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Biodiversity of endophytic mycoflora associated with medicinal plant *Geranium wallichianum* D. Don Ex-Sweet growing in Kashmir

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Fungal endophytes are the microorganisms living in a symbiotic relationship with their hostplants. These naturally occurring symbionts are abundant, have a huge biodiversity and provide a great deal of advantages to their host plants such as providing protection against pathogens and other biotic as well as abiotic stresses. In the present study, the diversity of fungal endophytes was examined from the root, stem, leaf and petiole of *Geranium wallichianum* D. Don Ex-Sweet, a medicinally important plant. A total of 74 endophytic fungal isolates were obtained from 120 plated samples of root, stem, leaves and petiole collectively. These fungi were identified on the basis of cultural and microscopic characteristics and 10 species of fungi belonging to 8 different genera viz; *Alternaria, Arthrinium, Aspergillus, Cladosporium, Colletotrichum, Drechslera, Fusarium* and *Nigrospora* were identified. The diversity deciphered in the current study can be utilized in assessing the potential of these fungal endophytes as biocontrol agents as they might be rich sources of a variety of bioactive secondary metabolites of commercial and medicinal importance

Keywords: Biocontrol, endophyte, fungi, secondary metabolite

INTRODUCTION

Endophytes are microorganisms residing inside the living plants without harming them. They spend their life cycle or a period of it, asymptomatically inside the healthy plant tissues (Singh and Dubey, 2015).These organisms harbor the host plant and concurrently help them to adapt to the varying biotic and abiotic stresses. Endophytes have been reported to be beneficial for the plants in manifold ways.Presently the endophytes are a targeted research area due to their ability to synthesize novel active leads with potential biological activity. However, the true potential of endophytes is yet under explored (Joseph and Priya, 2011).

A huge number of endophytes isolated from diverse medicinal plants are reported to produce a wide variety of metabolites which have proved as outstanding drug sources in the treatment against various diseases, and besides medicine, endophytes have potent implementation in food and agriculture industry (Strobel and Daisy, 2003). Endophyte research has shown a significant expansion in the recent years. Besides cataloguing new species, efforts are being made to understand the nature of endophyte-host interactions (Rodriguez, 2009) with emphasis on studying endophytes associated with the medicinal plants of known pharmacological value aiming the discovery of novel bioactive leads (Mitchell *et al*. 2008). Sampling endophytic fungal diversity and their characterization is a challenging task and equally promising in revealing the novel species, new bioactive compounds (Arnold and Lutzoni, 2007; Siebert, 2007) and metabolites produced by these fungal endophytes. This eventually leads to a better understanding of the ecological roles played by these beneficial microbes. A few studies on both endophytic and

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rhizospheric fungal diversity have been carried out in the natural habitat of the host plants (Samuels *et al.*2006; Cannon and Simmons, 2002 Ahmad *et al.* 2021a,b; Malik *et al.*2022).In the backdrop of this, our study is focused on characterizing the diversity of endophytic fungi associated with *Geranium wallichianum* D. Don Ex Sweet, a medicinal plant of Kashmir Himalaya. *G. wallichianum* used in the present study, locally known as 'Kawashud' or 'Ratanjot', is an endangered medicinal herb growing in Kashmir Himalaya, belonging to the family Geraniaceae (Shaheen*et al.*2017). This plant is known for its antimicrobial, antifungal and other medicinal properties (Ahmad *et al*. 2003;Shinwari and Gilani, 2003; Ismail *et al.*2012; Akhter and Bergmeier, 2013). Therefore, the current study was carried out with the objective of isolating and characterizing fungal endophytes from different plant parts of *G. wallichianum.*

MATERIALS AND METHODS

To realize the objective of the study entitled 'Biodiversity of endophytic mycoflora associated with medicinal plant *Geranium wallichianum* growing in Kashmir', the materials and methods followed are as under:

Sample collection

G. wallichianum grows in different regions of Kashmir valley such as Gulmarg, Drung, Pahalgam, Sinthan, Poonch etc. For the present study, fresh and healthy plants of *G. wallichianum* were collected from their natural habitat and brought to the laboratory of Plant Pathology and Mycology, Department of Botany, University of Kashmir for further observation. The plants were authenticated from the Centre for Biodiversity and Taxonomy, University of Kashmir and thereafter processed for the isolation of endophytes.

Isolation of endophytic Fungi

Endophytic fungi were isolated from the tissues of different plant parts taken from the healthy plants of *G. wallichianum* as per the protocol described by Petrini (1986). The tissue samples taken were that of roots, stems and leaves and petiole. The healthy plant tissues were washed thoroughly under tap water to remove soil and debris and then rinsed with sterile water twice. Afterwards, the tissues were surface sterilized under aseptic conditions. The immersion protocol followed was according to the sequence described; immersion of tissues in 70% Ethanol (v/v) for 1 min., immersion of tissues in 3% (v/v) sodium hypochlorite for 1 minute followed by rinsing with sterile water thrice, each time for 1 minute. The tissue samples were aseptically cut into smaller divisions, 5×5 mm each and then placed onto sterilized Potato Dextrose Agar plates that were supplemented with streptomycin to suppress bacterial growth. The PDA plates were then incubated at 28 ± 2^{0}C. From the third wash sterile water, aliquots were taken and plated onto sterilized PDA plates to ensure effective surface sterilization. The plates were checked for fungal growth after 72 hrs and the fungal tips of isolated fungal endophytes were transferred to fresh PDA plates to obtain pure fungal cultures.

Identification of fungal endophytes isolated in the study

The fungal endophytes isolated in this study were identified based on their cultural and microscopic characteristics using standard keys and manuals for fungal identification (Barnet and Hunter, 2000; Wantanabe, 2002; Dugan, 2006). This involved the observation and documentation of the growth patterns of the colony of endophytic fungi in PDA (Potato Dextrose Agar) medium, their colony morphology and microscopic features like shape and size of the spore and spore arrangement. These criteria were used in conjunction with the established fungal identification keys to determine the taxonomy of the isolated fungal endophytes.

RESULTS

Isolation and Identification of endophytic fungi

It was revealed from the results that out of 120 samples (30 each of root, stem, leaf and petiole) from *G. wallichianum* that were inoculated on fresh PDA medium, seventy-four of the inoculated samples showed fungal endophytic growth. A total of 10 fungal endophytic species belonging to 8 genera were isolated and identified from root, stem, leaves and petiole of *G. wallichianum.*

These species were identified as *Alternaria ricini* Sawada, *Alternaria tenuissima* (Kunze) Wiltshire, *Arthrinium phaeospermum* (Corda) M.B.Ellis, *Aspergillus oryzae* (Ahlb.) Cohn, *Aspergillus sydowii B*ainier & Sartory, *Cladosporium herbarum* (Pers.) Link, *Colletotrichum gleosporoides* (Penz.) Penz. &Sacc., *Drechslera avenae* (Eidam) Scharif, *Fusarium chlamydsporum* Wollenw. & Reinking, *Nigrospora sphaerica* (Sacc.) E.W. Mason.

Fungal endophytes isolated from the root tissue were divided into 3 genera viz. *Aspergillus, Fusarium and Arthrinium. Arthrinium phaoespermum* being the most abundant species isolated from the root of *Geranium wallichianum*. The fungal endophytes isolated from the stem account for a small proportion and were represented by 2 genera, viz. *Alternaria and Fusarium, Fusarium* being the dominant genus isolated from stem of *G. wallichianum*. The fungal endophytes isolated from leaf samples belonged to 4 genera namely *Alternaria, Cladosporium, Colletotrichum* and *Drechslera. Alternaria ricini* was the dominant species isolated from the leaf samples of *G. wallichianum. Nigrospora* was the only genus to be isolated from the petiole with a single species *Nigrospora sphaerica*.The various identification characters of the isolated fungi are as follows:

***Alternaria ricini* Sawada** (Fig. 1, i; 2, a)

Morpho-cultural characters: The colony is velvety, olivaceous green with a round border which is usually lighter in colour. It grows rapidly on PDA. Reverse is brown in colour with concentric ring pattern. The size of the colony reaches to a maximum of 9 cms upon incubation for 7 days on 25 °C.

Microscopic characters: The mycelium is branched and septate. Conidiophores are also branched, pale brown in colour and bear muriform or cylindrical conidia. Conidia are pale brown, solitary, narrow at the base with a round beak. The conidial length variesfrom 49.35 to 85.50 µm. The conidia show profuse septations both transverse (4-6) as well as longitudinal. Germ tubes are also seen emerging from the conidia.

***Alternaria tenuissima* (Kunze) Wiltshire** (Fig. 1,ii ; 2, b)

Morpho-cultural characters: The colony is downy, olive green in colour with granular grey surface and grows moderately on PDA. Reverse side is typically brown. Colony size reaches a diameter of 3 to 9 cms upon incubation at 25°C for 7 days.

Microscopic characters:*A. tenuissima* has profusely branched mycelium. Conidiophores are septate and brown in colour. The base of the conidium is round while it tapers towards the apex. The conidia are straight, curved or tapering to a beak and solitary or borne on short chains. The beak can be swollen or pointed at tip. The conidia have both transverse and longitudinal septa. The conidia are olivaceous brown in colour and their size ranges from 60–100 µm.

***Arthrinium phaeospermum* (Corda) M.B. Ellis** (Fig. 1, iii; 2, c)

Morpho-cultural characters: The colony is white, cottony, floccose, spreading and grows rapidly reaching a colony size of approx. 9 cms on PDA when incubated for 7 days at 25°C. The reverse is pale and turns to a tan brown colour as the colony matures. Brown spots are seen on the cottony aerial mycelium in a mature colony.

Microscopic characters: The mycelium bears septate hyphae and the lens shaped conidia are borne in clusters on pale conidiogenous cells. The conidia are small, ovoid to curved, 1 celled and brown in colour and 9–12 µm in diameter often seen with an equatorial germ slit.

***Aspergillus oryzae* (Ahlb.) Cohn** (Fig. 1, iv; 2, d)

Morpho-cultural characters: The colony is green and powdery in appearance and is sparsely spread on the PDA plate. Reverse of the colony is pale. It grows moderately when incubated at 25°C for 7 days.

Microscopic characters: The mycelium bears septate hyphae. The conidiophores are hyaline and terminate into a vesicle. The vesicle has

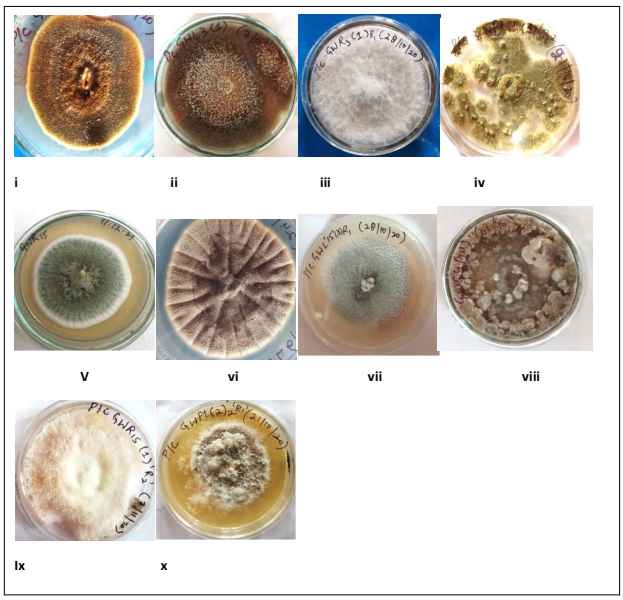

Fig.1. Morpho-cultural characteristics of fungal endophytes isolated from *G. wallichianum.* **(i)** *Alternaria ricini,***(ii)** *Alternaria tenuissima,***(iii)** *Arthrinium phaeospermum,***(iv)** *Aspergillus oryzae,* **(v)** *Aspergillus sydowii,* **(vi)** *Cladosporium herbarum,***(vii)***Colletotrichum gleosporoides,***(viii)***Drechsleraavenae,***(ix)** *Fusarium chlamydsporum,***(x)**,*Nigrospora sphaerica.*

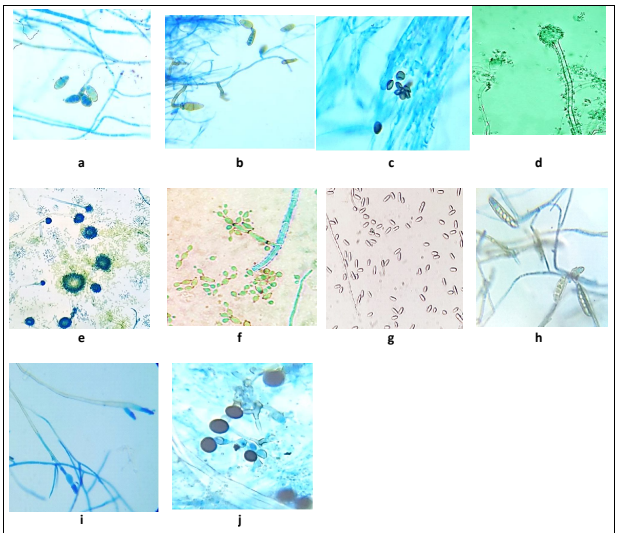

Fig.2: Microscopic characteristics of fungal endophytes isolated from *G. wallichianum.*
(a)*Alternaria ricini,* **(b)** *Alternaria tenuissima,***(c)** *Arthrinium phaeospermum,***(d)***Aspergillus oryzae,***(e)** *Aspergillus sydowii,* **(f)***Cladosporiumherbarum,***(g)***Colletotrichum gleosporoides,***(h)** *Drechslera avenae,* **(i)** *Fusarium chlamydsporum,***(j)**,*Nigrospora sphaerica.*

chains of round conidia attached to it. The conidia are small, round, 5-8 µm in diameter and appear greenish in colour when stained with lactophenol cotton blue during microscopic examination.

***Aspergillus sydowii* Bainier & Sartory** (Fig.1, v; 2, e)

Morpho-cultural characters: The colony is slow growing attaining a size of 1.5 – 2 cm in 7 days when incubated at 25°C in PDA. The colony is initially pale or cream coloured but develops dark green to blue green colour at maturity and has parallel ridges. Dark green to bluish green exudates appear in the center. The reverse side of the colony is pale initially but becomes reddish-brown as the colony matures.

Microscopic characters: Mycelium is septate. Conidiophores are thin, upright and hyaline. The conidiophores bear terminal vesicles that are densely covered in phialides bearing conidial chains. The conidial heads also appear bluish green in colour when stained with lactophenol cotton blue. The conidia are small, round and blue-green in colour and range from 2.5–4.0 µm.

***Cladosporium herbarum* (Pers.) Link** (Fig.1, vi; 2, f)

Morpho-cultural characters: The colony is rapidly growing, velvety in texture with parallel ridges giving it a crinkled appearance and olive grey in colour. The reverse is brown to black with a lighter edge. The colony reaches a size of 5-6 cm on PDA when incubated at 25°C for 7 days.

Microscopic characters: The mycelium consists of septate brown hyphae. The conidiophores are branching, dark and erect. Conidia are small pale brown to brown, elliptical to cylindrical in shape and one or two celled mostly. Conidia occur in branching chains and range from 4- 6 µm in length and 1-4 µm in diameter.

***Colletotrichum gleosporoides* (Penz.) Penz. & Sacc.** (Fig. 1, vii; 2, g)

Morpho-cultural characters: The colony is velvety and light grey in colour, rapidly growing upon incubation at 25°C for 7 days. The colony reaches a maximum of 7–8 cm in a week. The reverse is brown having a rough and wrinkled texture with cracks in the plate medium and has a spiral pattern.

Microscopic characters: The culture bears hyaline, septate and branched mycelium. Conidia

are hyaline, one celled, straight, cylindrical and obtuse at apices. The size of the conidia ranges from 10-15 µm in length and 5-7 µm in width.

***Drechslera avenae* (Eidam) Scharif** (Fig. 1, viii; 2, h)

Morpho-cultural characters: The colony is low, woolly, grey in colour with numerous whitish grey fluffy coremia, especially on the edges. The reverse is completely black or brown-black. The colony diameter reached an average of 6-8 cm on PDA.

Microscopic characters: The mycelium bears delicate hyphae. The conidiophores are pale brown in colour and bear conidia apically and laterally on fertile apical parts. The conidia are cylindrical to oblong in shape and are multidistoseptate with a protruding hilum. The conidia are approximately 45 x 20 µm in size and bear 5 to 6 distosepta.

***Fusarium chlamydosporum* Wollenw. & Reinking** (Fig. 1, ix; 2, i)

Morpho-cultural characters: The colony is cottony, pinkish with white aerial mycelium. The reverse side of the colony is carmine red in colour. The colony generally grew fast on PDA on incubation at 25°C for 7 days.

Microscopic characters: The mycelia were floccose and dense, interspersed with yellowish mycelium towards the center. Conidiophores are mostly short branched bearing microconidia. Macroconidia were not observed even after incubation for two weeks. However, chlamydospores are formed singly or in clusters at intercalary positions

***Nigrospora sphaerica* (Sacc.) E.W. Mason** (Fig. 1, x; 2, j)

Morpho-cultural characters: The colony is rapidly growing, greyish-white, floccose in texture and later develops black spots due to the development of conidia. The reverse is pale with interspersed black spots.

Microscopic characters: The conidiophores are short, simple or branched, hyaline, pale brown. The conidiophores bear single, terminal sub-globose or spherical dark black conidia apically. Each conidium is one-celled, jet black and shiny and smooth in appearance borne on a flat hyaline vesicle at the end of the condiophore, ranging on an average, 16-18 µm in diameter.

DISCUSSION

Our results clearly indicate that 10 genera of fungi constitute endophytic fungal association with the medicinal plant *G. wallichianum* growing in Kashmir Himalaya.In the present study, 10 species of fungal endophytes were isolated and identified based on cultural and microscopic characteristics. These fungal endophytic species were found associated with the different plant parts of *G. wallichianum*. The association of these endophytic fungi as found in the present study may have good impact on the growth and metabolism of the plant as has been reported in similar studies (Khan *et al*. 2012; Xia *et al*. 2015, Khalmuratova *et al*. 2015). Our studies are in conformity with Pelo *et al*. (2020), Qian *et al*. (2014), Silviera *et al*. (2020), Hanin *et al*. (2019) and Santos *et al*. (2015) who have reported association of fungal endophytes with medicinal plants. The association of important endophytic fungal species with *G. wallichianum* as revealed from the present study may influence the ecology and physiology of the host plant in a positive manner thereby increasing the growth of host plant by producing favourable hormones (Muhammad*etal.* 2015), helping in the accumulation of secondary metabolites (Parthasarathy, 2015), play positive role in nutrient cycling(Das and Varma, 2009; Lee *et al.* 2004) and act as strong biocontrol agents against plant pathogens which reduce medicinal plant biomass and cause diseases in medicinal and other plants (Bailey *et al.* 2008; Mejia *et al.* 2008; Hanada *et al.*2008; Paparu *et al.*2009 Ahmed *et* al. 2021; Malik *et al*. 2022). Our study will be helpful to provide information regarding the complex interactions taking place between the fungal inhabitants with their host plants and their impact on the ecology and physiological activity of the host plant. This study will help create a pool of fungal endophytes that can be further exploited

in assessing biocontrol activity of endophytic fungi besides helping in systematizing the asymptomatic fungal endophytes interacting with the different plant parts of *G. wallichianum.*

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DECLARATIONS

Conflict of interest: The authors declare no conflict of interest.

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