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ENDOPHYTIC FUNGI ASSOCIATED WITH TWIGS OF *BUDDLEJA ASIATICA* LOUR.

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ABSTRACT
Endophytic fungi were studied in twigs of *Buddleja asiatica* at two sites within Kathmandu city, Nepal. Sterile mycelia and five fertile taxa (*Alternaria, Fusarium, Epicoccum, Phoma* and *Cladosporium*) were isolated. *Alternaria, Fusarium* and sterile mycelia were isolated from both sites, whereas *Epicoccum, Phoma* and *Cladosporium* were site specific. Colonization frequency, isolation frequency and diversity of fungi were higher at Site II compared to site I.

Keywords: Endophytic fungi, *Buddleja asiatica*, Nepal, temperate.

INTRODUCTION
Endophytic fungi are an assemblage of microorganisms that chiefly belong to class ascomycetes of kingdom fungi. The organisms included in this group dwell inside the above ground tissue of plants [1], and have been reported from almost all climatic regions of the globe viz; tropical, temperate and alpine [2-5]. Different ecological factors such as season [6,7], nearby vegetation and humidity [7-9] influence the distribution of endophytic fungi in the host. Furthermore, levels of air pollution have also been shown to affect the assemblage of these microorganisms [10-12].
Diversity of fungal endophytes have been weakly explored in Nepal, a south Asian country located 28° 00' N and 84° 00' E. As per knowledge of the authors, few studies undertaken have focused mainly on alpine regions [13,14], with the tropical and temperate regions remaining to be explored.

The present investigation was thus carried out to understand the generic diversity of endophytic fungi in twigs of *Buddleja asiatica* growing in temperate region (Kathmandu capital city) of Nepal; and to compare the endophyte assemblages in samples collected from two different sites in the same region. *Buddleja asiatica* was selected for this study as it is endemic to Asia and can be found growing throughout the gap areas of the city.

MATERIALS AND METHODS
Site description and sample collection
Twig samples were collected from two different sites located about 22 km apart, in the month of August. The trees at the first site (Site I, "Thapathali area") were located in the middle of a busy traffic area with no neighboring vegetation. The second site (Site II, "Gaushala area") was situated about 3 km away from the nearest traffic area, and *Buddleja asiatica* trees in this site were located near forest with dense vegetation. For collecting twig samples three healthy looking trees were chosen from each site. From the middle portion of each tree, three mature twig

samples, each 10 cm long, with cracks on the bark were randomly cut. A total of 18 twig samples were collected from all sites, sealed in polythene bags and taken to the laboratory for further analysis.

Processing of twig samples

Twig samples were processed within 24 hours of collection. Each sample was randomly cut into ten 0.5 cm long segments, and surface sterilized by dipping in 75% ethanol for 1 minute, followed by 4% sodium hypochlorite for 3 minutes and finally in 75% ethanol for 30 seconds. The segments were then plated on petri dishes containing 2% malt extract agar (HiMedia Laboratories Pvt. Ltd., India) amended with a broad spectrum antibiotic (Broclox, Time Pharmaceuticals Pvt. Ltd., Nepal) (0.5 g l^{-1}). Rose Bengal (HiMedia Laboratories Pvt. Ltd., India) (0.03g l^{-1}) was also added to the media for suppressing growth of fast growing fungi [6]. The petri dishes were then sealed and incubated at 22±1°C until new fungal colonies stopped appearing from twig segments.

Subculturing and sporulation induction

Fungal colonies growing from twig segments were immediately transferred to petri dishes containing 2% malt extract agar. The plates were then incubated at 22±1°C and regularly checked for sporulation and purity of culture. Cultures that did not sporulate were transferred to low nutrient media (Oat meal agar), and further incubated at 22±1°C under 12 hour light/dark cycle (fluorescent light), and continuously monitored for sporulation.

Identification

Identification of fungal isolates was done based on morphology and spore characters [15].

Colonization and Isolation frequencies

Percent colonization frequency (%CF) of endophytic fungi at each site was calculated according to Petrini and Fisher [16]:

$$\% \text{ Colonization frequency} = \frac{\text{Total number of twig segments colonized}}{\text{Total number of twig segments in that sample}} \times 100$$

Isolation frequency of endophytic fungi at each site was calculated following Taylor *et al.* [6]:

$$\text{Isolation frequency} = \frac{\text{Total number of fungal isolates in a sample}}{\text{Total number of twig segments in that sample}}$$

Relative isolation frequency is used for species abundance, and was calculated as the number of twig segments colonized by a given fungus divided by the total number of segments infected [8].

Statistical analysis

Two-sample-proportion-z test was used to compare colonization frequencies of fungal endophytes from two sites. Mann-Whitney test was used to compare the number of fungal isolates recovered from two sites. For calculating diversity of endophytic fungal genera at each respective site, Shannon-Weaver index was used.

RESULTS AND DISCUSSION

Assemblage of fungal genera

A total of 206 fungal isolates were recovered from 180 twig segments. Some colonies did not sporulate despite attempts to induce sporulation. All non- sporulating colonies were categorized as sterile mycelia. *Alternaria, Fusarium* and sterile mycelia were isolated from both sites, whereas *Cladosporium*, *Phoma* and *Epicoccum* were site specific. The isolated fungal genera are common cosmopolitan endophytic fungi, and have been reported in twigs and leaves of plants from other temperate and tropical regions [6, 9, 11, 12, 17]. Only non-xylaracious fungal taxa were isolated in this study. This is a common trend seen in temperate plants; tropical plants on the other hand have more proportions of xylaracious fungi [18, 19]. Colonization frequencies and relative isolation frequencies of all the fungal genera are given in Table 1.

Table 1- Colonization frequencies (CF) and relative isolation frequencies (RF) of fungal endophytes from twigs of *Buddleja asiatica*

Fungal genera	Site I		Site II		Overall	
	CF (%)	RF (%)	CF (%)	RF (%)	CF (%)	RF (%)
Alternaria	31.1	50.9	56.6	59.3	43.8	56
Cladosporium	2.2	3.6			1.1	1.4
Epicoccum			6.6	6.9	3.3	4.2
Fusarium	37.7	61.8	42.2	44.1	40	51
Phoma			12.2	12.7	6.1	7.8
Sterile mycelia	21.1	34.5	18.8	19.7	20	25.5

Site analysis

There was significant difference in colonization frequencies between two sites, ($z = -5.598$, $P<0.01$) (Fig 1), site II had higher proportion of colonized twig segments compared to site I. There was no significant difference in the number of fungal isolates recovered from two sites (Mann-Whitney test, $P>0.05$), however greater number of isolates were recovered from site II compared to site I (Table 2). Diversity of fungal genera was also higher in site II compared to site I (Table 2).

Table 2- Isolation frequencies and diversity index of fungal endophytes at two different sites

Site	I	II
Total number of twig segments examined	90	90
Total number of fungal isolates recovered	83	123
Isolation frequency	0.92	1.36
Shannon-Weaver index	1.15	1.36

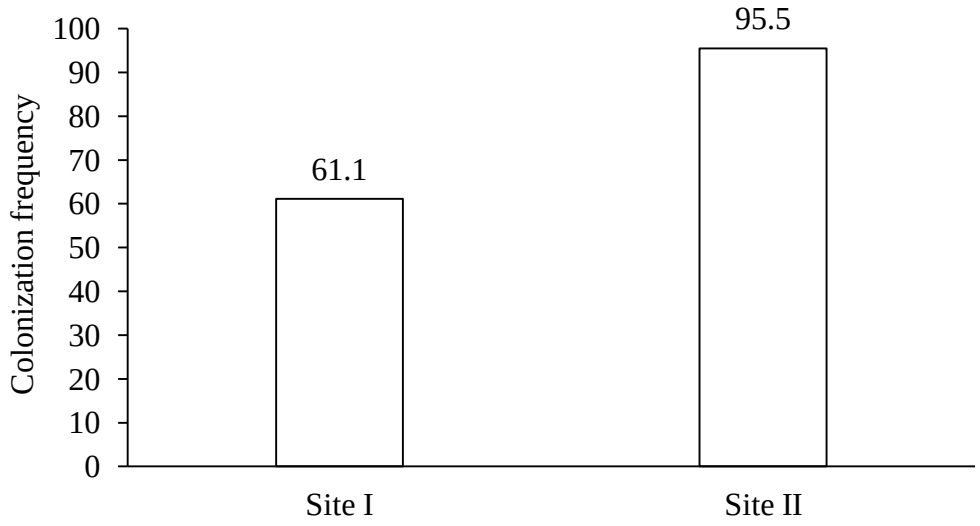

Figure 1 Overall colonization frequencies (%) of fungal endophytes at two different sites

Nearby vegetation affects potential inoculum sources, and humidity of the area. *Buddleja asiatica* trees at Site II were located near a forest; this might be one explanation for high colonization frequency, isolation frequency and diversity of fungal endophytes at this site. Several studies have reported site specific factors such as; nearby vegetation and humidity to influence assemblage of fungal endophytes [7, 8, 20]. Two trees sampled from site II were dwarf with twigs and leaves closer to the ground, which might be another factor for increasing colonization and isolation frequencies at this site. Toofanee and Dulymamode [9] reported high levels of endophyte colonization in leaves of *Cordemoya integrifolia* which were nearer to the ground compared to taller trees.

Along with dense nearby vegetation, site II was located farther away from vehicle emissions compared to site I, which might have reduced the effect of air pollution to a certain degree. This may have positively affected the colonization and isolation frequencies at this site. Air pollution has been reported to influence the assemblage of endophytic fungi inside the tissue of their host. Barengo *et al.*[12] reported low colonization frequency of *Ophiovalsa betulae* on twigs of *Betula pubescens* at high levels of NO_2 concentration. Similarly Kowalski and Gajosek [11] reported high levels of fungal colonization in stem (bark) of *Betula pendula* from pollution free zone compared to stems from low and high pollution zones.
Measurements of factors such as air pollution, humidity and percent cover of nearby vegetation could not be taken in this study, therefore, follow up studies is recommended for more concrete conclusions to be drawn.

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