Impact of Altitude on the Colonization Frequency of Endophytic Fungi Isolated from Rhododendron campanulatum D. Don of Sagarmatha National Park, Nepal

Prabina Rana1,2-3*, Chuenchit Boonchird3, Madan Koirala1 and Dinesh R. Bhuju1,2
1Central Department of Environmental Science (CDES), Tribhuvan University, Kirtipur, Nepal
2Nepal Academy of Science and Technology (NAST), Kathmandu, Nepal
3Department of Biotechnology, Mahidol University, Bangkok, Thailand

Received Date: June 28, 2017, Accepted Date: July 31, 2017, Published Date: August 10, 2017.

*Corresponding author: Prabina Rana, Biological Resources Unit, Faculty of Science, Nepal Academy of Science and Technology (NAST), Post Box 3232, Kathmandu, Nepal, Tel: +977-15-547-714, E-mail: prabinar2017@gmail.com.

Abstract
Endophytic fungi were isolated from leaf, stem, and root of Rhododendron campanulatum samples collected from 3950 masl, 4050 masl, 4150 masl, 4250 masl and 4350 masl of Pangboche of Sagarmatha National Park in eastern Nepal. Out of 1,080 leaf, stem and root segments, 186 fungal isolates were recovered. The overall colonization frequency was 17.22%. Highest colonization frequency was found in leaf (7.22%) followed by root (4.91%) and stem (5.09%) samples. The present investigation showed that the colonization frequency increased with the increase in altitude. Furthermore, the colonization frequency was higher at the tree line and above as compared to below it. Identification by microscopic morphology, the predominant fungi were Penicillium spp., Trichothecium spp., Alternaria spp. and Aspergillus spp. Isolation frequency of the predominant species varied along the altitudinal gradient.

Keywords: Tree line; Elevation gradient; Endophyte; Climate change; Fungi

Abbreviation
masl: Meter above sea level; mm: Millimeter; m: Meter; °C: Degree Celsius; m²: Square Meter; S: Seconds; Min: Minute; DNPWC: Department of National Park and Wildlife Conservation; EvK2CNR: Everest K2 National Research Council of Italy; %: Percentage; CF: Colonization Frequency; CR: Colonization Rate; IF: Isolation Frequency

Introduction
Studies along the altitudinal gradients in alpine ecosystems have shown that plant range are shifting in recent decades due to global warming [1–3]. The effects of global warming have been evident at tree line ecotone with species like Abies spectabilis showing upslope shift in the Himalayan region of Nepal [4].

The genus Rhododendron belongs to the family Ericaceae includes 1200 species distributed throughout Northeast Asia and Eurasia, Western Europe and North America [5–7]. Many of the Rhododendrons of high altitude regions are sensitive and may be susceptible to slight changes in temperature [8,9]. There are 32 species of Rhododendron which inhabit the montane and alpine zones [10,11] of Nepal. Rhododendron campanulatum is a small tree that can reach up to 6 m [12,13]. It grows at alpine and subalpine regions of Nepal and the local people frequently reported that this species has been seen colonizing upper altitude in recent years.

The air temperature falls by 0.6°C per 100 m on an average along the altitudinal gradient [14,15]. Since environment gradients are known to coincide with montane altitudinal gradients the distribution of a plant species could indicate the adaptability of the plant to the environment [16]. Therefore, changes due to climate could be easily observed in a mountainous environment along the altitudinal gradient [17].

Endophytic fungi are defined as fungi dwelling in healthy plant tissues without causing obvious symptoms in the plant [18]. All forest trees in the temperate zones are known to harbor endophytic fungi [19]. Studies on endophytic microbes show that they could influence plant distribution ecology, physiology, and biochemistry. Endophytic fungi can have great influence on the plant community structure by resulting in fitness to the plants under stress caused by increasing temperature and drought effects of global warming [20] allowing them to mitigate the impacts of environmental stresses. Hardly any studies have been geared to study the impacts of climate change on endophytic fungi. It is crucial to study the impacts of climate variability on the same host along the altitudinal gradient. The main aim is to study the response of endophytic fungi of R. campanulatum to altitudinal variations at the tree line of Nepal Himalaya.

Material and Methods
Sample Collection and Isolation

Samples were collected in April–May of 2012 from Pangboche of Sagarmatha National Park which is situated in eastern Nepal (Figure 1). Quadrats (10 m × 10 m) was laid from 3950 masl to 4350 masl at the difference of 100 m with 5 quadrats in each altitude. From each quadrat, three samples of leaf, stem and root were

Figure 1: Map showing the sample collection study plot at Pangboche, Sagarmatha National Park, Nepal.
collected from *R. campanulatum* for isolation of endophytic fungi. Isolation of endophytic fungi was carried according to the method of Hata et al. [21] with minor modifications. The collected samples were washed thoroughly with running water, air dried and cut into small pieces (4 mm diameter for leaf samples and 2–5 mm for root and stem samples). After which the samples were surface sterilized by sequential immersion in 75% ethanol for 1 min, 15% Hydrogen peroxide for 15 min, and 75 % ethanol for 30 s. After drying in blotting paper, the pieces were plated in PDA (Potato Dextrose Agar) slants amended with an antibiotic to suppress bacterial growth. The slants were incubated at 20 ± 2 °C until fungal growth appeared. Hyphal tips of emerging colonies were transferred to fresh PDA plates. To determine sterilization method was effective imprints of the cut fragments were made on PDA plate no fungal growth after incubation assured that the sterilization method was effective [22].

**Identification of Endophytic Fungi**

Endophytic fungi were identified on the basis of their morphological and microscopic characters using standard identification manuals [23].

**Colonization Rate (CF) and Isolation Frequency (IF)**

Colonization Frequency (CF %) /Colonization rate (CR) and Isolation frequency (IF) was calculated as described by Petrini et al. [24]. Colonization Frequency (CF) is equal to the number of segments colonized by an endophyte divided by the total number of segments analyzed × 100. Isolation frequency (IF) is the sum of isolation number of leaf, stem and root segments of a given taxon divided by the total number of segments × 100.

**Results and Discussion**

Endophytic fungi were isolated from leaf, stem, and root of *Rhododendron campanulatum* samples collected from 3950 masl, 4050 masl, 4150 masl, 4250 masl and 4350 masl from Pangboche of Sagarmatha National Park, Nepal. Out of 1,080 leaf, stem and root segments 186 fungal isolates were recovered. Some of the endophytic fungi identified morphologically are *Paeciliomyces* sp., *Epicoccum* sp., *Truncatella* sp., *Penicillium* spp., *Alternaria* spp., *Trichothecium* spp., *Aspergillus* spp. and *Fusarium* spp. The overall colonization rate was 17.22% and the colonization rate increased with altitude. Various studies show that the colonization rate or frequency varies among host plant; the colonization rate of *Tripterygium wilfordii* was 57.8% [25], *Boswellia serrata* was (11.46-24.1%) [26] and *Coscinium fenestratum* was 24% [27].

Highest colonization frequency was found in leaf (7.22%) followed by the stem (5.09%) and root (4.91%) samples (Figure 2a). Broad leaf characteristic of this species may have resulted in higher colonization frequency in the leaves. The colonization rate of endophytic fungi isolated from *Woodfordia fruticosa* was reported to be higher in leaf than in stem and roots [28]. Higher prevalence of endophytes in leaves than in branches of traditionally used medicinal plants in Malaysia were also reported [29]. Colonization frequency was influenced by altitude. Colonization frequency in leaf, stem and root was higher at the tree line (4150 masl) and

![Figure 2: (a) Colonization frequency of endophytes in stem root and leaf, (b) Colonization frequency along the altitudinal gradient, (c) Isolation Frequency of dominant strains and (d) Isolation frequency along the altitudinal gradient.](attachment:image-url)
above (4250 masl and 4350 masl) (Figure 2b). The frequency of endophytes varied over environmental gradients indicating that their distributions are affected by climate. Varying results have been observed in studies conducted to assess the response of fungal assemblage in the same host at different altitudes. Some studies have shown mixed response where the isolation frequency was not affected or increased or decreased with altitude [30,31]. A positive correlation between fine root endophytic fungi with altitude was observed in a study conducted in Norway [32]. The presence of endophytic fungi has been reported to be higher in plants in drought-stressed compared to mesic natural systems [33,34]. Plants have been reported to benefit to temperature rise in the presence of endophytic fungi than in its absence [35]. The present investigation showed that the colonization frequency increased with the increase in altitude. Furthermore, the colonization frequency was higher at the tree line and above as compared to below it. Increase in colonization rate at and above the tree line indicates that there could be a relation between higher colonization frequency and establishment and survival of R. campanulatum at the harsh terrain of tree line ecotone under the context of climate change. Further detail studies are required. Plants colonized by endophytes have greater resistance to disease and stress conditions as compared to non-colonized plants [36]. Endophytic fungi have played a significant role in assisting the plants to cope with stressed environmental conditions [37-40]. Endophytes may play various roles in natural ecosystems, therefore, climate induced changes could affect forest ecosystems [41,42]. Strong winds open canopy at the tree line ecotone may have favored the dispersal of fungal spores and endophytes could have helped in establishment and survival of the species facilitating its upward movement as observed by the local people.

Identification by microscopic morphology, the predominant fungi were Penicillium spp., Trichothecium spp., Alternaria spp. and Aspergillus spp. Isolation frequency of Alternaria spp. was highest followed by Trichothecium spp., Penicillium spp. and Aspergillus spp. (Figure 2c). These predominant species were present in all the altitudes studied. Altitude also had an impact on the isolation frequency of the predominant fungi (Figure 2d). The isolation frequency of leaf, stem, and leaf differed along the altitudinal gradient (Figure 2b) as reported by [39]. It was observed that the frequency of predominant fungi varied in leaf, stem, and root along the altitudinal gradient with higher frequency in leaf followed by root and the stem. The presence of Penicillium spp., Alternaria spp. and Aspergillus spp. was reported in Rhododendron anthropogon [43] and other plants [44]. Alternaria, Penicillium and Fusarium genera are characteristic of the Alpine region [30]. Other studies have also shown that a huge number of endophytes can be isolated from a given host but only a few species are dominant [45]. Acremonium sp., Alternaria sp., Colletotrichum sp., Fusarium sp. and Pestalopsis sp., were isolated from leaves of Quercus acuta from different altitudes from Mt Takao of Japan. In this study, only the Isolation frequency of dominant fungi was compared at different altitudes [31]. Another study focused on the comparison of fine root endophytic fungi along the altitudinal gradient [32]. Some endophytic fungi are common in leaf, stem, and root but others could be specific to the tissue type in different hosts [46].

**Conclusion**

Endophytic fungi have played a crucial role in assisting the plants to survive under stressed conditions allowing adaptation of plants to various climatic stress created by global climate change. In the present study, the overall colonization frequency was 17.22% and the colonization frequency increased with altitude. Highest colonization frequency was found in leaf (7.22%) followed by the stem (5.09%) and root (4.91%) samples. The colonization frequency in leaves, stem, and root was higher at the tree line and above as compared to below it. Increase in colonization frequency at and above the tree line indicates that there could be a relation between higher colonization frequency of endophytes and the establishment and survival of R. campanulatum at the harsh terrain of tree line ecotone under the context of climate change. However, the further detailed study is necessary.

**Acknowledgements**

The authors would like to express their sincere thanks to Ev-K2-CNRI and Nepal Academy of Science and Technology for funding their field works. Thanks also go to Central Department of Environmental Science, Tribhuvan University for supporting this research. Last but not least we would like to Department of National Park and Wildlife Conservation, for giving us permission to conduct this research.

**Conflict of Interest**

Authors declared that they have no conflict of interest.

**References**