# Selection of dark septate endophytes from *Ericaceae* plants to enhance blueberry (*Vaccinium corymbosum* L.) seedling growth

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**Abstract**
An experiment was conducted to find a beneficial root endophyte in order to enhance blueberry biomass accumulation as well as to confirm the role of dark septate endophyte (DSE) under axenic condition. The plant material for DSE isolation was selected from the *Ericaceae*. A total of 91 isolates were obtained from 300 root sections of *Rhododendron pulchrum*, *Rhododendron obtusum* and *Pieris japonica*. There were no significant differences observed after 10 weeks of inoculation on shoot length, root length and fresh weight of the seedlings, however there was a tendency of having longer root in the seedlings with some isolates especially with Pj029 (78.6 mm) and Pj022 (68 mm) compared with the control (51 mm). Nine putatively benefical endophytes were subjected to phylogenetic analysis. Based on the homology search the Rp005 isolate from *Rhododendron pulchrum* host was identified as *Heteroconium chaetospira*. Three other isolates namely, Rp022 and Rp011 isolates both from *Rhododendron pulchrum* host and Pj023 isolate from *Pieris japonica* host were confirmed as *Leptodontidium orchidicola*. These isolates have the ability to form inter- and intracellular hyphal structure within the host epidermal cell and assume to have bidirectional nutrient flow between the host and endophyte.

**Key Words**
Dark Septate Endophyte, *Ericaceae*, blueberry, colonization, intracellular structure

## Introduction
The dark septate endophytes (DSE) are broadly classified as conidial, sterile septate fungal endophytes that form melanised structures such as inter- and intracellular hyphae and microsclerotia in the plant roots and known to have affinities with ascomycetes (Jumpponen and Trappe, 1998). Despite the wide host preference of DSE, there are many basic questions that should be addressed (Jumpponen, 2001). The ecology of DSE is largely unknown and hypotheses are based on sparse evidence. The range and relative importance in *Ericaceae* are still unclear (Hambelton and Currah, 1997) and thus, research should focus on the functional aspects of the interaction between the two organisms involved in the association. In this study, a survey was conducted among some of the native *Ericaceae* species of the Japanese flora to isolate root associated fungal endophytes that probably can enhance the growth of blueberry seedling as an initial step toward to the better upland soil adaptability of blueberry. In addition, we examined the ability of these isolates to form intracellular structures with the host and we characterized the community structure of those endophytes.

## Methods
*Root sampling*
Root samples were collected from the following ericaceous species: *Rhododendron obtusum* (Lindl.) Planch. *Rhododendron pulchrum* Sweet and *Pieris japonica* (Thunb.) D. Don ex G. Don. in the Azalea-garden of Graduate School of Horticulture, Chiba University, Japan (35°46'N, 139°54'E). The sterilized root segments were placed in 90 x 15 mm Petri dishes filled with malt extract agar (MEA, 2%) and incubated at 18°C for 1 month. Only the dark, slow growing and septate endophytes were selected and subcultured to MEA media.

*Inoculation test*
The blueberry seeds were extracted from a commercial available frozen blueberry fruits. After the surface sterilization, the seeds were placed on PDA for germination and individually transplanted to 20 ml test tubes filled with 10 ml of modified Mitchell & Read media. Then, seedlings were inoculated with each of the 91 isolates and one additional known DSE *Heteroconium chaetospira* provided by Narisawa *et al.* (2000). Control tubes (non-inoculated) were also prepared containing only the host. Ten weeks after inoculation, the seedlings were destructively harvested to measure fresh weight and length of shoot and root. The roots were stained with trypan blue and determined root colonization by endophytes. Data on colonization intensity was measured using the modified method of Giovannetti and Mosse (1980).

*DNA extraction, sequencing and phylogenetic analysis*
The genomic DNA of isolates was extracted by FastDNA®Kit following the manufacture's instructions. The ITS region was amplified by ITS1F and ITS4 primers. The amplification was carried out in an automated PCR Thermal Cycler TP-600. The PCR product was purified with SUPREC™-PCR purification column. The sequencing reaction mixture was prepared with BigDye terminator cycle Sequencing Kit followed by the recommendation of manufacturer. ITS3 primer was used to span the 5.8S and ITS2 region. The sequencing data of isolates were subjected for homology search using the BLAST program through the GenBank database to match the closest sequence. Phylogenetic relation between the isolates of the present study and the closest sequences from related studies were combined to create a neighbor-joining tree by ClustalW. The neighbor joining tree was compiled by TREEVIEW. The confidence levels were calculated from 1000 replicates bootstrap samplings.

**Results**
*Inoculation test*
There were no significant differences observed after 10 weeks of inoculation on shoot length, root length and fresh weight of the seedlings, however there was a tendency of having longer root in the seedlings with some isolates especially with Pj029 (78.6 mm) and Pj022 (68 mm) compared with the control (51 mm). The highest colonization intensity was observed in Rp005 a *C. chaetospira* isolate (10.1%) followed by the Rp022 isolate (8.1%) and confirm this finding with the formation of ERM like intracellular structure within the epidermal cells in both strains.

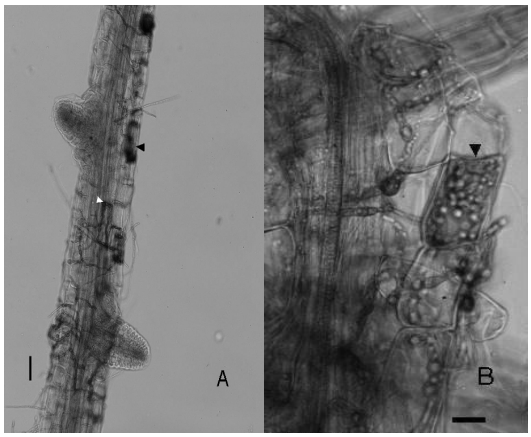


**Figure 1. (A) Chain-like hypha of Rp005 form intercellular (white arrowhead) and intracellular (black arrowhead) structures in the epidermal cells of blueberry (*Vaccinium corymbosum* L) seedling. Bar is 100µm. (B) Epidermal cells of blueberry (*Vaccinium corymbosum* L.) seedling heavily colonized (arrowhead) by hyphal complexes of Rp011. Bar is 30µm.**

*Phylogenetic analysis*
The highly diverse ITS2 region of the rDNA was used to determine relationships of isolates at species level. The neighbor joining tree clustered the isolates into three distinct clades. Because the internal branches having weak bootstrap support between clade I and the other two clades (clade II and clade III), indicating unrelated polyphyletic origin of isolates among the clade I and in the rest two clades. The Rp005 isolate have a high sequence similarity with *Cladophialophora chaetospira* (EU035406) and clustered in a strong bootstrap supported monophyletic clade (clade II) with other *C. chaetospira* strains including the *Heteroconium chaetospira*. The phylogenetic positions of Rp011, Rp022 and Pj023 were set to individual clade (clade III) having strong bootstrap support with a DSE of *Ranunculus adoneus* (*Ranunculaceae*), with an *Epacris* (*Ericaceae*) root associated fungi and with other *Leptodontidium orchidicola* strains. A strong bootstrap supported cluster was clamped the Ro012 with Ro024. Both show significant bootstrap support with *Cyphellophora* sp. Isolates Pj022 and Pj029 did not have any significant homology during BLAST search and their identity still unknown and will remain so until isolates will sporulate or until more taxa will be sequenced. Based on sequence similarity and phylogenetic analysis, five isolates were confirmed as DSE, including the *Heteroconium chaetospira* (syn. *Cladophialophora c*.) isolate derived from Narisawa *et al* (2000). The taxonomy of Rp005 with 100% sequence similarity confirmed as *C. chaetospira* isolate was from the host plant *R. pulchrum*. Moreover, distinctive colonization intensity was observed between these *H. chaetospira* (by Narisawa) and *H. chaetospira* Rp005 isolates 2.5% and 10.1 %, respectively. Usuki and

Narisawa (2004) found no statistical differences in the colonization intensity of axenic *R. obtusum var. kaempferi* (*Ericaceae*) seedlings inoculated with four strains of *H. chaetospira* (DSE) that varied between 14.5 to 19.5 %. Further, the same authors addressed that one strain of *Oidiodendron maius* (ERM) produced significantly higher percentage of intercellular structure (31.8%) compared to the above mentioned four *H. chaetospira*. Similarly to our study, seedling fresh weight of *Rhododendron obtusum var. kaempferi* did not show any significant difference. Dalpé (1986) found a similar percentage of colonization by five *Oidiodendron* strains that ranged between 3-21% in axenic blueberry (*Vaccinium angustifolium* Ait.) experiment after 60 days of inoculation. Narisawa (2007) observed that the *H. chaetospira* is a commonly occurring DSE, in contrast with what was previously reported. Other more frequently isolated DSE species such as *Phialocephala fortinii* and *L. orchidicola* have faster growth rate, and thus, make it more difficult to detect and isolate *H. chaetospira*. In this study, both *H. chaetospira* isolates have the ability to form intracellular structure in blueberry host that resembled ERM coil. The formation of intracellular structure in *Ericaceae* by *H. chaetospira* was previously reported in axenic *Rhododendron. obtusum var. kaempferi* (Usuki and Narisawa (2004), however, the source of Rp005 isolate derived from *R. pulchrum* in our study, indicates the first isolation of *H. chaetospira* from an *Ericaceae* in Japan. Interestingly, there was no any report about DSE species in a complete endophytic survey of *R. obtusum var. kaempferi* at approximately 30 km NE from the sample site of the current study (Usuki *et al.,* 2003).

**Table 1. Results of inoculation test. Effects of different *Ericaceae* derived endophytes on the root lenght, shoot lenght, fresh weight, formation of intracellular structure and colonization intensity of blueberry (*Vaccinium corymbosum* L.) seedlings 10 weeks after inoculation.**

| Isolates† | Shoot lenght (mm) | Root lenght (mm) | Fresh weight (mg) | Formation of intracellular structure | Colonization intensity (%) |
|---|---|---|---|---|---|
| Rp005 | 28.3 a | 51.6 a | 65.0 a | + | 10.1 a |
| Rp011 | 41.0 a | 43.3 a | 48.5 a | + | 1.8 cd |
| Rp022 | 29.6 a | 48.3 a | 61.6 a | + | 8.1 a |
| Ro012 | 46.6 a | 50.3 a | 70.1 a | + | 5.2 bcd |
| Ro024 | 38.3 a | 49.6 a | 56.4 a | - | 0.0 d |
| Ro034 | 36.0 a | 58.3 a | 69.5 a | - | 0.0 d |
| Pj022 | 46.0 a | 68.0 a | 93.4 a | - | 0.0 d |
| Pj023 | 39.6 a | 63.3 a | 65.4 a | + | 1.2 d |
| Pj029 | 48.3 a | 78.6 a | 66.1 a | + | 4.1 abc |
| H.chaetospira | 47.6 a | 57.6 a | 63.6 a | + | 2.5 bcd |
| Control | 35.6 a | 51.0 a | 76.0 a | - | - |

* same letter means not significantly different at $P \leq 0.05$ based on Tukey Test
†Rp=*Rhododendron pulchrum*, Ro=*Rhododendron obtusum* Pj= *Pieris japonica*.

The Rp022, Pj023 and Rp011 isolates set in a monophyletic position in clade III which dominated by *L. orchidicola*. The high percentage of sequence similarity with other strains of *L. orchidicola* (98%) obtained from BLAST search among GenBank data and strong bootstrap support of the clades suggested the conspecificity of these three isolates with *L. orchidicola*. The *L. orchidicola* recovered from both *Rhododendron pulchrum* (Rp011 and Rp022 isolates) and *Pieris japonica* (Pj023 isolate) in our study confirmed the previous findings of Midgley *et al.* (2004) where they find two different species of neighboring Ericaceae host plants (*Woollsia pungens* and *Leucopogon parviflorus*) sharing same endophyte. Inspite of the worldwide distribution of *Ericaceae*, only 25 species has been mentioned as host of DSE (Hambelton and Currah 1997, Currach and Tsuneda, 1993, Ahlich and Sieber 1996). Majority of the hosts were sampled in alpine heath, a stable sand dune and an ombrotrophic *Sphagnum* bog in the Canadian Rocky Mountains (Hambelton and Currah 1997), a *R. obtusum* plant in Tottori, Japan and in a coniferous forest in Switzerland, Central-Europe (Ahlich and Sieber, 1996). All sampled sites were covered by ericaceous shrub understory. Solely the *Phialocephala fortinii* represented the DSE in all sites irrespective of host plants or geographic location. The Rp005 isolate *(H. chaetospira)* from *R. pulchrum* host the first report of a *Heteroconium* strain derived from an *Ericaceae* in Japan. The Rp022 and Rp011 isolates from *R. pulchrum* and Pj023 isolate from *P. japonica* host the first *L. orchidicola* strains from *Ericaceae*.

## Conclusion

Our result suggests that both *H. chaetospira* (Rp005) and *L. orchidicola* (Rp022, Pj023 and Rp011) have the

ability to form inter- and intracellular structures in axenic blueberry seedlings together with their enzymatic abilities (Fernando and Currach, 1995) and in broader perspective, possibly act on the part of ericoid mycorrhiza and make them a good candidate for further container experiments with blueberry plants in order to clarify the function in host-endophyte continuum.

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