Orchid endophytes and their effect on growth in *Vanilla planifolia* Andrews

Hongos endófitos de orquídeas y su efecto sobre el crecimiento en *Vanilla planifolia* Andrews

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Abstract

Endophytes are microorganisms that grow inside plant tissues without causing symptoms of disease which roots are associated with, among others, potential benefits in the defense against pathogens and increased nutrient availability. In the present study, endophytes from roots of orchids of the genus *Vanilla* in wild state were isolated, in order to determine their effect on plant growth of *V. planifolia* when inoculated into the substrate. The results showed that variables such as biomass, root length and height of the plant are affected by the inoculation of these endophytes. The effectiveness of these fungi on the plant protection or growth stimulation contributes to the generation of tools for the use of bio-inoculants. This will reduce the use of chemical inputs and promote environmentally friendly practices in farming systems of vanilla.

Key words: Ceratobasidium, fungal endophytes, growth, inoculum, *Vanilla planifolia*, *Xylaria*.

Resumen

Los endófitos son microorganismos que crecen dentro de los tejidos vegetales sin causar síntomas de enfermedad. Aquellos asociados a las raíces tienen, entre otros posibles beneficios la defensa contra patógenos y un aumento en la disponibilidad de nutrientes. En el presente estudio se aislaron hongos endófitos de raíces de orquídeas del género *Vanilla* en estado silvestre, con el fin de determinar su efecto sobre el crecimiento de plantas de *V. planifolia* cuando se inocularon en el sustrato. Los resultados mostraron que variables como biomasa aérea, longitud de raíces y altura de la planta son afectadas por la inoculación de estos endófitos. La efectividad de estos hongos sobre la protección de plantas o la estimulación en el crecimiento contribuye a la generación de herramientas para el uso de bioinocu- lantes. De esta forma se reduce el uso de insumos químicos y se promueven prácticas amigables con el ambiente en sistemas de cultivo de vainilla.

Palabras clave: Ceratobasidium, crecimiento, hongos endófitos, inóculo, *Vanilla planifolia*, *Xylaria*.

Introduction

Literature on endophyte fungi and mycorrhizal forming fungi in orchids is much related and sometimes is impossible to discuss these associations separately. Endophyte fungi are microorganisms that grow into plant tissue without causing damaging symptoms and, recently, they started to be recognized (Bayman and Otero, 2006; Brundrett, 2006; Stone *et al.*, 2000). Endophytic association described where is living a microorganism (Brundrett, 2006; Stone *et al.*, 2000), without assuming or excluding the possibility of a benefit for both parts. To the contrary, the concept of mycorrhizal is functional and describes a commonly mutualistic relation (Smith and Read, 1997; Rasmussen, 2002; Cameron *et al.*, 2006, 2007).

Recent studies (Bayman and Otero 2006; Chen *et al.*, 2011, 2012; Singh *et al.*, 2011; Xing *et al.*, 2011) demonstrated the large diversity of fungal endophytes that do not form mycorrhizal associated with roots and aerial part of orchids. These studies highlight the role of these microorganisms in plant protection against pathogen attacks, by means of secondary metabolites or by better nutrition through nutrient availability (Schulz, 2006). They also mention, not only the benefits for the plants but, the potential of these endophytic organisms and their enzymes and secondary metabolites, for example, in fuel industry (Singh *et al.*, 2011) and as antibiotics (Xing *et al.*, 2011).

However, these studies let open the lack in knowledge on the implications of these associations and brought into discussion the need to know deeply these interactions and, their importance on the physiology of both, the host plant and the endophyte (Bayman *et al.*, 1997) and their functional meaning. Therefore, a better understanding of the function of this organism in nature could lead to develop technologies for species conservation, competitive crops and friendly methods with the environment (Yuan *et al.*, 2009). Bayman *et al.* (2011) and Schulz and Boyle (2006) proposed the possibility that mycorrhizal and fungal endophytes can protect host plants against pathogen attacks or generate some kind of resistance to stress factors.

Some species of the genus *Vanilla* have economical importance being the second most expensive spice, after saffron, and the flavoring most used in food industry (Havkin-Frenkel *et al.*, 2011; Bucellato, 2011). In Colombia is registered the presence of *V. planifolia* Andrews, specie of high economical importance in several departments of the country such as Antioquia, Chocó, Valle del Cauca and Bolívar (Ledezma *et al.*, 2006; Misas, 2005; Ordóñez *et al.*, 2012); however, there is few knowledge on nutrient requirements and associations of this specie with endophytic microorganisms. For this reason, this study has the objectives of determining the endophytic fungi associations present in *Vanilla* sp. and studying their potential on *V. planifolia* seedling growth under greenhouse conditions based on the inoculation of fungi isolated from other orchids and wild vanilla.

Materials and methods

Vanilla sp. plants to isolate fungal endophytes were collected in the Atlantic Coast near Morrosquillo gulf and Montes de Maria (Sucre), Sierra Nevada de Santa Marta (Magdalena), San Pedro de Urabá; San Luis; San Jerónimo and Porce (Antioquia); Yopal (Casanare); Serranía de la Macarena (Caquetá); Buenaventura and surroundings (Valle del Cauca) (Table 1). In each location at least three vanilla plants were selected that had good growth and without any visual symptoms of disease or nutritional deficiencies. For each plant root samples of approximately 20 cm length were collected and placed on 10 g of adjacent soil. Root were exposed trying to avoid their altera-

Table 1. Collection sites where *Vanilla* sp. in wild state were registered, Colombia.

Location	Department
Porce	Antioquia
San Jerónimo	Antioquia
San Luis	Antioquia
San Pedro de Urabá	Antioquia
Yopal	Meta
Serranía de la Macarena	Sucre
Golfo de Morrosquillo	Sucre
Buenaventura	Valle del Cauca
S. N. de Santa Marta	Magdalena

tion, cut with pruners, packed in hermetic closure plastic bags, properly labelled and moved fast to the lab on styrofoam boxes; there, they were washed with tap water, superficially disinfected with 70% ethanol for 1 min, 3% sodium hypochlorite for 30 s and, finally washed three times with sterile distilled water (Otero *et al.*, 2002). For sowing 2 mm cuts were done with sterile surgical blades. On Petri dishes were sowed in triplicate eight root fragments. Media used for isolations was potato dextrose agar (PDA), supplemented with 50 µg/ml of penicillin and streptomycin sulfate before incubation in the dark at 28 °C for eight days.

Molecular identification was performed using the obtained colonies by sequencing the ITS regions according to the protocols of the Cellular and Molecular Biology lab of the Universidad Nacional de Colombia - Medellín (data not shown).

Inoculum preparation

From the Petri dishes, were previously fungal endophytes were isolated; a 0.5 x 0.5 cm piece of inoculum was taken and placed on an Erlenmeyer containing liquid potato dextrose. Then it was placed on a shaker at 120 rpm at room temperature in the dark. After eight days of culture, with a home mixer disinfected with 70% alcohol for 1 h and 4% sodium hypochlorite for 30 min, fungi was liquefied till getting fine particles. 250 ml of liquefied were obtained and volume was completed till 600 ml on a plastic container.

For the inoculation process, a total of 500 cm^3 wood chips and vermicompost on a ratio 1:1 were used per pot, they were autoclaved at 15 psi, 120 °C for 60 min. these pots were filled with 500 cm^3 of substrate and 20 ml of inoculum were added in the top. Finally, pots were covered with sterile newspaper and left at room temperature for seven days till the fungi grew in this substrate.

Application of inoculum to the plants

V. planifolia plants were obtained from cuttings obtained in the nursery located in San Pedro de Urabá (Antioquia) and property of Bioandes C.I Ltda. Each plant selected had at least seven internodes (approximately 20 cm length), that were previously disinfected of fungal pathogen and no pathogen preexisting in the roots and aerial part. This procedure was performed fumigating the aerial part with the fungicide Antracol® 2% (Propined 700g/kg) and Mertec® 1% (Thiabendazole 500g/l) once per week for three weeks. After eight days of the inoculation the fungi had already grown, thus, in each pot a cutting was sowed placing at least an internode into the substrate. Plants were tied up to the artificial tutor (wood sticks 70 x 4 x 1 cm) with sisal ropes. Finally, pots were covered with chopped quartz to get a protective layer against insect and other agents' entry. Inoculation process was repeated twice: at assembly and after 45 days.

Six fungi from the genus *Rhizoctonia* were used coming from orchids plants from Valle del Cauca, obtained by Valadares (Valadares, 2009): P-17, P-18, P-19 and P-20 of *Psigmorchis pusida* and I-1, I-2 of *Ionopsis utricularoides*; 12 fungal endophytes isolated in this study from wild *Vanilla* spp. and a control where the soil was sterile and without inoculation (Table 2). The assay was established in the greenhouse under ceiling shade of the Bioandes C.I Ltda. Company in the town of Sopetrán (Antioquia) with an average temperature of 25 °C and relative luminosity of 9-11%.

Biomass and growth

To measure biomass and total growth, it was harvested four plants of six months per treatment. Total aerial biomass (MA -g) and terrestrial root mass (MR –g) were obtained by weighting the plant material dried on oven at 60 °C for four days. Root length (LR -cm) was determined by measuring the main root and all the secondary and tertiary roots. To determine the foliar area (AF –cm^2) a portable meter LI-3000C was used and, for total height (AL -cm) the plant length at harvesting time was measured from the base till the growing apex.

Experimental design

Treatments were distributed on a complete randomized array with four replicates. Dependent variables were evaluated to comply with the assumptions (Hoshmand, 2006) and the analysis was done on the SAS 9.2 software for Windows.

Results

Collected information on the distribution spots of wild *Vanilla* sp. in Colombia allow the identification and visit to nine places located in multiple departments (Table 2). Collected plant roots belonged to wild individuals without signals of disease.

By sequencing ITS regions (data not shown), done by following the protocols of the Molecular and Cell Biology Lab of the Universidad Nacional – Medellín, it was possible to identify inoculated fungi that belong to typical endophyte genus of orchids and other plants (Picture 1). It was not possible the identification of V-10 and V-13 isolates due to repeated DNA contaminations.

In Table 3 are observed the net values for each one of the biomass variables. After validating the assumptions, results indicate significant differences in: plant height (P = 0.0002), root length (P = 0.0213), root mass (P = 0.0173) and aerial mass (P = 0.0431); while for leaf area there were no differences (P = 0.1148).

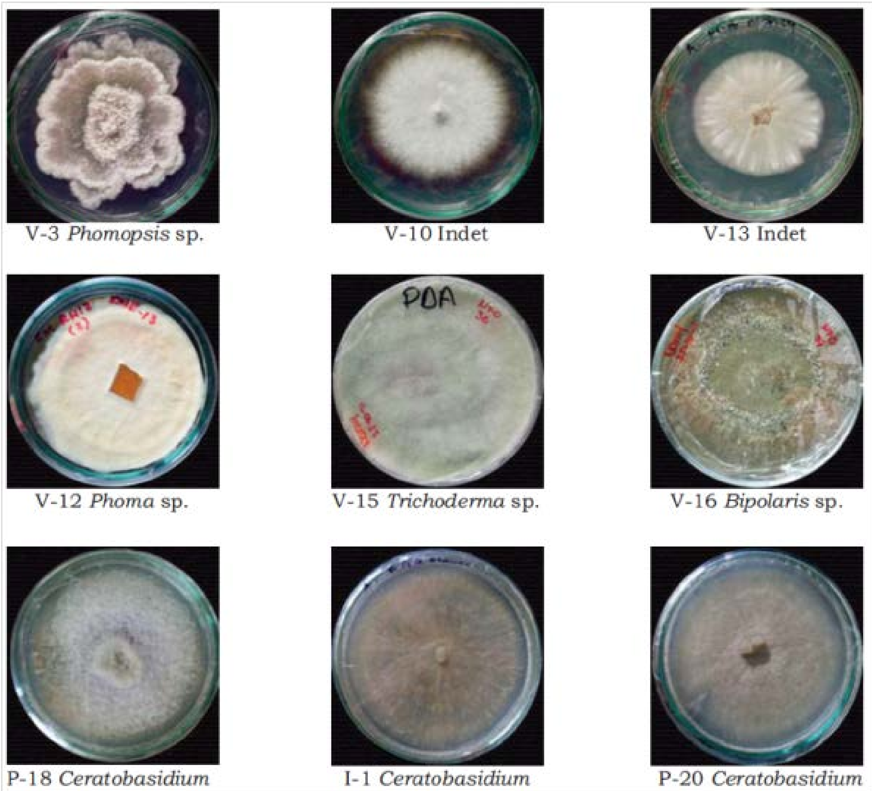
Discussion

Additional to mycorrhizal formation fungi, the fungal endophyte group associated to other plant organs has gain a lot of interest due to multiple ecological functions (Brundrett 2006; Yuan *et al.*, 2009; Bayman and Otero, 2006). However, most of the studies have been focused more on the identification of these fungi than in their possible roles on orchid nutrition, defense against pathogens, or in adaptive processes to stress factors (Gamboa-Gaitán, 2006; Ordóñez, 2012).

Used fungi in this experiment (I-1, I-2, P-17, P-18, P-19 and P-20), obtained from Valadres (2009), belong to the *Ceratobasidium* group of the genus *Rhizoctonia* considered as endophytes that form mycorrhizal in orchids in Australia (Warcup and Talbot, 1967; Otero et al., 2011), Scotland (Warcup and Talbot, 1967), Puerto Rico (Otero *et al.*, 2002, 2004, 2007; Porras-Alfaro and Bayman, 2007), and

Table 2. List of endophytes, hosts and origin locations of the inoculated fungi in the *Vanilla planifolia* seedling growth experiment.

Code	Host	Location
I-1	*Ionopsis utricularoides*	Buenaventura (Valle del Cauca)
I-2	*Ionopsis utricularoides*	Buenaventura (Valle del Cauca)
V-3	*Vanilla* sp.	San Luis (Antioquia)
V-4	*Vanilla* sp.	San Pedro Urabá (Antioquia)
V-6	*Vanilla* sp.	Porce (Antioquia)
V-8	*Vanilla* sp.	Buenaventura (Valle del Cauca)
V-9	*Vanilla* sp.	Montes de María (Sucre)
V-10	*Vanilla* sp.	San Pedro Urabá (Antioquia)
V-11	*Vanilla* sp.	San Pedro Urabá (Antioquia)
V-12	*Vanilla* sp.	San Pedro Urabá (Antioquia)
V-13	*Vanilla* sp.	San Pedro Urabá (Antioquia)
V-14	*Vanilla* sp.	Morrosquillo gulf (Sucre)
V-15	*Vanilla* sp.	Montes de María (Sucre)
V-16	*Vanilla* sp.	Golfo de Morrosquillo (Sucre)
P-17	*Psygmorchis pusida*	Buenaventura (Valle del Cauca)
P-18	*Psygmorchis pusida*	Buenaventura (Valle del Cauca)
P-19	*Psygmorchis pusida*	Buenaventura (Valle del Cauca)
P-20	*Psygmorchis pusida*	Buenaventura (Valle del Cauca)
Control		



Picture 1. Colony morphology of some inoculated isolates in *Vanilla planifolia* plants.

Colombia (Mosquera-Espinosa, 2010). P-18 isolate, obtained from Valadares (2009), had a significant effect on the variable plant length; in contrast, the other isolates were not noticeable on the other biomass variables. This indicates that, possibly for the rest of fungi, the nutrient input is not enough to increase growth rates or that for this stage on plant growth this association is not fundamental, as it is on seed germination, and it is facultative for stages where the plant is photosynthetically active (Porras-Alfaro and Bayman 2007).

In biomass variables like plant and root length, and root mass, the best treatments were V-13, V-11 and V-9, the latest two are members of the Xylariaceae family. This group was identified by Bayman *et al.* (1997); Chen *et al.* (2011, 2012); Xing *et al.* (2011); and Yuan *et al.* (2009) as orchid endophyte and was isolated mainly from leaves and roots. This family is characterized for having saprophyte organisms and orchid endophytes like *Lephantes*, *Dendrobium*, *Sobralia*, *Maxillaria*, *Psychilis* and *Epidendrum* (Bayman and Otero, 2006) as in other plants as in *Guarea guidonia* Meliaceae (Gamboa-Gaitán and Bayman 2001), among others, (Davis *et al.*, 2003). Similarly, they are common inhabitants of wood and substrates in decomposition (Whalley, 1996). In vitro studies demonstrated that both, *Xilaria* and species of the genus *Hypoxilon*, are capable of producing enzymes like calases, cellulases, cellulo-biohydrolases and cellulo-deshydrogenases involved in degradation of lignin and cellulose, mainly components of substrates used in orchid culture (Whalley 1996; Pointing *et al.*, 2003). In this way, degradation of woody materials as wood chips and leaf litter, substrates in which vanilla grows, contributes to the solubilization and availability of nutrients for the plant, improving the nutrition on the growth media. Studies showing that Xylariaceae family species can give benefits living like endophytes are scarce. The application of inoculum with endophytes of the genus *Xylaria* reduces effectively the damage caused by pathogens in *Theobroma cacao* (Yuan *et al.*, 2009). Studies

Table 3. Biomass variables measured on *Vanilla planifolia* plants inoculated with different fungi.

Code	Endophyte	MA (g)	AL (cm)	AF (cm^2)	LR (cm)	MR (g)
Ctrl		1.8902	15.5	160.5975	109.55	0.253
I-1	*Ceratobasidium*	1.6612	12.7	129.375	102.6	0.2642
I-2	*Ceratobasidium*	2.8325	34	194.61	161.3	0.456
V-3	*Phomopsis* sp.	2.3995	29.2	173.14	142.475	0.3972
V-4	Indet.	2.6255	24.7	199.275	138.65	0.4757
V-6	*Phomopsis* sp.	2.1345	30.5	170.715	133.275	0.3675
V-8	Indet	1.9145	23.5	130.245	155.9	0.4477
V-9	*Hypoxylon* sp.	2.4005	30.25	173.7075	187.85	0.4907
V-10	Indet.	3.1382	32.5	210.3125	221.975	0.64
V-11	Xylariaceae	2.3477	34.75	181.495	106.875	0.3237
V-12	*Phoma* sp.	2.6252	25.25	171.685	210.375	0.648
V-13	Indet.	2.82	39.25	205.415	163.15	0.4652
V-14	*Phomopsis* sp.	2.1462	17.25	142.903	98.9	0.296
V-15	*Trichoderma* sp.	1.7465	15.75	252.1652	100.175	0.2742
V-16	*Bipolaris* sp.	1.9552	20.25	122.625	100.025	0.292
P-17	*Ceratobasidium*	2.0082	21.25	132.185	142.05	0.3707
P-18	*Ceratobasidium*	2.7707	34.25	169.1675	150.35	0.473
P-19	*Ceratobasidium*	2.0297	15.75	126.0325	77.925	0.2225
P-20	*Ceratobasidium*	2.268	20	145.4325	153.875	0.416
F<0.005		0.0431	0.0002	0.1148	0.0213	0.0173

Notations: MA(g) = aerial mass; AL(cm) = plant height; AF(cm^2) = leaf area; LR(cm) = root length; MR(g) = root mass.

of Davis et al. (2003) showed and characterized secondary metabolites that include antimycotics and antibiotics that control plant and human pathogens. Whalley and Edward (1998) characterized as main metabolites the following: dihydroisocoumarins and derivates, succinic acid and derivates, sesquiterpenes alcohols, butyrolactones, cytochalasins, naftalene derivates and long chain fatty acids.*Phomopsis* groups (V-3, V-6, and V-14), *Bipolaris* (V-16), *Phoma* sp. (V-12) and *Trichoderma* (V-15) did not show a noticeable behavior on the evaluated variables and correspond to previously reported orchid endophytes in the genus *Dendrobium* (Chen et al., 2012), *Stelis*, *Lepanthes*, *Maxillaria*, *Epidendrum* (Bayman and Otero, 2006) and *Odontoglossum* (Singh et al., 2011).

Although the findings on the possibility that some inoculated fungi can act as potential pathogens, in this experiment vanilla seedlings did not show any symptoms of organ (roots, stem or leaves) damage, probably because in these cases the inoculated fungi work as decomposition agents on the used substrate, wood chips and vermicompost, typical from places were vanilla roots are found. In the same manner, inoculation of organisms of this group could not only favor the plant on nutrient availability but also on secretion of enzymes and secondary metabolites to avoid or biocontrol (Ordóñez, 2012).

Mosquera-Espinosa (2010) demonstrated for the first time the use of *Rhizoctonia* binucleated (*Ceratobasidium*) isolates obtained from orchids as a potential biocontroler of R. solani, a rice pathogen, and other pathogens like *Fusarium* spp., *Phytophthora* spp. and *Pythium* sp., as a promising alternative for biocontrol strategies within an integrated management program.

On the other hand, fungal endophytes can act as latent pathogen agents during long periods constituting asymptomatic interactions, therefore the microorganisms in that situation can be defined as temporal endophytes, which has been observed in other plants (Schulz and Boyle, 2006; Gamboa-Gaitán, 2006). However, by mutation, environmental changes, nutrient status or plant age, a latent endophyte can become pathogen or vice versa (Ovando *et al.*, 2005; Lana *et al.*, 2011). For that reason, it is important to recognize the physiological effects of fungal endophytes on plants and the potential of these microorganisms on plant protection and nutrition improvement.

Conclusions

- In this study the growth and development rates of *V. planifolia* plants were stimulated by three fungal endophytes isolated from wild vanilla plants and other orchids. Additionally, it was observed the diversity of fungal endophytes associated with vanilla roots, some of them reported as pathogens or beneficial.

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