



Aerial Nodulation in *Hippophae salicifolia* D. Don (Eleagnaceae) and its Ecological and Medical Implications in the Alpine Regions of Sikkim Himalaya

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Abstract

Seabuckthorn (Eleagnaceae), used in different countries for the production of several medicines, cosmetics and food products. It is rich in vitamin C, vitamin A, E and K, protein, organic acid, carotenoids, flavonoids and steroids. Simultaneously, *Hippophae* L. is actinorhizal plants and fix N_2 in symbiosis with *Frankia*. *Hippophae salicifolia*, *H. rhamnoides* and *H. tibetana* occur naturally in the Himalaya, of which *H. salicifolia* is endemic to Indian Himalaya. Surveys were conducted to study ecology of *Hippophae salicifolia* in its distribution ranges in Lachen and Lachung valleys of North Sikkim (India). We recorded the occurrence of aerial nodules in *Hippophae salicifolia* located at Teeling, Lachung. Microscopic observations showed *Frankia* in the aerial nodules of *Hippophae salicifolia*. We suggest that development of aerial roots around wound sites and crevices on the trunk (rhytidome) paved the way for aerial nodulation in the species. We report the first aerial nodulation in *Hippophae salicifolia* from Himalaya.

Since *Hippophae salicifolia* is a pioneer species to colonise N₂ poor soils and exposed surfaces, its application in checking land erosion and retention of soil fertility is of paramount importance in the Himalayan ecosystems.

Keywords: Actinorhizal Plants, *Hippophae salicifolia*, Aerial Nodules, *Frankia*, Sikkim Himalaya.

Introduction

Seabuckthorn (*Hippophae* sp) has been used as food, beverages and medicine by the humans since decades, especially in Russia and China. Sea buckthorn has attracted attention world over due to its nutritional and medicinal values. The diversity in biochemical and nutritional expressions shown by this plant are due to its distribution, origin, climate and methods of extraction. Bioactive compounds like ascorbic acid, carotenoids, tocopherols and phenols are antioxidants, and compared with other fruits. Seabuckthorn has been demonstrated having anti oxidant, anti bacterial, anti ulcer, anti cancer, anti hypertensive, anti inflammatory activities [1].

The genus *Hippophae* L. (Family: Eleagnaceae), commonly known as sea-buckthorn, is a dioecious, actinorhizal and wind pollinated thorny shrub or small tree [2-4]. A total of 7 species and ten sub-species are distributed worldwide, of which 6 species are restricted to Asia and one species, viz., *Hippophae rhamnoides* L. is distributed in the Northern Hemisphere ecosystems ranging from Asia to Europe [5-7]. Several studies have reported that *Hippophae rhamnoides* L. was introduced to South and North America [8-12]. All the species of *Hippophae* are diploids with the chromosome number $2n = 24$ [13-14]. Generally, sea-buckthorns are fast growing woody plants, which are important for both economic and ecological purposes in the ecosystems of the northern prairies and temperate and subalpine regions of the Himalaya due to its ability to fix atmospheric nitrogen symbiotically with *Frankia* bacteria [15-17]. *Frankia* (Frankiaceae) is a common soil actinomycete, which infects a diverse range of woody plant species forming dinitrogen-fixing root nodules. The symbiotic relationship of sea-buckthorns-*Frankia* helps soils maintaining high N₂ content and fast growth rates in low nutrient and/or disturbed habitats [17,18].

In Indian Himalaya, three species of *Hippophae*, viz., *H. salicifolia* D. Don, *H. rhamnoides* L. sub-species *turkestanica*, and *H. tibetana* Schl. are naturally distributed in the temperate and alpine regions of Ladak, Himalchal Pradesh, Uttarkhand, Sikkim and Arunachal Pradesh, of which *H. salicifolia* is endemic to the Indian Himalaya [2, 18, 19]. In Sikkim Himalaya, *Hippophae salicifolia* D. Don occurs naturally in Lachen and Lachung valleys of North Sikkim ranging from 2300 to 3500 m elevations. It prefers to grow along the riversides and on non-riverine fragile slopes in these valleys. Furthermore, field observations in Lachen and Lachung valleys have shown that sea-buckthorns can be planted by stem-cuttings.

Normally, nodulation in *Hippophae* species takes place in root system within soil and fixes atmospheric nitrogen with the help of *Frankia* bacteria resulting in fertility of soil [20-22]. However, a strange type of nodulation, i.e., aerial nodulation was reported by many workers, such as, Arora [23] reported on aerial nodulation in *Aeschynomene* spp., followed by Yatazawa and Yoshida [24] and Alazard [25] in the same species. Dreyfus *et al* [26] reported the aerial nodulation in *Sesbania* species. Walter and Bien [27] published another type of aerial nodulations on the adventitious roots of *Pentaclethra macroloba*. Prin *et al* [28] reported the occurrence of the first actinorhizal stem nodulation in *Casuarina cunninghamiana*.

In the present study, we report the first aerial nodulation on the trunk of *Hippophae salicifolia* from the Indian Himalaya. Field investigations and laboratory results confirm the presence of *Frankia* bacteria in the aerial nodules. Further, we explain the possible reasons of aerial nodulation in *Hippophae salicifolia* and its ecological importance to further the study.

Materials and methods

Study area

In the present investigation, we considered the natural distribution range of *Hippophae salicifolia* in Lachung and Lachen valleys, which are located in North Sikkim in the Eastern Himalayan Region of India (Figure 1). The landscape in Lachung and Lachen valleys is nestled within the cap of colossal mountain peaks and characterised by mountainous terrain with the elevation starts from around 2300 m amsl. Primarily, the temperate flora dominate

the lower stretch and the subalpine flora predominate the upper reaches in the region. The Teesta river flows through the Lachen valley with Zemu river as the largest tributary, on the other hand in Lachung valley, the Lachung river with several small tributaries trickles down leading to make the confluence with the Teesta river at Chungthang. Comparatively, Lachung valley has higher number of human population than the Lachen and they are settled in small colonies in the valleys. Majority of Lachungpa and Lachenpa of Bhutia community, the original (local) inhabitants of Lachung and Lachen valleys, respectively, are farmers and their economy used to depend on pastoralism before the tourism industry came into existence in Sikkim. However, pastoralism still practices in these valleys. In addition to local community, defence personnel of India have also established many posts in the valleys.

Sampling

We undertook explorative surveys to locate *Hippophae salicifolia* in Lachung and Lachen valleys during 2010-2012. We recorded the population of *Hippophae salicifolia* in Chaten, Lachen, Zema I, Zema II, Zema III and Kalep in Lachen valley and from different locations including Teeling in Lachung valley. For each location, we covered approximately 300 to 400 hectare area both on the riversides and non-riversides of the valleys.

We recorded a few aerial nodules on the trunk of *Hippophae salicifolia* tree in Teeling area, which was located around 2-3 km away from Lachung (Figure 2). After locating the first aerial nodule, we inspected nearly all individuals of *Hippophae salicifolia* from all locations to further record the aerial nodule. Our extensive physical verifications in Lachen and Lachung valleys showed that only one *Hippophae salicifolia* tree was found to bear two bunches of aerial nodules. The tree bearing aerial nodules was located between 27° 47' 32" and 27° 43' 57" N latitude and 88° 33' 07" and 88° 33' 13" E longitude. The girth of the aerial nodules bearing tree trunk was measured about 3.8ft and found to be approximately 40-50 years old.

A portion of aerial nodules was carefully removed from the trunk of *Hippophae salicifolia* with the help of a pointed knife and forceps. The collected nodule samples were immediately placed in glass vials (Borosil) containing DPM (defined propionate minimal medium) of Baker and O'Keefe [29] and stored in ice box. The samples were labelled properly and transported them as early as possible at Biotechnological Application and Research Centre laboratory, Sikkim State Council of Science and Technology, Sajong (Rumtek), East Sikkim for further study.

Histological study

Thin transverse sections of the aerial nodule samples were made in grease-free clean slides with the help of razor blades. The sections were satined with the methylene blue dye and observed under the light microscope (Leica).

Isolation of Frankia from aerial nodules and its in vitro culture

Soils adhered in the nodule samples were removed with the help of a paint brush; the lobes were separated and brushed carefully in a running tap water. The nodule samples were again washed with sterile distilled water adding a drop of tween-20 per litre and it was done for 15 minutes with constant shaking. Sterilization of the nodule sample was carried out by dipping it in 0.01% HgCl₂ and shook for 1 minute. The procedure was repeated for three times. The nodule samples were further sterilized with 30% hydrogen peroxide for 1-2 minutes and rinsed with sterile distilled water for three times. Final sterilization of the nodule sample was done with 70% alcohol for 1 minute and rinsed with sterile distilled water. The sterilized nodule samples were placed on the sterile petriplate, cut into 3-4 small pieces and placed into the DPM medium. The cultures were incubated at 28° C without light in the incubator (REMI) and left for observations at regular intervals.

Results

In situ observations on aerial nodulation

For the first time, we recorded the aerial nodulation on the trunk of *Hippophae salicifolia* from the Himalaya. After thorough examination of all the individuals of *Hippophae salicifolia* naturally inhabiting in Lachen and Lachung valleys, North Sikkim, we found only a single plant that bore two bunch of aerial nodules in the cavity at bifurcate site of the tree (Figure 3). The cavity at bifurcate site where the aerial nodules recorded was found to be partially covered with decayed leaf litters and other humus and organic matters. On close observation, we revealed that the aerial nodules were formed on the arial roots developed at the site (nodulation site) of bifurcate tree trunk. The periphery of the actively growing nodules was surrounded by aged-old dead and decayed nodules (Figure 3). Investigations for locating more aerial nodules, we recorded a bunch of hanging nodules right above the

previous nodules on the same tree trunk (Figure 4). The aerial nodules were formed on a short and sturdy aerial root developed on crevices of the rhytidome. The nodule was found to be developed on the tree trunk at about 1.5 m height above the soil surface. However, further investigations in the same locality did not record the occurrence of aerial nodules in *Hippophae salicifolia*.

The tree bearing aerial nodules was located on the side of a free-flowing stream in Teeling, near Lachung valley and the main associate species of the tree recorded were *Abies* sp., *Tsuga* sp., shrubby *Rhododendron* spp. *Arisaema* spp., *Primula* spp., etc.

The formation of aerial nodules is reported to be the first of its kind in *Hippophae* and has generated a curiosity for locating more aerial nodules in the genus from other distribution ranges.

Histological observations

The present study was aimed to confirm the presence or absence of infecting microorganism in aerial nodules of *Hippophae salicifolia* and not to record detail structures of the nodules. Morphologically, the aerial nodules showed bigger in size than the root nodules. Histological examinations of the transverse sections revealed the presence of many infected cells in the parenchyma cells under the light microscope (Figure 5). The infecting microorganisms demonstrated the characteristic features of actinimycete similar to *Frankia* consisting of colonies with hyphae and sporangia. A preliminary observation showed similarity in histological features between aerial and the root nodules. However, the transverse sections could not reveal the presence of vesicles in the aerial nodules under the light microscope.

In vitro culture observations

In order to confirm the presence of *Frankia* strains in the infected aerial nodules of *Hippophae salicifolia*, we inoculated the nodules in DPM medium for *in vitro* culture of *Frankia* strains. After 30 days of inoculation, a small and cottony flex like floating structure was observed in the media. The frankial growth was further sub-cultured in the same fresh media within 7-8 months. After staining the frankia cells with methylene blue, many interwoven hyphal colonies of *Frankia* were observed under the light microscope. We

recorded visible hyphae, sporangia and vesicles from the samples cultured on media that demonstrated the presence of *Frankia* in the aerial nodules of *Hippophae salicifolia* (Figure 6). Sporangia were septate and spherical. We recorded two types of sporangia, viz., the terminal and intercalary sporangia in the hyphae. The average diameter of vesicles was recorded to be of 3.0 μm and the sporangia recorded the average diameter of 10 μm .

Discussion

Aerial nodulation and nitrogen fixation

In the genus *Hippophae*, aerial nodulation is a rare phenomenon. Recently, we recorded the aerial nodules on the trunk of *Hippophae salicifolia* tree. This is the first record of aerial nodulation in *Hippophae salicifolia* from the Himalaya. Our observations in *in situ* habitats showed that all the aerial nodules were formed on the lateral roots developed on the trunk of a tree, which was approximately of 40/50 years old. Interestingly, the tree had well developed thick rhytidome (bark) with crevices and humus and organic matters mainly of lichens and mosses adhered to it, which suggests that they help retain moisture for a long period of time and maintain a good humidity throughout the year. The presence of such moisture keeping elements might have triggered to develop lateral roots in the crevices that led to the creation of micro-environment favorable to form aerial nodules in the old tree. Similar observation is reported in *Cassurina* spp. by Prin *et al* [28]. In the first aerial nodule (Figure 3), the recently developed white fresh nodules in the cavity were covered by the outer-layered dead and decayed nodules, which can be seen as stack of black masses. Presence of such dead and decayed nodules clearly demonstrates that nodules were formed years ago. Prin *et al* [28] have also reported the deposition of aged-old degraded and decayed nodule mass around the fresh aerial nodules in *Cassurina cunninghamiana*. However, the second aerial nodule was found to be hanging on a short and sturdy lateral root developed in the crevices of rhytidome covered by mosses. Studies on *Cassurina cunninghamiana* have reported that sufficient rainfall, air humidity and presence of rhytidome are important factors responsible for inducing aerial nodulation [28]. It is known that root nodulations normally occur for symbiotic nitrogen fixation in actinorhizal plants; however, some plant species have evolved aerial nodulations to establish microsymbiotic relationships with the host plants in response to local and flooding environment [27,30].

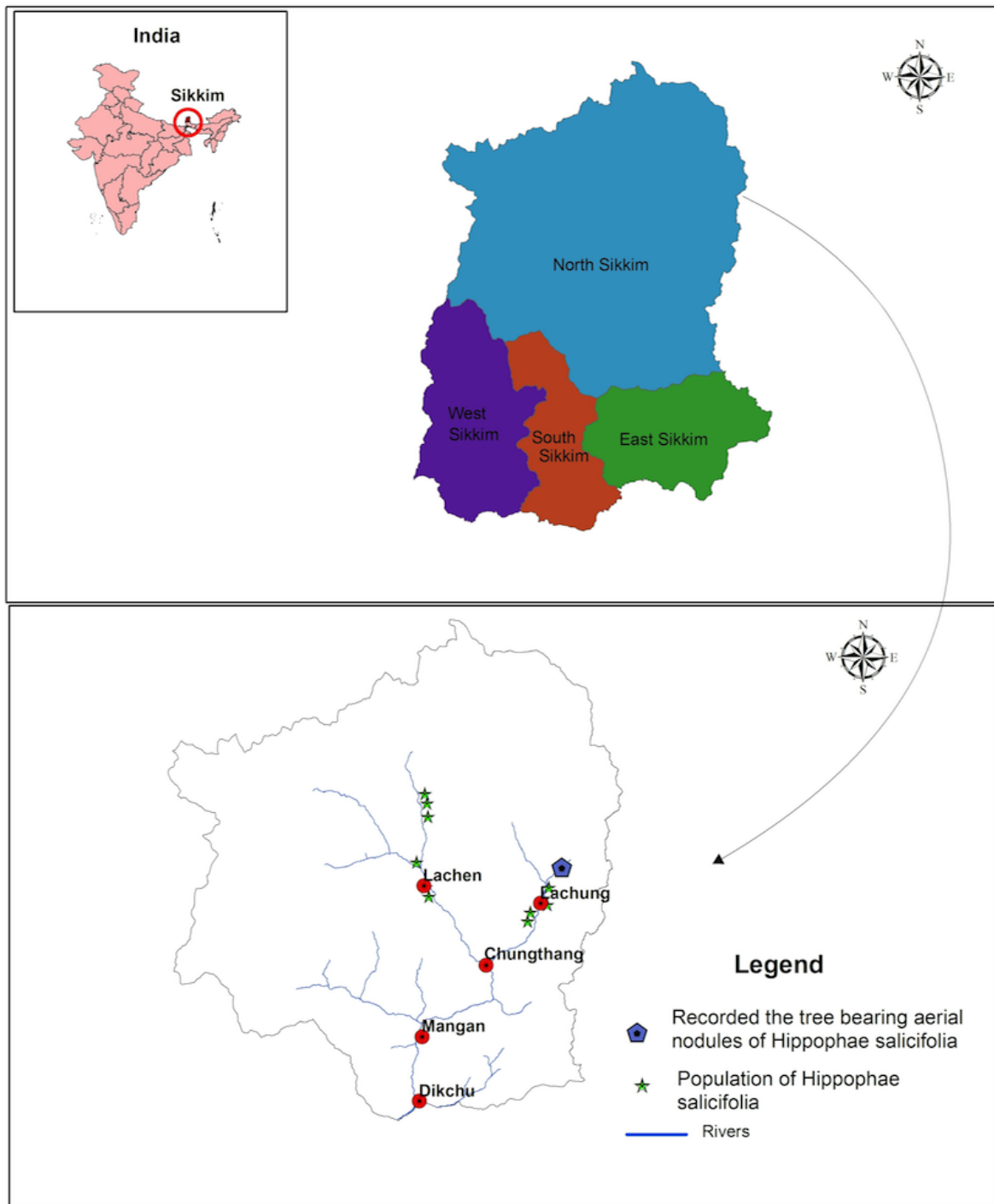


Figure 1: The map showing distribution of *Hippophae salicifolia* L. (Eleagnaceae) in Lachen and Lachung valleys of North Sikkim (India). Locations of *Hippophae salicifolia* population are marked with * sign. Lachen valley recorded the higher number of *Hippophae salicifolia* population and their population sizes.

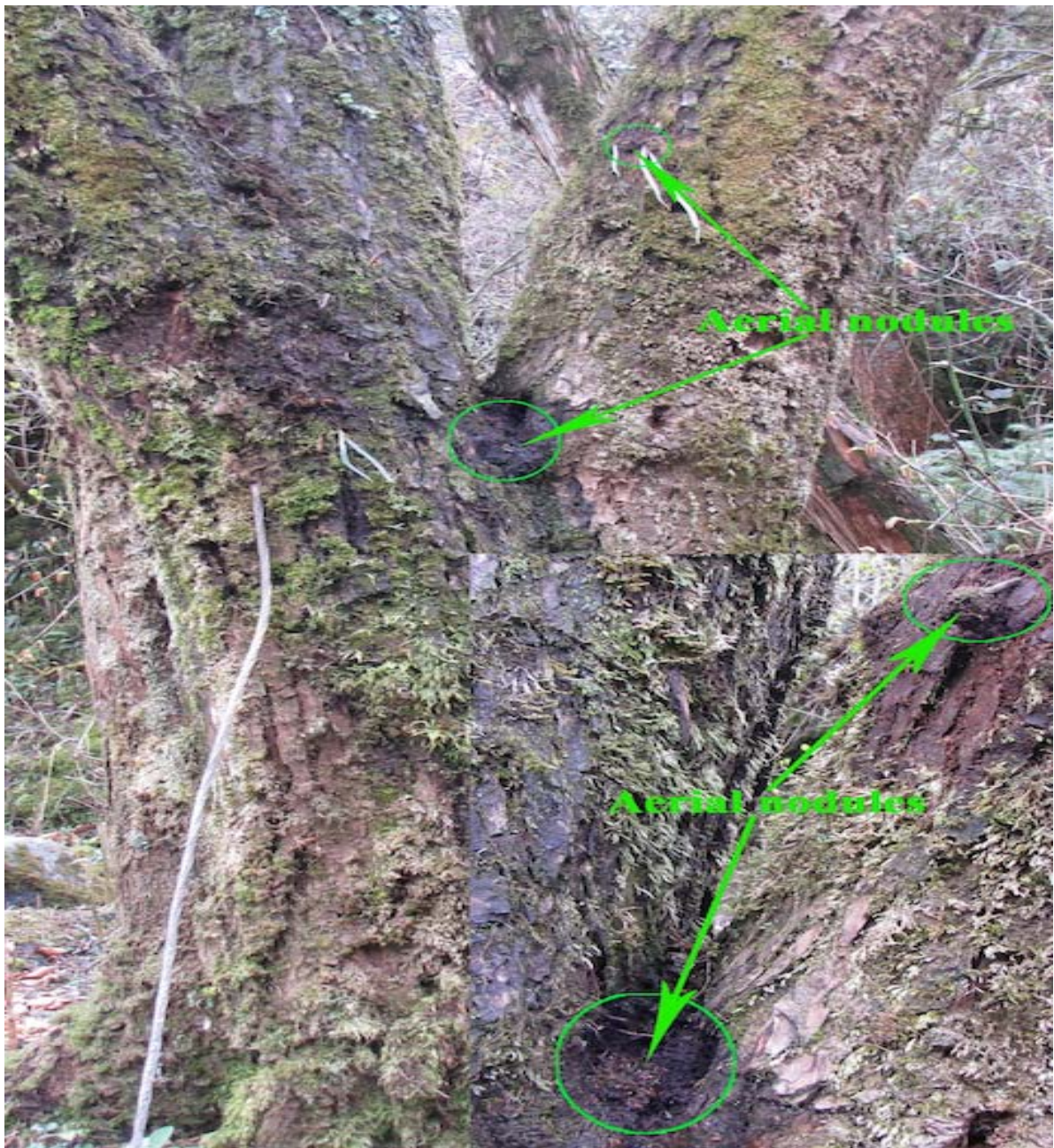


Figure 2: An aged-old tree trunk (rhytidome), approximately 40-50 years old, bears aerial nodules at the bifurcate site and on the trunk (rhytidome) of *Hippophae salicifolia*. Aerial nodules were formed on the aerial roots developed from the wound sites and crevices on the trunk. The tree was located at Teeling, 2-3 km away from Lachung, North Sikkim.



Figure 3: Aerial nodules at the bifurcate site of trunk recorded in *Hippophae salicifolia*. Recently developed aerial nodules in the centre (see inset) are surrounded by the black mass of old, dead and decayed aerial nodules.

Since the population of *Hippophae salicifolia* naturally colonise in nitrogen poor loose, marginal and flooded soils in cold temperate and sub-alpine regions of Lachen and Lachung valleys in North Sikkim, aerial nodulation probably allows plants of *Hippophae salicifolia* to fix N_2 more efficiently in such nitrogen-poor environments as has been documented on stem nodulation in legumes [31-33]. In the other hand, Ladha *et al.* [34] have suggested that stem (aerial) nodulation in actinorhizal plants seems to be a clever evolutionary adaptation to waterlogged conditions. *Frankia* infected stem (aerial) nodules are reported in *Casuarina equisetifolia*, *C. cunninghamiana* (Casuarinaceae) and *Hippophae salicifolia* (Eleagneceae).

The aerial nodulation occurs only in a few aged-old host plants, which suggests a host genetic specificity for the establishment of symbiotic relationships [28].. However, close



Figure 4: A sturdy aerial root developed from crevices on the trunk (rhytidome) of *Hippophae salicifolia* bearing aerial nodules. The hanging aerial nodule was covered by lichens and mosses suggesting they help retain moisture for a longer period in the nodule.

examinations at the nodulation sites of the aged-old *Hippophae salicifolia* tree clearly demonstrate that the aerial nodules develop on aerial roots and do not attach with the stem (trunk) surface. We also observed in Lachen and Lachung valleys that vegetative propagation can be achieved by planting stem-cuttings, which undoubtedly shows the evidence of root formation from cut area on stem. It is most likely that the aerial roots developed at around wound sites on the rhytidome of *Hippophae salicifolia* and initiated the aerial nodulation creating same micro-habitats as the root nodulation using the organic matter and humus

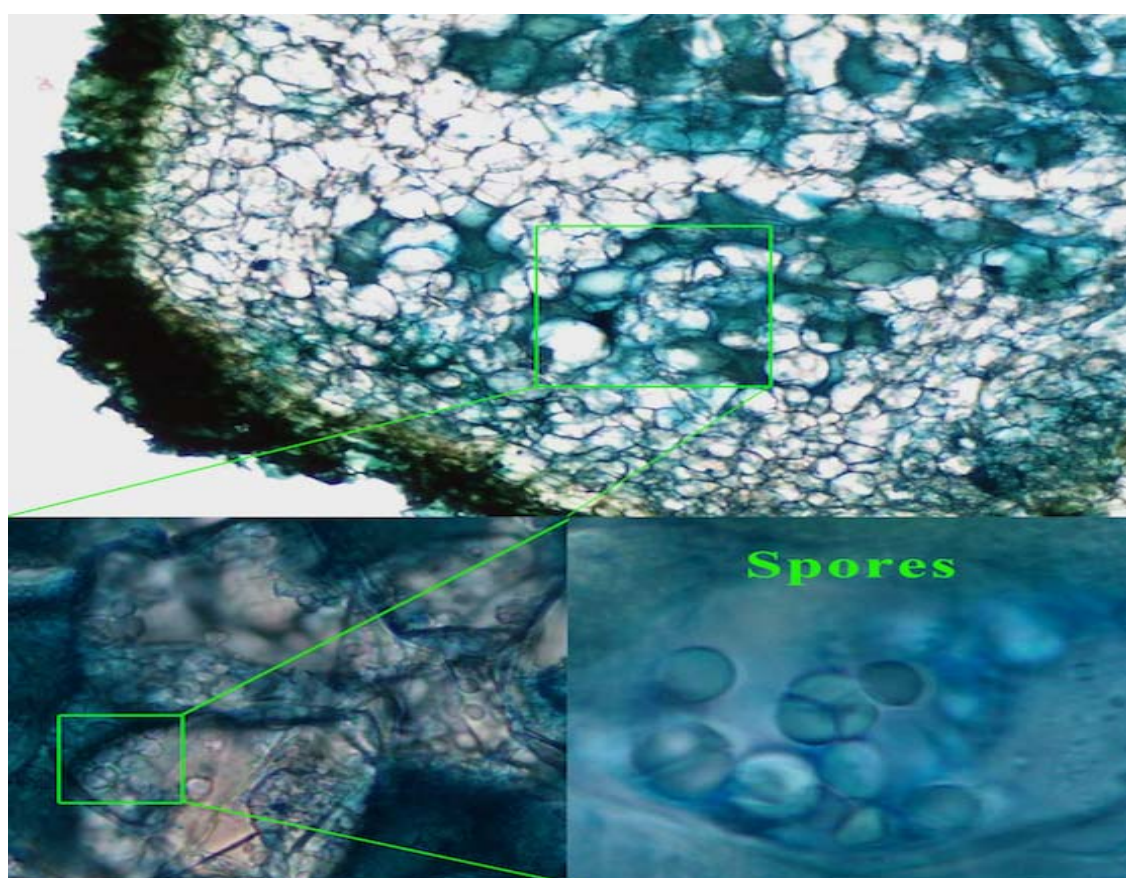


Figure 5: A portion of the transverse section of aerial nodule showing the plant cells infected with *Frankia* under the light microscope. Histological observations did not reveal the structure of hyphae, vesicle and sporangia, which are the characteristics of the *Frankia*. However, many young spores were recorded within plant cells (see enlarged view) suggesting the presence of *Frankia* in the aerial nodules.

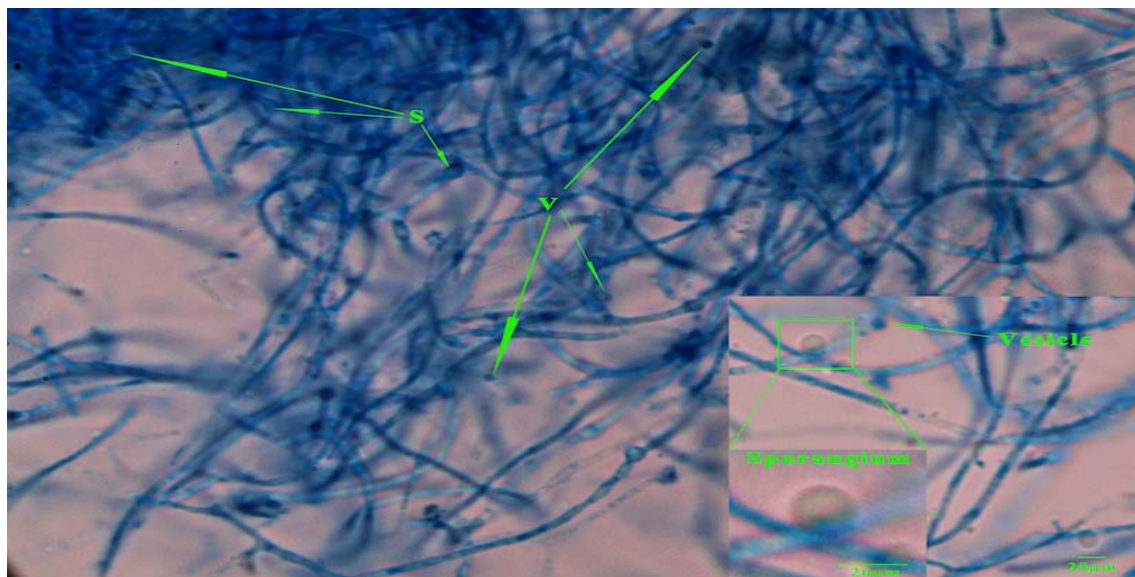


Figure 6: Microscopic observations of the *Frankia* grown on *in vitro* media (DPM) showing the characteristic features of *Frankia*, viz., hyphae, vesicles and sporangia. The results confirm the presence of *Frankia* in the aerial nodules of *Hippophae salicifolia*.

resources from lichens and mosses grown on the trees, which substantiates the role of external factors than the specificity of host-genetic effects. Based on these preliminary examinations, we further hypothesise that older actinorhizal plants develop more aerial nodules at wound sites than the younger individuals to evolve an adaptive strategy for fixing N_2 and readily available it to the aging aerial parts of

the plant for better functions, because supplying N_2 from root nodules that have become weak and lost its vital functions is a tedious journey. This assumption is substantiated by the production of small quantum of root nodules in the older trees of *Hippophae salicifolia* (personal observation). However, detail studies on genetics and ecological aspects of aerial nodulating organisms and host-plants and the relation between them are needed to test many hypotheses on the symbiosis of actinorhizal plants.

Characterization of Frankia

Frankia strains generally infect root nodules in actinorhizal plants. In contrast, we report here

the *Frankia* infected aerial nodules in *Hippophae salicifolia*, which is not common in the genus. In *Hippophae salicifolia*, aerial nodules are similar in morphology and structure to root nodules except the former is bigger in size, which upholds the observation reported in *Aeschynomene fluminensis* [35]. The occurrence of larger aerial nodules may help in enhancing N₂ fixation, and thereby increase the stem growth in *Hippophae salicifolia*.

In the infected cells of aerial nodules, septate sporangia are evident but not hyphae and vesicles in the cross sections. Studies have reported that hyphae and vesicles are encapsulated with plant cell wall-like material and remain outside the host plasmalemma [36,37]. It is further reported that, within the nodule, *Frankia* penetrate cell walls producing hyphae and spread disorganized in host cells [38]. However, microscopic examinations of the infected tissue of aerial nodules cultured on media show hyphae, sporangia and vesicles, which clearly demonstrates the characteristic structures of *Frankia*. Sporangia are septate, spherical and multilocular in *Hippophae salicifolia*. Studies have shown that *Frankia* strains possess septation in hyphae and produce both terminal or intercalary multilocular sporangia [39,40]. In culture, multilocular sporangia are produced by all *Frankia* strains [39,41-42]. Furthermore, sporulation in some nodules in *Myrica* and *Alnus* [43,44] suggesting that septate enlarged into multi-locular sporangia comprising of many spores in the nodules of *Hippophae salicifolia*. *Frankia* bear large, spherical and septate vesicles in *Hippophae salicifolia* similar to *Alnus* and *Elaeagnus*, wherein *Frankia* are large, spherical and multiseptate vesicles [45,46]. The diameter of vesicles in *Hippophae salicifolia* is within the range as reported on *Frankia* of *H. rhamnoides* [47]. It is well established fact that vesicles are the most definitive character of *Frankia*, which clearly confirms the presence of *Frankia* strains in the aerial nodules in *Hippophae salicifolia*.

Ecological implications

Actinorhizal plants bear *Frankia* infected nodules and the presence of host plants is essential in maintaining and amplifying *Frankia* populations except in a few cases. These plants are useful for reforestation and reclamation especially of marginal soils [18]. Among the actinorhizal plants, sea-buckthorns are a wind pollinated angiosperm [48] and it colonizes quickly in new soils and exposed surfaces [18]. Sea-buckthorns are reported to have shown

superior properties in afforestation, soil conservation and waste land reclamation compared to other actinorhizal plants because of its extensive root system [49]. Several studies suggest that sea-buckthorn trees have potential applications in reclaiming and conditioning soil, producing timber and pulp, and acting as nurse, windbreak, ornamental and fuel wood plants [50,51].

Actinorhizal plants serve as pioneer species in successional plant community development [40]. For example, temperate and sub-alpine environment of the Himalayan mountain ecosystems allow the generous growth of actinorhizal symbiosis, and use of the actinorhizal plants can definitely contribute to the pressing problem of deforestation in the region. Sea-buckthorns, which do not require chemical fertilizers and are able to colonize successfully even in marginal soils, are fast-growing N₂ fixing trees and these can be the best choice of checking land erosion and retain the soil fertility. The recent earthquake in 18th September, 2011 with the epicentre near the base of the Mt. Kanchendzonga has caused extensive landslides in the region. Post earthquake observations in these two valleys have revealed that the formation of *Hippophae* seedling colonies in the exposed surface areas (personal observation). This clearly reflects the ecological significance of *Hippophae* in the temperate and alpine ecosystems of the Himalaya. Studies have also reported that trees with aerial nodules fix N₂ for the whole life and are assumed to be efficient soil improvers. Our preliminary *in situ* observations have already shown that aerial roots can be developed around wound sites on the trunk of *Hippophae salicifolia*, which suggests that production of aerial nodulating trees of *Hippophae* spp. is achievable in near future. However, a detail study is also required on *Frankia* strains infecting aerial nodules because these strains can do a good job of producing natural bio-fertilizer to other plants.

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