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RESEARCH ARTICLE

Host tree specificity and seed germination of *Dendrobium aphyllum* (Roxb.) C.E.C. Fisch. in Sri Lanka

H.B. Chathurika Harshani[1], S.P. Senanayake[1*] and H. Sandamali[2]
[1] *Department of Botany, Faculty of Science, University of Kelaniya, Kelaniya.*
[2] *Institute of Fundamental Studies, Hantana Road, Kandy.*

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Abstract: Tree species can be considered as hosts for many epiphytes and the orchids, as epiphytes, display a biased distribution among hosts. It is possible that the barks of these hosts have some chemical and physical characteristics that influence the seed germination of orchids. The dependence on symbiotic fungi for seed germination is also a crucial factor for orchid distribution. *Dendrobium aphyllum* is an orchid species with restricted distribution in the sub - montane region of Sri Lanka. The aims of this study were (1) to determine the host tree specificity of the epiphytic *D. aphyllum* using physical and chemical bark characteristics of the host, (2) to isolate and identify the symbiotic fungi in *D. aphyllum* roots and (3) to determine the effect of tree bark extracts on symbiotic seed germination of *D. aphyllum.* Methanol, dichloromethane, hexane and water were used to extract the chemical compounds in tree barks. Aqueous and organic bark extracts of non host species (*Erythrina* sp*., Erythrina berteroana, Eurya accuminata, Semecarpus marginata, Symplocaceae cochinchinensis*) inhibited the seed germination of *D. aphyllum.* However, the effect of host tree species (*Eurya ceylanica, Actinodaphne stenophylla, Eurya* sp., *Antidesma zeylanicum, Citrus maxima, Semecarpus nigra-viridis* and two *Syzygium* spp.) on seed germination fluctuated with the concentration of host bark extracts. The physical characteristics of bark such as texture, peeling behaviour and thickness showed a positive correlation with the host tree specificity of *D. aphyllum*. Symbiotic fungus *Rhizoctonia* sp. was isolated as the associate in *D. aphyllum* roots.

Keywords: *Dendrobium aphyllum,* host tree specificity, seed germination.

INTRODUCTION

Potentially, any tree species can provide micro environments suitable for epiphyte survival (Callaway *et al*., 2001). It would therefore be expected that epiphytes would inhabit any suitably located tree, given adequate seed availability. However, it has been observed that epiphytes display a biased distribution towards some host species (Valencia-Diaz *et al*., 2010). Therefore it is possible that the barks of these host species or non host species contain some chemicals that can induce or inhibit the seed germination of epiphytes. Not only the chemical characteristics, but also the physical characteristics of the bark such as texture, peeling behaviour and thickness may influence the survival of epiphytes. Besides, the plant-plant interactions regulate the structure and composition of plant communities. Allelopathy is a chemical interaction in which plants or micro-organisms inhibit or stimulate the biological functions of other biological systems through the release of secondary metabolites to the environment (Valencia-Diaz *et al*., 2010).

Orchidaceae is the world's largest plant family with estimates of more than 25,000 species (Dearnaley, 2007). Many of the orchids are expensive and difficult to cultivate because the germination of seeds is not possible due to the shedding of the fruits before the attainment of maturity, lack of mycorrhizal association, inadequate nutrition, etc. Most of the orchids contain a few-celled embryos at the time of seed maturation and its proper development takes place only during the germination of seeds (Dutta *et al*., 2011). However, as the seeds do not have sufficient reserved food material (lacks endosperm) to facilitate growth of the embryo during germination, they have to depend on some external sources for nutrients to make the undifferentiated

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* Corresponding author (priyangi@kln.ac.lk)

embryo to develop into a protocorm (Richardson *et al*., 1992). The association with mycorrhizal fungi helps to draw the nutrients such as carbon, nitrogen and phosphorous for orchids. Fungal endophytes in such associations are believed to provide simple sugars and other nutrients required for seed germination by breaking down starch. Therefore, only 2 - 5 % of seeds germinate in the environment, which is very less in comparison with time (Arditti, 1967).

Dendrobium aphyllum is an orchid species found in Sri Lanka with restricted distribution, hence in need of conservation. Therefore, it is important to study the relationship between the host tree specificity and the seed germination of *D. aphyllum* to explore the potential of *in vitro* cultivation.

In many studies (Arditti, 1967; Valencia-Diaz *et al*., 2010) allelopathy was considered as the mechanism to explain host–epiphyte relationships. They have only examined the effects of compounds of bark extracts on seed germination of orchids. However, the allelopathic effect can be confounded with the lack of mycorrhizae during the germination process. Therefore, the main objective of the present research was to determine the relationship between the host tree specificity and the seed germination of *D. aphyllum* by using the chemical and some of the physical characteristics of tree barks under symbiotic conditions.

METHODS AND MATERIALS

Collection site and sampling

The Knuckles Mountain Range, a montane forest, in Sri Lanka was selected as the sampling site for the collection of tree barks, seeds and roots of orchid species. The mean annual precipitation lies between 3000-5000

Table 1: Study sites and characteristics of host and non host tree species

Site	Plot	Species	GPS value	Elevation ± SE
Deanston	1	Host-1 *Eurya ceylanica* Wight Non host-1 *Semecarpus marginata* Thwaites	07°31’42 “N, 080°44’17 “E	1100 ± 8 m
	2	Host-2 *Actinodaphne stenophylla* Thwaites Non host-2 *Eurya accuminata* DC.	07°31’08 “N, 080°43’43 “E	1113 ± 8 m
Deanston	1	Host-3 *Eurya* sp. Non host-3 *Erythrina berteroana* Urb.	07°36’40”N, 080°45’12 “E	1210 ± 8 m
	2	Host-4 *Antidesma zeylanicum* Lam.	07°31’08 “N, 080°44’42 “E	1218 ± 8 m
Gammaduwa	1	Host-5 *Citrus maxima* Burm.	07°24’05 “N, 080°48’02 “E	1378 ± 8 m
		Host-6 *Semecarpus nigra-viridis* Thwaites	07°24’35 “N, 080°48’20 “E	1379 ± 8 m
Dotulugala	1	Host-7 *Syzygium* sp.1 Non host-4 *Symplocaceae cochinchinensis* Lour.	07° 33’ N 080° 85’ E	1177 ± 8 m
Corbet’s gap	1	Host-8 *Syzygium* sp.2 Non host-5 *Erythrina* sp.	07°22’25” N 080°50’31” E	1225 ± 8 m

mm and the mean annual temperature ranges between 5.5 - 35 °C (www.knucklesrange.org/forest.html).

The Knuckles montane forest area was selected because epiphytic orchids can be found frequently in this area. Five study sites (Figure 1) were selected randomly in the montane forest area and two plots were demarcated within each of the five sites, which have trees with epiphytic *D. aphyllum.* The trees that had *D. aphyllum* epiphytes were considered as host trees and the trees, which did not have orchid epiphytes within an area of (10 ×10 m^2) were considered as non host trees (Table 1).

Bark collection and extraction of secondary metabolites

Within the period of January to May 2011, the barks from 8 trees of host species and 5 non host species were collected from 5 sites at 2 m height. Herbarium specimens of the host and non host trees were also prepared for the identification of tree species. Barks were cleaned in order to eliminate external agents such as invertebrates, detritus, dust and lichens. The bark physical characteristics such as texture and peeling behaviour were noted before cleaning and the bark thickness was measured by using a Vernier caliper (minimum scale, 1/20 mm). Subsequently the barks were air dried until a constant dry weight was obtained. Dry bark was ground to < 3 mm in an electric mill (KOHINOOR, IS: 4250). The resulting dry bark powder samples (approximately 200 g per species) were stored in darkness at −15 °C until use.

Due to the lack of knowledge about the putative allelopathic effect of hosts and non hosts, the aqueous extracts were obtained by percolating (20.0 mL each time) 10.0 g of dry and ground bark of each host species three times with distilled water. The leachates were strained through two layers of filter paper (Whatman No. 2) and lyophilized under vacuum at −20 °C (freeze-dry) until complete dryness was achieved. Later, the dry weight was measured (Sartorius BP 6100, RDM marketing, UK) and the percentage yield of each extract per host was calculated using the following equation.

$$\text{Percentage yield} = \frac{\text{WE} \times 100}{\text{WDM}}$$

WE = Weight extracted, WDM = Weight dry material

A general screening was carried out, where 90.0 g (30.0 g for each organic solvent) of dry bark material per host species was subjected to extraction by maceration at room temperature with organic solvents. After shaking the material overnight, the leachates were strained through two layers of Whatman No.2 filter paper.

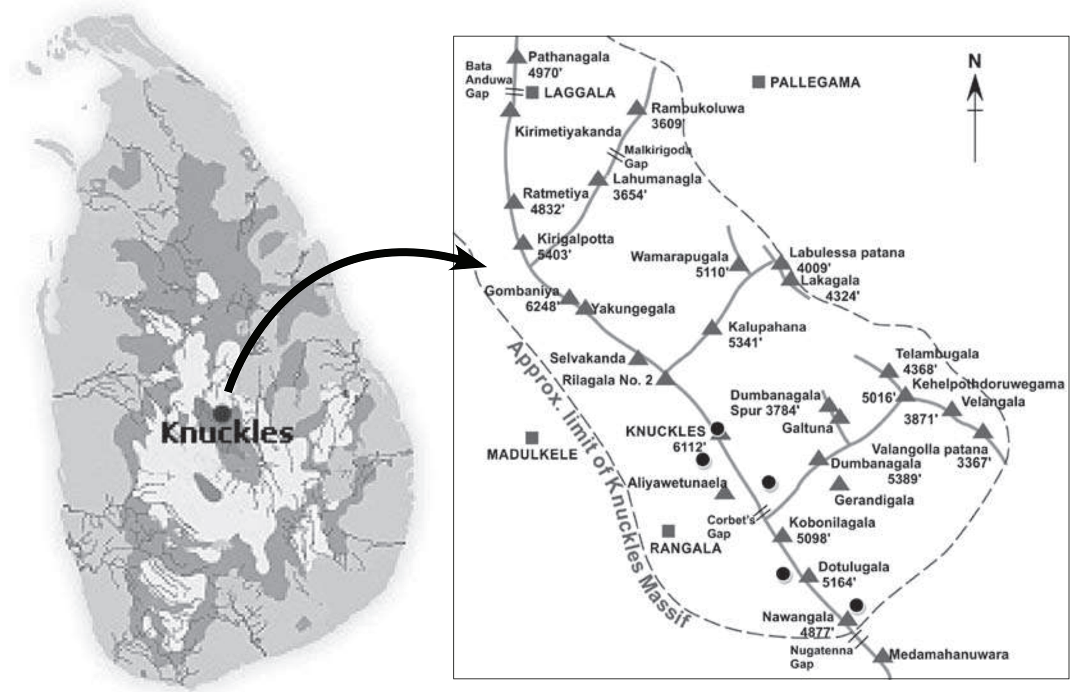

Figure 1: Map of the Knuckles mountain range in Sri Lanka indicating 5 study sites (●)

Hexane, dichloromethane and methanol were chosen as organic solvents as they have different polarities and as a consequence can carry a broad spectrum of compounds with different chemical characteristics and allelopathic potential (Bhath *et al.,* 2005). Hexane and dichloromethane carry water insoluble compounds or those with low water solubility (i.e. terpenoids). The lipophilic properties of terpenoids allow them to get into the cell membrane and reach the target site. On the other hand, higher-polarity compounds like phenolics are obtained by methanol (Valencia-Diaz *et al*., 2010). After filtration, the extracts were concentrated under vacuum (Rota-evaporator Buchi R-200) at 40 °C for hexane and dichloromethane, and at 46 °C for methanol. Dry extracts were stored in a cold environment. Percentage yield of each extract per host was calculated using the above equation.

Seed collection

The germination trials with aqueous extracts and organic extracts were undertaken with seeds collected in May 2011. Seed capsules of *D. aphyllum* were collected from 3 individual plants. The seeds were removed from capsules once dehiscence occurred in a sealed container to avoid contamination. The seeds were then randomized (mixed) and stored for further use.

Root collection

Healthy roots of *D. aphyllum* were collected from the same locations that were used to collect the bark samples. They were collected, wrapped in tissue paper, put in plastic bags and kept in an ice box during transportation to the laboratory.

Fungal isolation

Mycorrhizal fungi were isolated using a modification of the Masuhara Katsuya methods (Athipunyakom *et al*., 2004). The root segments were treated with 5 % sodium hypochlorite (NaOHCl) for surface sterilization. After shaking for 5 min, they were rinsed 3 times with sterile distilled water. The roots were then cut into longitudinal and cross sections and observed for the presence of hyphal coils (pelotons) on a glass slide under a compound microscope in sterile conditions. The epidermal layer was then removed to avoid contamination. Due to the difficulty of transferring a single peloton, small root segments (2mm length) were placed on 1/6 NDY medium containing 100 mg/L streptomycin and 50 mg/L tetracycline in a petridish and incubated in the dark at 28 °C. After 3 to 7 days incubation, the hyphal tips were transferred onto a potato dextrose agar (PDA) medium.

Fungal identification

The macroscopic features examined were, colony growth pattern, colour, and mycelia formation. The fungal growth was observed from the colony on PDA. For microscopic examination, fertile hyphae were mounted in sterile water on a microscopic slide, covered with a cover slip and examined under a light microscope. Fungal identification was carried out according to fungal identification keys and pictorial guides stated in Athipunyakom *et al.* (2004) and Sneh *et al.* (1996).

Preparation of concentration series from bark extracts

A concentration gradient (0.01, 0.1, 1, 10, 100 and 1000 μgmL^{-1}) of the original extract of aqueous and organic (hexanic, dichloromethanic and methanolic) extracts from all host and non host species were used. In preparation of the concentration series for aqueous extracts, sterile distilled water was used as a solvent and as the control. For organic extracts, dimethyl sulphoxide (DMSO - $(CH3)_2SO$) at a maximum concentration of 1 % (v/v) was used as the solvent and as the control. Results of the present study showed that DMSO has no significant effect on seed germination of *D. aphyllum* when compared with distilled water treatment (p = 0.874, mean ± SE, 25.45 % ± 1.4 % germination in water vs. 25.08 % ± 1.7 % germination in DMSO).

In vitro seed germination with symbiotic fungi

Initially, an experiment was carried out to test the effect of fungal species that were isolated previously on the germination of *D. aphyllum* seeds. Three plates of Dixon's Oat medium were inoculated with agar blocks of the isolated fungal colony. Another three plates of Dixon's Oat medium were used as the control without inoculation. They were allowed to incubate in the dark at 28°C for 30 days and the seed germination percentage was calculated. A significant increase of the germination was observed in the treatment with the above isolated fungus when compared to the control (p = 0.04, mean ± SE, 9.293 ± 3.12 % germination in control vs. 21.693 % ± 1.56 % germination with fungus). Therefore, the above isolated fungus was used as the symbiotic fungus for further steps.

DMSO was the solvent used for organic extracts. Therefore, another experiment was carried out to determine the effect of DMSO on seed germination. Three plates of Dixon's Oat medium with seeds and the isolated mycorrhizal fungus were utilized for this purpose. Agar blocks inoculated with 1mL of sterile distilled water was taken as the control. This was allowed to incubate in the dark at 28 °C for 30 days. Then the germination percentage was calculated.

In vitro symbiotic seed germination was tested using a modified method of Zettler and McInnis (1993). For this purpose, the plates were prepared with Dixon's Oat medium. The above prepared concentrations were applied into plates using a sterile pipette. 1 mL of each concentration of each extract was applied before adding the seeds. Mature *D. aphyllum* seeds were taken from the stored container and put in sterile water. 0.5 mL of sterile water containing seeds (counted using compound microscope; approximate value = 54) was pipetted on to a 1×4 cm filter paper strip (Whatman No.1) in a petri dish. The filter paper was placed on solidified Dixon's Oat medium with bark extracts. All these steps were done under sterile conditions in a laminar floor cabinet. All the plates were allowed to incubate in the dark at 28 °C for 30 days. Three replicates were done for each concentration of each host. After 7 days of inoculation the colony diameters of symbiotic fungus were measured under different concentrations of different host bark extracts.

In order to exclude the effects of factors other than allelopathy (i.e. non-viable seeds or dormancy) from the calculation, the percentage inhibition of seed germination was calculated with respect to the control (which consisted of distilled water without extract for aqueous extracts and DMSO without extract for organic extracts). Percentage inhibition of seed germination was therefore calculated after 30 days according to the following equation:

$$\frac{X_0 - Xi}{n} \times 100$$

Where, X_0 is the mean number of germinated seeds in the control, Xi is the number of germinated seeds under a specific concentration (i), and n is the number of seeds in an experimental unit (approximate value = 54). In this equation a positive value indicates inhibition, a negative value means promotion of the germination and zero indicates complete absence of bark host effect (Valencia-Diaz *et al*., 2010).

Separation of chemical compounds in bark extracts

The thin layer chromatography were developed for all three organic extracts (hexanic, dichloromethanic and methanolic) for each tree species. A small amount (5-10 µL) was spotted on 9 × 1 cm pieces of (20 × 10 cm) pre-coated thin layer chromatographic (Silica gel 60-F-254) plates (0.5 mm thickness, Merck Germany). For methanolic extracts - acetic acid: chloroform (1:9) solvent system, for dichloromethanic extracts dichloromethane: methanol (99:1) solvent system and for hexanic extracts-hexane: ethyl acetate (1:1) solvent system were used. All TLC plates were observed under UV light and the R_f values were calculated for each component using the following equation (Harborne, 1998).

$$R_f \text{ Value} = \frac{\text{Distance from baseline travelled by solute}}{\text{Distance from baseline travelled by solvent (solvent front)}}$$

Statistical analysis

Bark thickness of the host tree species and non host species were subjected to one –way ANOVA and the means were compared using Tukey' s multiple comparison test. The mean percentage germination of *D. aphyllum* seeds with mychorrizae and control treatment were also compared with Tukey's multiple comparison test. The means of germination under DMSO and water treatments were also compared with Tukey's multiple comparison test. Data for all analysis were tested for normality. The effect of different bark extracts on seed germination was compared using principal component analysis. Statistical analysis was done using the MINITAB-14 statistical software package.

RESULTS

Physical characteristics of host tree barks

The statistical analysis confirmed that there was no significant difference ($p > 0.05$) between the mean bark thickness of host 1, host 2, host 3, host 4, host 5 and host 8. But the mean bark thickness of host 6 and host 7 was significantly different ($p < 0.05$) from other host species and also they were significantly different from each other. Considering the bark characteristics of host tree species, most of them had rugose (rough, wrinkled) barks. Only host 4 and host 6 reported smooth bark surfaces. But all host species had non peeling barks (Table 2).

Statistical analysis showed that there was no significant difference between the mean bark thickness of non host 2 and non host 4. However, the mean bark thickness of non host 1, non host 3 and non host 5 were significantly different from the other host species and also they were significantly different from each other. Considering the bark characteristics of non host tree species, most of them had smooth barks. Only non host 1 and non host 4 had rugose bark surface (Table 3).

Table 2: Bark physical characteristics of host tree species

Species	Bark characteristics	Mean bark thickness (mm)
Host-1	Rugose, non peeling	1.15 ^{a}
Host-2	Rugose, non peeling	1.13 ^{a}
Host-3	Rugose, non peeling	1.13 ^{a}
Host-4	Smooth, non peeling	1.11 ^{a}
Host-5	Rugose, non peeling	1.10 ^{a}
Host-6	Smooth, non peeling	1.32 ^{b}
Host-7	Rugose, non peeling	1.01 ^{c}
Host-8	Rugose, non peeling	1.08 ^{a}

Each data point represents the mean of three replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test.

Table 3: Bark physical characteristics of non host tree species

Species	Bark characteristics	Mean bark thickness (mm)
Non host-1	Rugose, peeling	0.63 ^{a}
Non host-2	Smooth, peeling	0.41 ^{b}
Non host-3	Smooth, peeling	0.25 ^{c}
Non host-4	Rugose, non peeling	0.43 ^{b}
Non host-5	Smooth, non peeling	0.68 ^{d}

Each data point represents the mean of three replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test.

Chemical characteristics of host tree barks

Percentage yield of host and non host bark extracts

The aqueous extracts of barks showed the maximum percentage yield for all host and non host tree species. Methanolic extracts showed the second highest and hexanic extracts showed the minimum percentage yield (Figures 2 and 3).

Effect of symbiotic fungi on seed germination

Fungal isolation and identification

When the cross sections of roots were stained with cotton blue in lacto phenol stain, blue colour stained cells were observed in the cortex of *D. aphyllum.* High magnification (×1000) of compound microscopy showed pelotons of symbiotic fungi in the root cortex. The presence of pelotons is a characteristic feature of orchid roots (Figure 4).

On PDA, the colony reached 4.5 cm in diameter after 5 days of incubation at 28 °C and was observed as white to cream, cottony, submerged with glabrous surface and concentric zonation. There was usually a clear distinction between the long straight main hyphae and short lateral hyphae that branch repeatedly and form chains of monilioid cells. Another characteristic of many of these mycelia was that in pure culture, some hyphal tips coil into peloton-like structures as they do within the orchid cells (Figure 5). According to Athipunyakom *et al.,* (2004) and Sneh *et al.* (1996), the symbiotic fungus associated with *D. aphyllum* was identified as *Rhizoctonia* sp.

Percentage seed germination of D. aphyllum with symbiotic fungus

There was a significant increase ($p < 0.05$) in the mean percentage seed germination of *D. aphyllum* with symbiotic fungus compared to the control (Table 4). Therefore this isolated fungus was used as the symbiotic fungus for further steps.

Table 4: The Effect of symbiotic fungus on seed germination of *Dendrobium aphyllum*

Treatment	Mean germination %
Control (without fungus)	9.29 ^{a}
With fungus	21.69 ^{b}

Each data point represents the mean of three replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test.

There was no significant difference ($p > 0.05$) in the mean percentage seed germination of *D. aphyllum* between distilled water treatment and the DMSO (dimethyl sulfoxide) treatment (Table 6). Therefore, distilled water was used to dissolve the aqueous bark extracts and DMSO was used to dissolve the organic bark extracts (methanolic, dichloromethanic and hexanic).

Seed germination of D. aphyllum in methanolic, dichloromethanic, hexanic and aqueous bark extracts of host trees

For 0.01 μgmL^{-1} and 0.1 μgmL^{-1} concentrations, all host tree species showed promotion of germination in methanolic extracts, but for other concentrations the host species showed inhibition, promotion or no difference when compared to the control (Figure 6a).

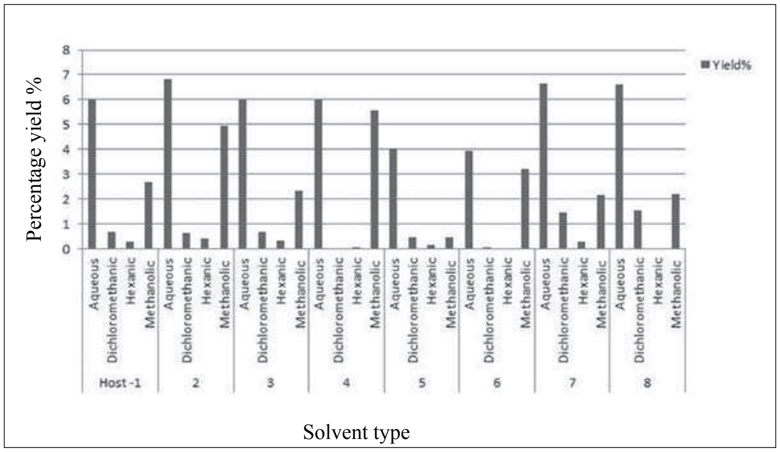

Figure 2: Percentage yield of bark extracts from different solvent types (aqueous, dichloromethanic, hexanic, methanolic) for host species

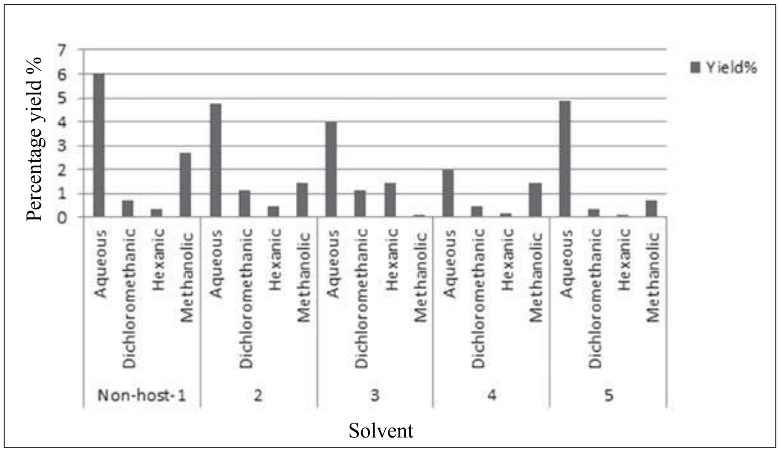

Figure 3: Percentage yield of bark extracts from different solvent types (aqueous, dichloromethanic, hexanic, methanolic) for non-host species

Table 5: Percentage seed germination of *Dendrobium aphyllum* in distilled water treatment and the DMSO treatment

Treatment	Mean germination %
Control (distilled water)	25.45 ^{a}
DMSO	25.08 ^{a}

Each data point represents the mean of three replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test.

In dichloromethanic extracts also 0.01 µgmL^{-1} and 0.1 µgmL^{-1} concentrations of all host species showed promotion of germination. The highest promotion of germinations were shown by host 1 and host 3 in 0.10 µgmL^{-1}concentration and host 5 and host 6 in 0.1 µgmL^{-1} concentration of dichloromethanic bark extracts (Figure 6b).

When considering the hexanic extracts, 0.01 µgmL^{-1} and 0.1 µgmL^{-1} concentrations showed promotion of germination for all host species. The highest promotion of germination was showed by 1000 µgmL^{-1} concentration of host 5. Host 1, host 3 and host 7 showed promotion of germination in all concentrations studied (Figure 6c).

The highest inhibition was shown in 0.1 µgmL^{-1} of host 8 in aqueous extracts. Host 7 in 0.01 µgmL^{-1}, host 5 in 10 µgmL^{-1} and host 3 in 100 µgmL^{-1} concentrations had neither inhibition nor germination (Figure 6d).

Seed germination of D. aphyllum in methanolic, dichloromethanic, hexanic and aqueous bark extracts of non host trees

In methanolic extracts, 1000 µgmL^{-1} concentration showed the maximum inhibition of seed germination

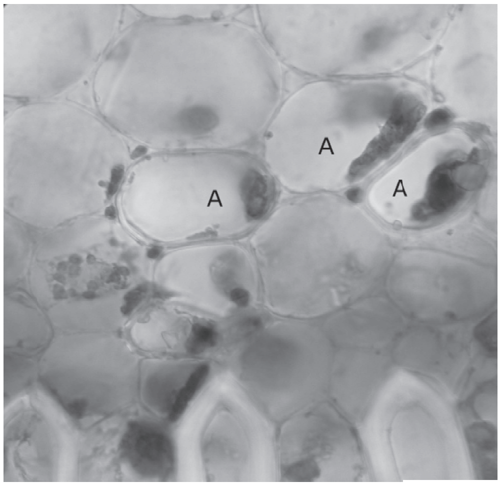

Figure 4: A cross section of *Dendrobium aphyllum* root. A- Root cortical cells containing pelotons of symbiotic fungi, stained with cotton blue in lacto phenol (compound microscopy × 1000)

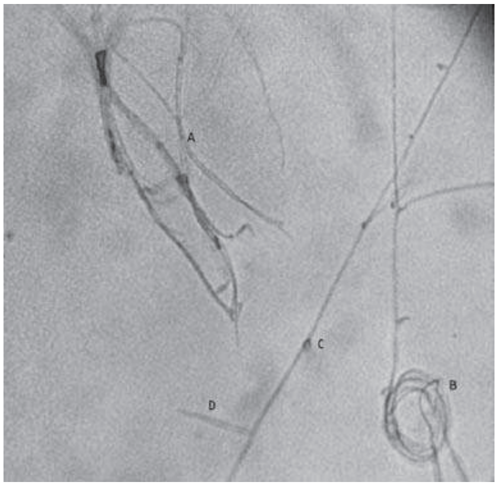

Figure 5: Hyphae of *Rhizoctonia* sp. A - Hyphae: B - Hyphal tips coiled into peloton-like structures: C - Long straight main hyphae: D - Short lateral hyphae (compound microscopy × 400)

for all non host tree species when compared to the other concentrations. The minimum inhibition was shown by the 0.01 µgmL^{-1} concentration of methanolic bark extraction. When increasing the concentration of methanolic bark extraction for all non host tree species, the inhibition also increased (Figure 7a).

For all concentrations, most of the non host species showed inhibition of germination under dichloromethanic extracts. However, for 0.01 µgmL^{-1} and 0.1 µgmL^{-1} concentrations, non host 4, and for 0.01 µgmL^{-1}, non host 5, showed neither inhibition nor germination. Inhibition of germination was increased when increasing the concentration of bark extraction. The highest inhibition was shown by non host 1 for all concentrations and the maximum of the inhibition was showed in 100 µgmL^{-1} and 1000 µgmL^{-1} concentrations (Figure 7b).

All non host species showed inhibition of germination for most of the concentrations in hexanic bark extracts and showed their maximum inhibition in 1000 µgmL^{-1} and minimum in 0.01 µgmL^{-1} concentrations. The inhibition of seed germination was increased with the increasing concentration of hexanic bark extracts (Figure 7c).

In aqueous extracts, all non host tree species showed inhibition of germination for all concentrations. The highest percentage inhibition was shown in 1000 µgmL^{-1} concentration of non host 2 while the lowest was shown in 0.01 µgmL^{-1} concentration of non host 3 (Figure 7d).

Effect of methanolic, dichloromethanic, hexanic and aqueous bark extracts of host trees on symbiotic fungus

The colonies of symbiotic fungus were observed after 4 days of inoculation in each concentration of bark extracts of all host species. It was noted that the colonies have taken 7 days to reach a measurable diameter. Mean colony diameters of symbiotic fungus in different bark extracts ranged between 0.5 ± 0.1 cm to 4.5 ± 0.1 cm (Table 6).

Effect of methanolic, dichloromethanic, hexanic and aqueous bark extracts of non host trees on symbiotic fungus

The symbiotic fungal colonies were observed in each concentration of all non host species after 5 days of inoculation and they were also measured in the 7th day after inoculation. The mean colony diameters of symbiotic fungus ranged between 2.3 ± 0.1 cm to 0.4 ± 0.1 cm (Table 7).

DISCUSSION

Vascular epiphytes are an essential component of forest vegetation, in terms of both species diversity and their role in forest ecosystem functions. However, the mechanisms that maintain epiphyte diversity, especially for orchid epiphytes are not well known. According to Tremblay *et al.* (1998) the host tree specificity in orchids has rarely been observed. Catling *et al*. (1986) reported that orchids appear to be generally non-specific in their choice of host trees. Although a few orchids are potentially host restricted, Tremblay *et al*. (1998) and Verga-Torres *et al*. (2010) reported that in the tropical dry forest of San Andres de la Cal, the low abundance of epiphytes coupled with the high concentration on a few host species, suggests the host limitation. Callaway *et al*. (2002) investigated the species-specific relationships among

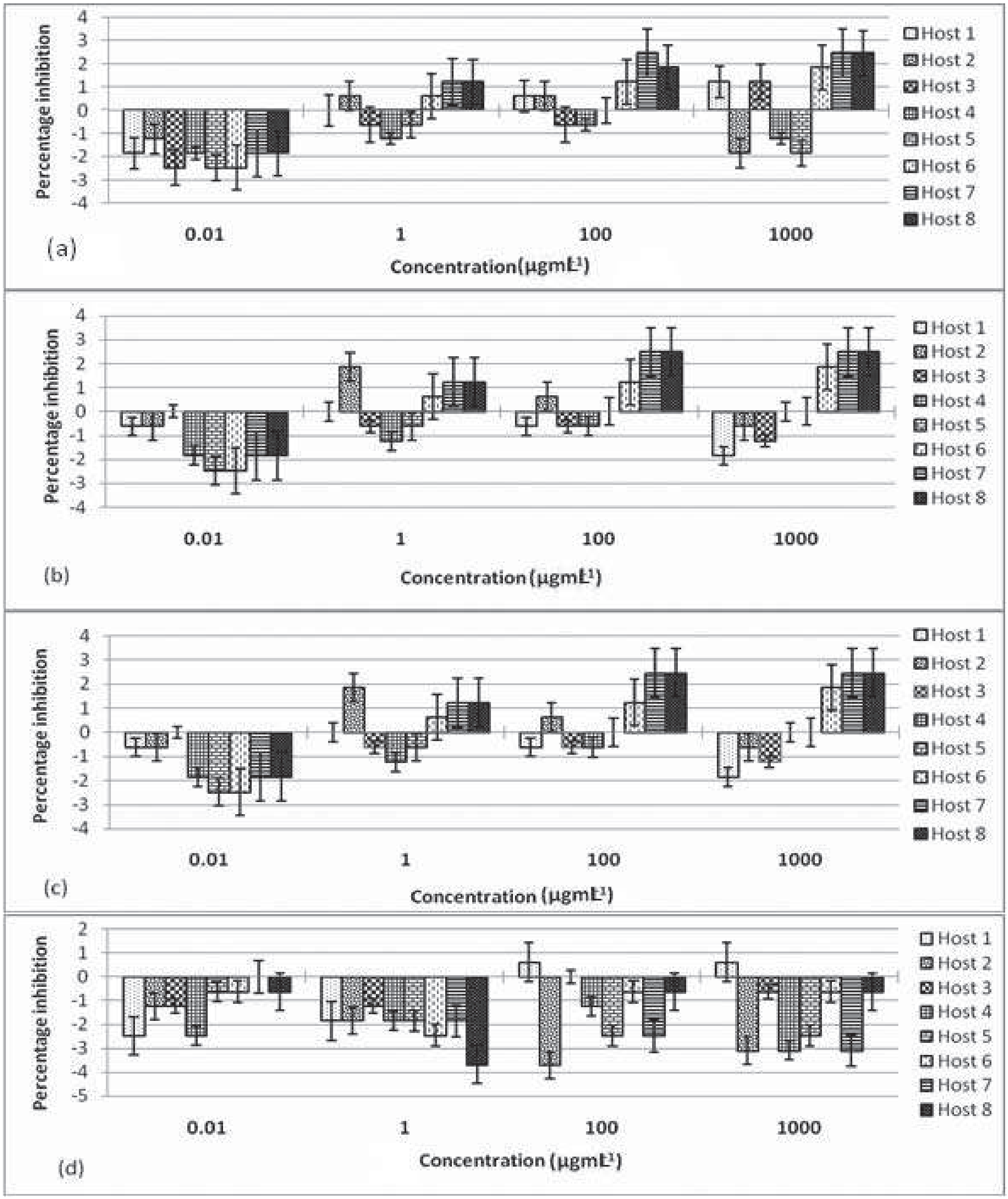

Figure 6: *Dendrobium aphyllum* seed germination under different concentrations (0.01, 1, 100, 1000 μgmL^{-1}) of host bark extracts (a) methanolic; (b) dichloromethanic; (c) hexanic; (d) aqueous

two species of vascular epiphytes and ten host tree species in a coastal plain forest in the southeastern United States. According to Akiko *et al.* (2008), in forests, the distribution pattern of vascular epiphyte diversity is affected by two major processes: dispersal and establishment.

Physical characteristics of tree barks

According to the present study, most of the host trees for *D. aphyllum* had non peeling barks. A rugose texture of the bark was seen in most of the host trees; only two species out of the eight host species studied had smooth textured

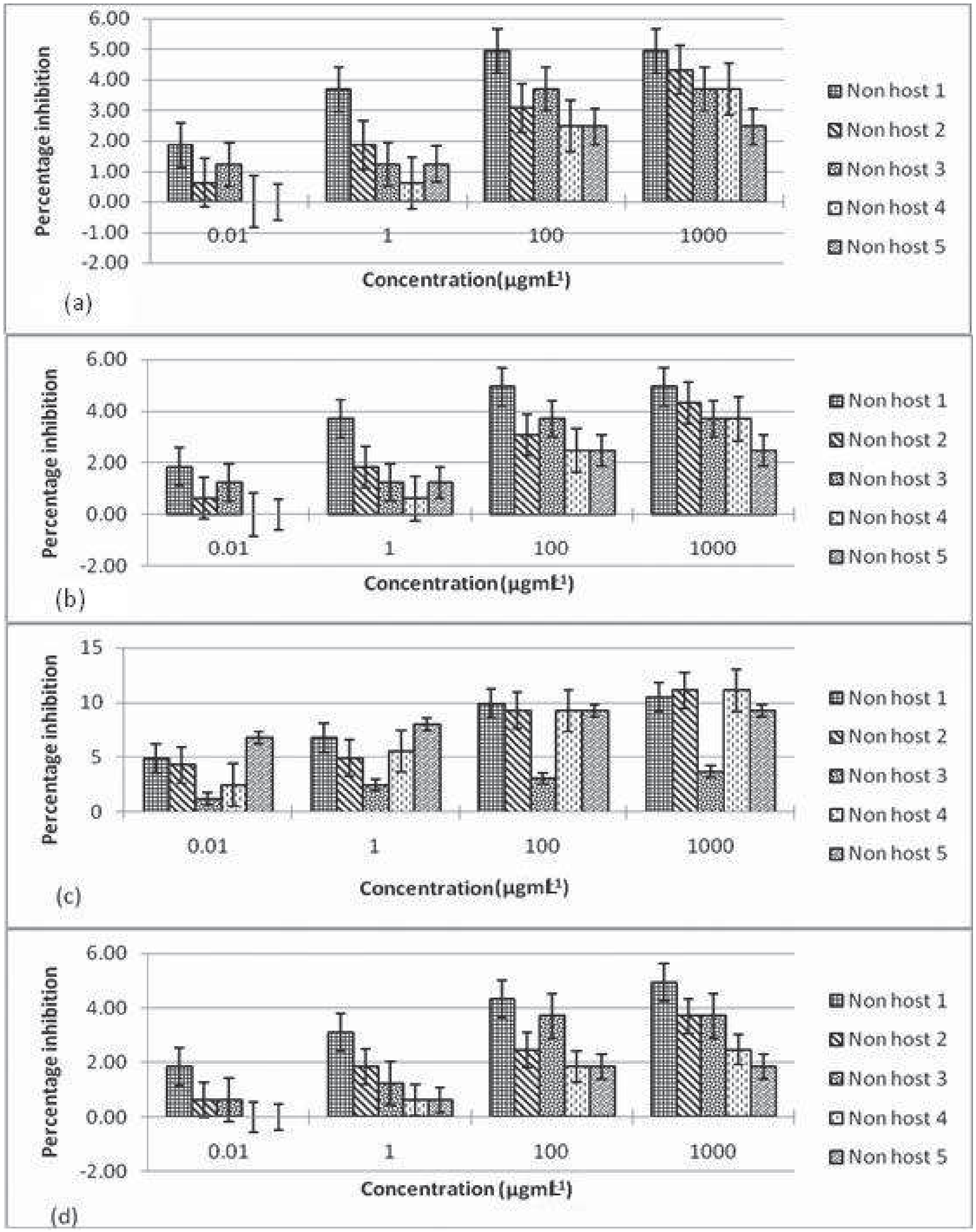

Figure 7: *Dendrobium aphyllum* seed germination under different concentrations (0.01, 1, 100, 1000 µgmL^{-1}) of non-host bark extracts (a) methanolic; (b) dichloromethanic; (c) hexanic (d) aqueous

bark. However, the texture of these trees may change due to the presence of mosses and other epiphytes. In all host species that were considered, the surface of the bark was moist due to the presence of mosses. Tremblay *et al.* (1998) have found a positive association between moss cover and also the influence of substrate water availability on the establishment of *Lepanthes caritenensi*.

The bark thickness of the host species also is an important parameter. According to the present study, although the bark thickness varied among the eight host species and five non host species, the bark thickness of the host species was significantly ($p < 0.05$) higher than that of the non host species. Thick barks of host trees may provide more suitable substrate for the epiphyte survival than the thin barks. According to Verga - Torres *et al.* (2010), tree size and bark characteristics explain the presence of epiphytes on trees. Generally large adult trees with a non-peeling,

Table 6: Symbiotic fungal colony growth under different concentrations (0.01, 1, 100, 1000 µgmL^{-1}) of host bark extracts (methanolic, dichloromethanic, hexanic, aqueous)

Host species	Mean colony diameter in methanolic bark extract (cm) ± SE			
	0.01 µgmL^{-1}	1 µgmL^{-1}	100 µgmL^{-1}	1000 µgmL^{-1}
Host 1	4.0 ± 0.1	2.0 ± 0.1	1.7 ± 0.1	1.5 ± 0.1
Host 2	3.0 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	3.5 ± 0.1
Host 3	4.4 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	1.5 ± 0.1
Host 4	4.0 ± 0.1	3.5 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
Host 5	4.5 ± 0.1	3.2 ± 0.1	2.0 ± 0.1	3.5 ± 0.1
Host 6	4.4 ± 0.1	1.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Host 7	4.0 ± 0.1	1.0 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Host 8	4.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
Host species	Mean colony diameter in dichloromethanic bark extract (cm) ± SE			
	0.01 µgmL^{-1}	1 µgmL^{-1}	100 µgmL^{-1}	1000 µgmL^{-1}
Host 1	3.0 ± 0.1	4.5 ± 0.1	2.0 ± 0.1	1.5 ± 0.1
Host 2	3.0 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	2.5 ± 0.1
Host 3	2.0 ± 0.1	3.0 ± 0.1	2.5 ± 0.1	3.4 ± 0.1
Host 4	4.0 ± 0.1	3.4 ± 0.1	2.0 ± 0.1	3.1 ± 0.1
Host 5	4.5 ± 0.1	3.2 ± 0.1	2.0 ± 0.1	3.5 ± 0.1
Host 6	4.3 ± 0.1	2.6 ± 0.1	2.0 ± 0.1	3.0 ± 0.1
Host 7	4.0 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Host 8	3.8 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
Host species	Mean colony diameter in hexanic extract (cm) ± SE			
	0.01 µgmL^{-1}	1 µgmL^{-1}	100 µgmL^{-1}	1000 µgmL^{-1}
Host 1	3.2 ± 0.1	4.4 ± 0.1	1.9 ± 0.1	1.4 ± 0.1
Host 2	3.1 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	3.2 ± 0.1
Host 3	4.3 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	1.5 ± 0.1
Host 4	4.1 ± 0.1	3.6 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
Host 5	3.8 ± 0.1	3.1 ± 0.1	2.3 ± 0.1	3.5 ± 0.1
Host 6	4.2 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.1
Host 7	4.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Host 8	4.0 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Host species	Mean colony diameter in aqueous bark extract (cm) ± SE			
	0.01 µgmL^{-1}	1 µgmL^{-1}	100 µgmL^{-1}	1000 µgmL^{-1}
Host 1	4.5 ± 0.1	4.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Host 2	4.3 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
Host 3	4.4 ± 0.1	4.5 ± 0.1	2.0 ± 0.1	1.7 ± 0.1
Host 4	4.0 ± 0.1	4.5 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
Host 5	3.5 ± 0.1	4.2 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
Host 6	3.4 ± 0.1	4.5 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
Host 7	3.0 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	4.0 ± 0.1
Host 8	3.0 ± 0.1	4.0 ± 0.1	0.8 ± 0.1	0.6 ± 0.1

Table 7: Symbiotic fungal colony growth under different concentrations (0.01, 1,100, 1000 μgmL^{-1}) of non host bark extracts methanolic, dichloromethanic, hexanic, aqueous)

Non host species	Mean colony diameter in methanolic bark extract (cm) ± SE			
	0.01 μgmL^{-1}	1 μgmL^{-1}	100 μgmL^{-1}	1000 μgmL^{-1}
Non host 1	1.5 ± 0.1	1.6 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
Non host 2	1.3 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.5 ± 0.1
Non host 3	1.3 ± 0.1	2.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Non host 4	1.0 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	1.7 ± 0.1
Non host 5	1.0 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	2.0 ± 0.1

Non host species	Mean colony diameter in dichloromethanic bark extract (cm) ± SE			
	0.01 μgmL^{-1}	1 μgmL^{-1}	100 μgmL^{-1}	1000 μgmL^{-1}
Non host 1	1.4 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Non host 2	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
Non host 3	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.5 ± 0.1
Non host 4	1.7 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Non host 5	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.5 ± 0.1

Non host species	Mean colony diameter in hexanic bark extract (cm) ± SE			
	0.01 μgmL^{-1}	1 μgmL^{-1}	100 μgmL^{-1}	1000 μgmL^{-1}
Non host 1	1.5 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Non host 2	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	0.5 ± 0.1
Non host 3	1.4 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
Non host 4	1.2 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Non host 5	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.6 ± 0.1

Non host species	Mean colony diameter in aqueous bark extract (cm) ± SE			
	0.01 μgmL^{-1}	1 μgmL^{-1}	100 μgmL^{-1}	1000 μgmL^{-1}
Non host 1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
Non host 2	1.2 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	3.5 ± 0.1
Non host 3	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
Non host 4	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
Non host 5	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.5 ± 0.1

thick and fissured bark should harbour more epiphytes than small hosts with a peeling, thin and smooth bark.

When comparing the taxonomy of host tree species and non host species of the present study, it was seen that they belonged to different unrelated taxa. *Eurya* and S*yzygium* were the most abundant host genera. Therefore *D. aphyllum* was not restricted to a single host taxon and has shown a range of host species. Verga - Torres *et al.* (2010) suggested that the vascular epiphyte composition and abundance may be influenced by forest host composition and this is supported by the results of the present study.

When considering non host species, most of them had smooth barks and they are peeling. Five non host species was observed and three of them had smooth bark surfaces while only one non host species had a rugose, non peeling bark though it did not contain the *D. aphyllum* epiphyte. Castro-Hernandez *et al.* (1999) have found that thick and porous bark is a determinant factor in epiphytic seedling survival. Zimmerman and Olmsted (1992) have found that hosts with peeling barks contain a lower epiphyte species richness than non peeling bark hosts, possibly because a peeling bark could increase the seedling mortality. This experimental evidence supports the findings of the present study. However in contrary to the finding of the present study, Verga-Torres *et al.* (2010) concluded that neither host size nor obvious bark characteristics can be used to predict epiphyte distribution. Additional to bark texture and behaviour, other bark properties such as chemical composition may be determining epiphyte colonization.

Chemical characteristics of tree barks

According to the results of the present study, it was found that there was no significant difference ($p > 0.05$) between the percentage seed germination of *D. aphyllum* under distilled water treatment and the DMSO treatment. These results support the previous findings of Valencia-Diaz *et al*. (2010), who found that there was no significant difference in seed germination of *Tillandsia recurvata* under the above two treatments. Therefore, DMSO was employed for extracts that were non water soluble and distilled water was employed for extracts, which were water soluble.

As mentioned elsewhere hexane, dichloromethane and methanol were chosen as organic solvents, because they have different polarities and they can carry a broad spectrum of compounds with different characteristics and with allelopathic potential (Bhath *et al.,* 2005). Hexane and dichloromethane carry water insoluble compounds or those with low water solubility while higher polarity compounds like phenolics are obtained by methanol. Terpenoids, alkaloids and phenolics are principal chemical groups that have been related with allelophathic activity (Koffi *et al*., 2010). Valencia-Diaz *et al*. (2010) have reported that there was a positive correlation between the concentrations of flavonoids and the inhibition of seed germination of *Tillandsia recurvata*, an epiphytic bromeliad.

Percentage yield of host bark extracts

In the present study, aqueous extracts of barks showed the maximum percentage yield for all host tree species. Methanolic extracts showed the second highest while hexanic extracts often showed the minimum percentage yield. The percentage yield gives an idea on the extraction efficiency of these four solvents. Due to the different efficiencies of different solvents when extracting plant chemicals, a concentration series was used. Therefore the effect of different efficiencies in extracts was minimized. As the germination behaviour in response to each extracts could not be predicted the concentrations used were chosen to cover a logarithmic range.

Seed germination of *D. aphyllum* in methanolic, dichloromethanic, hexanic and aqueous bark extracts of host and non host trees

When comparing the results of the inhibition of germination of *D. aphyllum* seeds under different concentrations of the host and the non host bark extracts, the present study showed some correlations. During the inhibition of seed germination under different concentrations of the methanolic extracts, different host species behaved in different manner. When comparing all host tree species host 6, host 7 and host 8 showed the same pattern but in different quantities. In low concentrations (0.01 and 0.1 μgmL^{-1}), all host species showed promotion of germination. But the inhibition of seed germination of all non host species under methanolic extracts showed a correlation with the concentration. When increasing the concentration, inhibition also increased for all non host species, but in different quantities.

When comparing all host tree species for inhibition of seed germination under different concentrations of dichloromethanic extracts, host 6, host 7 and host 8 showed the same pattern but in different quantities. In low concentrations (0.01 and 0.1 μgmL^{-1}), all host species showed promotion of germination. The overall pattern of inhibition and promotion of germination in different concentrations was approximately similar in methanolic and dichloromethanic bark extracts.

There was no significant correlation in the inhibition of seed germination under different concentrations of hexanic extracts, for the host species studied. However, when comparing all host tree species, host 6, host 7 and host 8 showed the same pattern but in different amounts. Promotion of germination was not observed in low concentrations of hexanic extracts.

All non host species showed inhibition of germination under aqueous bark extracts and all host species showed promotion of germination (except 100 μgmL^{-1} and 1000 μgmL^{-1} concentration of host 1). The percentage inhibition also increased with the increasing concentration for all non host species.

According to the present study, different concentrations have different effects on the inhibition of germination of *D. aphyllum* seeds. However, the overall effect of organic solvents was not considered in this study due to limited time duration. Therefore this study has to be extended to assess the overall effect of organic bark extracts on seed germination.

Thin layer chromatographic (TLC) analysis was carried out in order to separate and analyze the chemical composition of bark extracts of different host species. When considering the TLC analysis, a range of chemical components were extracted using different organic solvents. TLC of methanolic bark extracts showed the presence of more chemical components than dichloromethane and hexane extracts whereas a lesser number of components were extracted in hexane. This can be related to the results that were obtained from the percentage yield of organic solvents, where the highest percentage yield was shown for methanol. Therefore it was concluded that the methanolic extract contains the highest number of chemical components when compared to dichloromethane and hexane. Normally methanolic extracts contain polar compounds due to its polarity and this suggests that these plant barks contains more polar compounds than non-polar compounds.

Despite the relatively high volume of research, only one previous study has approached allelophathy as a mechanism to explain host-epiphyte relationships (Valencia-Diaz *et al.,* 2010). This examines the effects of compounds in the bark of Mexican *Quercus* spp. on the germination of *Encyclia tampensis*, an orchid from Florida. However, the authors have not taken into consideration that orchid seed germination depends on the action of mychorrizas.

Effect of symbiotic fungi on seed germination

In fact, the orchid germination and establishment can also be influenced by the interaction with insects and other organisms that has an effect on the symbiotic fungus (Flores-Palacios & Ortiz-Pulido, 2005). Researchers have demonstrated that *in vitro* seed germination of some orchid species could easily be carried out with specific fungi isolated from the orchid symbiotic fungi (Vujanovic *et al.,* 2000).

According to the present study, pelotons of symbiotic fungi were observed in the cortex of *D. aphyllum* roots. However, according to Suarez *et al*. (2006), the distinction between endophytic fungi inhabiting only the velamen or the root surface and mycorrhiza forming fungi colonizing the cortical tissue was mostly unclear. Salifah *et al*. (2011) found thirty-one different isolates of endophytic fungi from root tissues of the vegetative plants of *Grammatophyllum speciosum*, *G. scriptum* and *G. stapeliifllorum*. The genera with the most frequencies were *Fusarium* and *Trichoderma,* which were mostly originating from *G. speciosum* and *G. stapeliiflorum,* respectively. Dutta *et al*. (2011) investigated the *in vitro* multiplication and protocorm development of *D. aphyllum* seeds, but that was not a symbiotic germination.

Rhizoctonia sp. was isolated and identified from *D. aphyllum* roots in the present study. It was also associated with a statistically significant increase ($p < 0.05$) of the germination of *D. aphyllum* seeds. Therefore it was inoculated as symbiotic fungus for seed germination. The identification of seven genera and fourteen species of mycorrhizal fungi from eleven terrestrial orchids by Athipunyakom *et al.* (2004) supports the findings of the present study. The characteristics of *Rhizoctonia repens* studied by Athipunyakom *et al.* (2004) are much similar to the symbiotic fungus that was isolated in the present study. However the ultra-microscopic features and nuclear staining of the fungi were not examined in the present study. Further study of the fungus is needed to identify such proportion exactly. Shan *et al.* (2002) isolated twenty *Rhizoctonia* like fungal strains from the roots of a terrestrial orchid species, which also support the evidence from the current investigation.

DNA-based fungal identities of mycobionts from tropical terrestrial and epiphytic orchids, the host range, and the inclusion of data from several ecological parameters are still needed to determine whether the association strategies differ between epiphytic and terrestrial orchids or between temperate and tropical habitats. According to Agustini *et al.* (2009), orchids inoculated by fungi isolated from other plants did not show any positive effect on seedling development. But *Dendrobium* seedlings inoculated with symbiotic fungi isolated from other orchids showed a better growth than the non-inoculated ones. This suggests that genus *Dendrobium* is non-specific for the symbiotic fungus with respect to seed germination. *In vitro* culture systems also indicate that *Fusarium* sp. and *Pyrenochaeta* sp. enhance the seedling growth of *D. loddigesii* (Chen *et al*., 2010).

Effect of methanolic, dichloromethanic, hexanic and aqueous bark extracts of host and non host trees on symbiotic fungus

It was observed that the mean colony diameter values fluctuate among concentrations of bark extracts as well as among tree species. After 4 to 5 days of inoculation, fungal colonies were observed in all concentrations of host and non host bark extracts. This indicates the suitability of the bark extracts for the fungal growth to a certain extent.

Fungal colonies in non host bark extracts were small in size with respect to the colony diameters. This suggests the prevalence of a negative effect of non host bark extracts on the growth of symbiotic fungal colonies. Hence, the inhibition of germination of *D. aphyllum* seeds can be influenced by the effect of bark extracts on symbiotic fungus.

Generally, the plant extracts contain a high range of secondary metabolites. When considering the plant secondary metabolites there may also be an effect on symbiotic fungi. According to Horace (1985), the lower and higher plants are sources of secondary metabolites that have allelo-chemical effects in plants, fungi, bacteria and vertebrates. Arditti (1967) suggested that it is possible that some bark compounds favour fungi beneficial to the orchid, by selective inhibition of others. It also may contribute to the host limitation of orchids. When considering the reasons behind some trees becoming non host for the orchids, the effect of these secondary metabolites not only on seed germination but also on symbiotic fungi associated with orchids is also crucial. Therefore further studies are needed for the determination of the effect of secondary plant metabolites on the symbiotic fungal survival and the germination of orchids on hosts.

The findings of this study can be used to conserve rare orchid species by identifying the factors affecting their germination. This study can be extended to quantitatively identify the ideal chemical composition needed for better growth of orchid seeds *in vitro*. That may be of vital importance for orchid growers and for those in the orchid industry as well as for the conservation of rare and endemic orchid species.

CONCLUSION

The findings of the present investigation concluded that there is a correlation between bark physical characteristics and the host specificity of epiphytic *D. aphyllum.* The inhibition of seed germination of *D. aphyllum* can be correlated with the chemical composition of the non host barks, but no significant correlation was found in the host barks. Therefore, the chemical composition may not be the only influential factor for the inhibition or promotion of seed germination of *D. aphyllum*. Some other factors such as the water holding capacity of the bark, bark stability, presence of facilitators and microclimate also may contribute to the distribution of *D. aphyllum. Rhizoctonia* sp. was identified as the symbiotic fungus associated with *D. aphyllum* roots, which has an effect on increasing the germination of *D. aphyllum* seeds.

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