Apiospora sp. (D) with Cladosporium sp. and (E) Unidentified Isolate 2 at 7 DAI



(Raut et al., 2021). An indirect mode of antagonism is further elucidated through the release of *C. cladosporioides* of a compound called cladosporin, which inhibits the growth of test pathogens by more than 90% (Wang et al., 2013). In addition, metabolites and volatile organic compounds released by some strains are associated with the robust growth of tomato seedlings (Raut et al., 2021; Amatuzzi et al., 2018). In general, the mode of antagonism by endophytes is through competition, antibiosis, and parasitism (Taping et al., 2023).

Conclusions

Fungal endophytes were successfully isolated from asymptomatic potato leaves collected from Buguias, Benguet. The isolates were identified through morphological and cultural characterization as *Apiospora* sp. and *Cladosporium* sp., while the other 2 isolates remained unidentified. These two isolates belong to the phylum Ascomycota, where a majority of reported fungal endophytes belong. The pathogenicity result confirms the association of endophytic fungi on asymptomatic potato leaves without necessarily causing disease symptoms. The dual assay result demonstrated the potential antagonistic ability of the endophytes against *Fusarium* sp., as evident by their low to moderate levels of antagonism.

Recommendations

This study was limited to the isolation of potato leaf endophytes from Lamot, Natubleng, Buguias, Benguet, but more endophytes could be discovered when the sampling site is widened. The limitation of morpho-cultural identification is further acknowledged; hence, molecular identification is recommended to confirm the identity of the isolates. While tested endophytes were found to be non-pathogenic and showed antagonism *in vitro*, this needs validation in a greenhouse experiment. Also, the extraction and study of the endophytes' bioactive metabolites is suggested.

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