How significant are endophytic fungi in bromeliad seeds and seedlings?

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How significant are endophytic fungi in bromeliad seeds and seedlings? Effects on germination, survival and performance of two epiphytic plant species

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Abstract

In bromeliads, nothing is known about the associations fungi form with seeds and seedling roots. We investigated whether fungal associations occur in the seeds and seedling roots of two epiphytic *Aechmea* species, and we explored whether substrate and fungal associations contribute to seed germination, and seedling survival and performance after the first month of growth. We found a total of 21 genera and 77 species of endophytic fungi in the seeds and seedlings for both *Aechmea* species by Illumina Miseq sequencing. The fungal associations in seeds were found in the majority of corresponding seedlings, suggesting that fungi are transmitted vertically. Substrate quality modulated the germination and growth of seedlings, and beneficial endophytic fungi were not particularly crucial for germination but contributed positively to survival and growth. Overall, this study provides the first evidence of an endophytic fungal community in both the seeds and seedlings of two epiphytic bromeliads species that subsequently benefit plant growth.

Keywords: *Aechmea*, Bromeliads, Endophytic fungi, *Fusarium* spp., Germination, Survival, *Trichoderma* spp., Vertical transmission

1. Introduction

Seed germination and establishment of most epiphytic plant species is strongly affected by heterogeneous biotic and abiotic conditions at the microhabitat scale (Mondragón and Calvo-Irabien, 2006; Toledo-Aceves and Wolf, 2008; Goode and Allen, 2009; Mondragón et al., 2015). One would expect germination proportions to be generally low and highly variable in space and time because most seeds would fall on unsuitable arboreal habitats (Zotz, 2016). Abiotic factors that play an important role in germination success include: exposure to incident radiation (Pinheiro and Borghetti, 2003; Klekailo et al., 2012; Marques et al., 2014; Leroy et al., 2017), temperature (Montes-Recinas et al., 2012), water availability (Bader et al., 2009; Cavalcante et al., 2010; Mantovani and

Iglesias, 2010), the nature of the substrate (Hietz et al., 2012; Leroy et al., 2017), and elevation and/or location in the canopy (Winkler et al., 2005). Water and nutrient availability are important factors for the epiphytes, because these plants have no direct contact with terrestrial soil, and seedlings desiccate faster than adults (Schmidt and Zotz, 2001; Zotz and Hietz, 2001; Mondragón et al., 2004).

The role of biotic factors, such as fungal associations, should not be overlooked for epiphyte establishment (Whigham et al., 2008). Given the benefits of fungal associations in nutrient-limited habitats (Smith and Read, 2010), such as tree canopies, seedlings of vascular epiphytes would be expected to be highly assisted by mycorrhizal and/or non-mycorrhizal fungi. However, little is known about the associations that fungi form with epiphytic seedlings in the forest canopy (Whigham et al., 2008; Zotz, 2016), and the diversity and ecological roles of fungal endophytes remain largely unclear (Bayman and Otero, 2006; Novotna et al., 2018). Previous studies have largely focused on orchid seed germination and population establishment, since the existence of suitable fungal partners is vital for this family (e.g., Dearnaley et al., 2012; McCormick and Jacquemyn, 2014). Numerous studies have shown that the roots of tropical epiphytic orchids harbor diverse fungal taxa, including mutualistic mycorrhiza and diverse endophytic non-mycorrhizal fungi (e.g., Otero et al., 2007; Kartzinel et al., 2013; Novotna et al., 2018). Mycorrhizas and endophytes have also been found in various epiphytic angiosperms such as Araceae, Clusiaceae, Ericaceae, and Piperaceae (Nadarajah and Nawawi, 1993; Rains et al., 2003). In epiphytic bromeliads, it has been hypothesized that interactions with fungi may not occur, or may be limited, because of less developed root systems or roots that are not the primary organs of water and nutrient uptake (Benzing, 2000; Whigham et al., 2008).

Bromeliads are one of the most diverse groups of epiphytes found in the entire tropical and subtropical zones of the Americas. The family comprises *ca*. 3140 species and 58 genera distributed between three subfamilies (Bromelioideae, Tillandsioideae, and Pitcairnioideae (Crayn et al., 2004; but see Givnish et al., 2011 for recent systematic updates). Bromeliad species are found in extreme

conditions of moisture availability (from rain forests to arid coastal sands), elevation (from sea level to ca. 4000 m a.s.l), and exposure to sunlight (fully exposed to shaded forest understory), reflecting their success in dispersing and adapting to a wide variety of habitats and environments. Bromeliads exhibit a variety of water and nutrient uptake mechanisms, including absorptive soil root systems (terrestrial bromeliads), and the leaf water-absorbing trichomes (unique to bromeliads) which reduce roots to a purely mechanical support function, attaching the plant to the substrate (Benzing, 2000). Mature, epiphytic bromeliads are adapted to period of drought having water-storing tanks formed by overlapping leaf bases (Benzing, 2000), by developing crassulacean acid metabolism (CAM) photosynthesis (Crayn et al. 2004), or by using leaf water-absorbing trichomes which reflect sunlight (Pierce, 2007). Seedlings of some C_3 tank-forming bromeliads (from the Tillandsioideae subfamily) do not form tanks until they are 1-5 years (Zotz et al., 2004; Meisner and Zotz, 2012) so periods with little to no water and nutrients are likely to affect seedlings to drought stress more severely than adults (Hietz and Wanek, 2003). In contrast, *Aechmea mertensii*, a CAM tank-forming bromeliad from the Bromelioideae subfamily, is totally devoid of leaf water-absorbing trichomes at the seedling stage and depends on its roots for water and nutrient absorption (Petit et al., 2014; Leroy et al., 2017). Leroy et al. (2017) showed that this species performs better when growing in rich substrate indicating a significant nutritional role of the roots. However, while fungal associations were observed microscopically on either terrestrial (mycorrhizas) or epiphytic (dark septate endophytes) adult bromeliad roots (Allen et al., 1993; Rabatin et al., 1993; Rowe and Pringle, 2005; Grippa et al., 2007; Lugo et al., 2009), there have been no reports of fungal colonization of bromeliad seeds and seedlings (Whigham et al., 2008). Indeed, we still know very little about bromeliad germination and seedling ecology.

In this context, the purpose of this study was two-fold. First, to investigate whether fungal associations occur in the seeds and seedling roots of two *Aechmea* species which are CAM tank-forming at adult stage, and to determine the specific richness of fungal communities (i.e., alpha diversity). Second, to explore the role of roots in water and nutrient uptake, and whether fungal

associations contribute to seed germination, and seedling survival and performance. To this end, we set up a greenhouse experiment to grow the seeds of *Aechmea aquilega* and *A. mertensii* on substrates of two qualities (nutrient rich and poor) and on two fungal associations (high and low). Fungal associations were achieved by reiterative use of fungicide to reduce colonization by any mycorrhizal and endophytic fungi. We compared seed germination success, and seedling survival and performance, for all treatments, using a set of eight leaf/root traits which reflect plant growth and resource uptake. Additionally, we extracted fungal DNA of seeds and seedling roots to characterize fungal communities by Illumina Miseq sequencing. We hypothesized that if *Aechmea* seedlings depend on their roots for mineral acquisition, seedlings might have fungal associations, and that nutrient-poor substrate should decrease germination proportion, and seedling survival and performance. We also hypothesized that if fungal associations are required for *Aechmea* seedling growth, the use of fungicide should reduce the survival and performance of the seedlings.

2. Materials and methods

2.1 Bromeliad species

Aechmea aquilega and *Aechmea mertensii* are tank-forming bromeliads from the subfamily Bromelioideae with CAM photosynthesis pathway (Crayn et al., 2004). While *A. aquilega* occurs as an epiphytic, lithophytic or secondary terrestrial bromeliad (Talaga et al., 2015), *A. mertensii* is specifically found in complex associations with arboreal ants known as ant-gardens (Leroy et al., 2009; Orivel and Leroy, 2011). The mature fruits of both species are fleshy and indehiscent at maturity. While the fruits of *A. aquilega* contain numerous seeds (> 100 seeds per fruit, pers. obs.), fruits of *A. mertensii* average 10 seeds per fruit (Leroy et al., 2012).

For *A. aquilega*, two inflorescences of the same patch were collected, in November 2016, in the city of Sinnamary, French Guiana (05°22’39’’N, 52°57’35’’W). For *A. mertensii*, the seeds were collected, during the same period, from four individuals (of the same maternal plant) growing in the greenhouse at the campus Agronomique in Kourou, French Guiana (05°09’46’’N, 52°38’33’’W). A

total of 1200 mature seeds were used for each bromeliad species from a low number of inflorescences to limit the likelihood of maternal effect.

2.2 Experimental procedures

To determine the effects of substrate quality, fungicide, and their interactions on germination, and seedling survival and performance, of both species, we set-up a 2-month experiment in a greenhouse at the Campus Agronomique in Kourou (see also Leroy et al. (2017) for the influence of environmental conditions on the germination and growth requirements). Environmental HOBO sensors were used to measure light, temperature and relative humidity in the greenhouse (model UA-002-64, HOBO Pendant Tem Light – 64k and model U23-001, HOBO Pro V2 Temp/RH Data logger, Amanvillers, France). During the 2-month experiment, the mean relative humidity was 77.89 ± 0.45% (min-max: 44.31-98.95%), the mean temperature was 29.5 ± 0.12°C (min-max: 24.36-39.14°C), and the daily mean light intensity was 41419.72 ± 1638.13 lux (*ca*. 75% reduction compared to the light intensity outside the greenhouse).

We selected two different substrates: compost (nutrient-rich), and white sand (nutrient-poor). Four different treatments were set-up: (T1) white-sand + compost (i.e., nutrient-rich substrate), (T2) white-sand (i.e., nutrient-poor substrate), (T3) sterilised white-sand + sterilised compost + fungicide (i.e., nutrient-rich substrate without fungal association), and (T4) sterilised white-sand + fungicide (i.e., nutrient-poor substrate without fungal association). For T3 and T4, the white-sand and compost were autoclaved at 121°C for 20 min. Two 2 x1.2 m trays were installed in the greenhouse to separate T1 and T2 from T3 and T4 to avoid any contamination when applying the fungicide. For each tray, 20 Petri dishes (90 x 14.2 mm) per treatment were completely filled with the corresponding substrate and with autoclaved filter paper (Whatman No. 1) at the bottom.

The seeds were used immediately after their removal from the fruit as they are non-dormant (Benzing 2000). The seeds were sterilized using the following method: immersion in a 70% alcohol solution for two minutes, rinsed twice in sterilized distilled water, immersion in commercial bleach

(5% NaOCl) for 30 min, and rinsed three times in sterilized distilled water. We distributed the seeds from different fruits of a single inflorescence among all of the treatments equally to control maternal effect. A total of 15 seeds were sowed per Petri dish and 20 replicates were used for each of the 4 treatments (i.e., 300 seeds per treatment). A mosquito net enclosure was installed above all Petri dishes for each treatment to prevent herbivory. Then, the seeds were manually sprayed twice a day with 2 µm-filtered rainwater to eliminate microorganisms while keeping all nutrients. To prevent any fungal colonisation on T3 and T4 we spread an aqueous solution of Thiabendazole [2-(1,3-thiazol-4-l) benzimidazole] at 1 g. L^{-1} once a week over two months. Thiabendazole is a systemic fungicide widely used as a post-harvest and pre-packing and transportation treatment (e.g., Khan et al., 2001).

2.3 Fungicide efficiency

The fungicide efficiency was tested by comparing fungal root colonisation of five seedlings per treatment. Fresh roots were washed with tap water and then stained with NaOCl-acid fuchsin-glycerin following Kormanik & Mc Graw (1982). Root colonization was quantified with an optical microscope at x 40 magnification using the magnified intersections method (see McGonigle et al., 1990). Both hyphae and vesicles were considered and pooled for the calculation of the endophytic fungal colonization (% root length) according to the treatment. Additionally, at the end of the experiment, we tested for phytotoxic effects of the fungicide on seedling foliage by spraying Thiabendazole once a week for 4 weeks on the remaining non-treated seedlings. The fungicide did not affect the vegetative structure and growth of the seedlings.

2.4 Seed germination, and seedling performance and survival

All Petri dishes were inspected every day to count the germinated seeds. The seeds were considered germinated when the radicle protruded by at least 2 mm through the seed coat. The percentage of final germination was calculated as the number of seedlings divided by the number of seeds. Then, up to 20 seedlings per treatment were harvested 15 d and 30 d after first germination to evaluate

their performance. At the same time, seedling survival was recorded every day for 60 d after the seeds were sown in the Petri dishes.

For each seedling harvested, we measured a set of eight key leaf and root traits reflecting plant growth and resource uptake that are good predictors of plant performance (Poorter and Bongers, 2006). The leaves were separated from roots and immediately weighed with a precision balance to obtain the total fresh mass (LFM). Then, the longest leaf length (LLmax), total leaf area (LA), and stomatal density (SD) were measured. To estimate the LA and LLmax a digital photograph was taken of all the leaves of each seedling and of the root system with a Nikon Coolpix 4500 digital camera (macro mode was used when necessary). The lengths and leaf area were then measured using image analysis software (Image-ProPlus, Media Cybernetics, Silver Spring, MD, USA). SD was determined for the abaxial and adaxial surfaces of the longest mature leaf from imprints made using transparent nail varnish. The number of stomata per mm^2 was then recorded using an inverted microscope (Olympus-BX51) to examine each preparation in three to five randomly selected areas measuring 1 mm^2 each. Then, all leaf lamina and roots were dried at 60°C for 72 h to obtain the total leaf dry mass (LDM or biomass) and the total root dry mass (RDM or biomass). The leaf to root ratio (L:R ratio) was calculated as LDM/RDM. The leaf mass per area (LMA, g m^{-2}) was calculated as the ratio between the LDM and the LA, and the leaf water content (LWC, %) was calculated as (LFM-LDM)/LFM x 100.

2.5 Data analysis

All statistical analyses were conducted using R, version 3.4.4 (R Development Core Team, 2015), and were evaluated at a 95% confidence level. The results are presented as means ± SE throughout the text.

Seed germination followed a binomial distribution and so lacked the properties of linearity and additivity. Logistic regressions were used to determine which of the factors (i.e. substrate and fungicide) affected seed germination probabilities. Data were analysed using a generalized linear model for binomial responses with a logit link function. We considered time as a continuous variable

and plant identity (i.e., seed origin) as a random factor to control for maternal effect. Data from final germination were arcsin transformed before analysis to achieve homogeneity of variance (Bartlett test). Two-way ANOVA was used to test for the effect of substrate, fungicide, and their interactions in seed germination success and in seedling survival percentages. When significant, ANOVA was followed by Tukey's tests (Honestly Significant Differences, HSD) for *a posteriori* testing of multiple means.

Kaplan-Meier curves were used to display the proportion of seedlings surviving over time (Kaplan and Meier, 1958). We used the survfit function and additional graphical options in the *survival* package. The monitoring of surviving seedlings was stopped after 12 weeks, but it cannot be assumed that there was no further mortality. Hence, we used a Cox proportion hazards model to assess differences in seedling survival between all treatments (Therneau and Grambsch, 2000). The Cox proportional hazard model was also appropriate because some seedlings were harvested before they died, which caused left censoring. Probability of survival was modelled over time as a function of substrate and fungicide with plant identity as a random factor. The Cox survival analysis was run using the coxph function in the *survival* package.

Three-way ANOVA was used to examine the effect of substrate, fungicide, time, and their interactions on the eight dependent variables (i.e. LL_{max}, TLA, LDM, RDM, L:R ratio, LMA, LWC, and SD) with seed ID as a random factor. Data were log or rank transformed, when necessary, to satisfy the assumptions of the ANOVA. When significant, ANOVA were followed by Tukey's tests (HSD) for *a posteriori* testing of multiple means.

2.6 Molecular fungal identification

All seeds from four *A. mertensii* fruits and two fruits of *A. aquilega* (i.e., from the same plants used for the germination experiment) were directly immerged in a CTAB 2X Buffer (100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM Na2 EDTA, 2% CTAB) and kept at 4°C. For both species, the entire root system of five 15 d seedlings for T1, T2, and T3 (in T4, the roots have not developed, see Results section)

were gently washed under tap water, dried with a paper towel and directly immersed in a CTAB 2X Buffer, and kept at 4°C until DNA extraction.

The total DNA was extracted from the seeds and the roots using the commercial DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The barcode used was the Internal Transcribed Spacer 1 (ITS1) of the ribosomal DNA. The fungal library was obtained with the ITS5/5.8SF (5'-GGAAGTAAAAGTCGTAACAAGG-3'/5'-CAAGAGATCCGTTGTTGAAAGTK-3') primer set (Epp et al., 2012; White et al., 1990). Amplifications were carried out in 20 µl reactions containing 10 µl of Mastermix Amplitaq Gold of 5 U/µl (Fisher Scientific), 1 µl of 5 µM for each primer, 5 µl of 5% PVP (PolyVinyl Pirrolidone, SIGMA), 1 µl milliQ water and 2 µl of template DNA. The PCR was performed on a DNA Engine Peltier thermal cycler (BIO-RAD, PTC-200, Foster City, CA, USA) with a program consisting of initial denaturation at 95°C for 10 min, 40 cycles of repeated reactions (denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1min) and final elongation of 7 min at 72°C. Post-cycling, samples were held at 4°C. PCR products were loaded onto a 1 % standard agarose gel for electrophoresis stained by 5% (v/v) GelRed (Biotium, Hayward, USA). Unsuccessfully amplified samples were subjected to multiple amplifications at various DNA concentrations. After quantification with the QUBIT 2.0 (Thermo Fisher Scientific, Waltham, Ma, USA), the purified PCR products were mixed in equimolar amounts to prepare sequencing libraries. The libraries were paired-end sequenced using the Illumina MiSeq technology (2 x 300bp) by Fasteris (Plan-les-Ouates, Switzerland).

MiSeq sequence libraries were built by tag detection with a personal batch. They were analysed with a custom automated pipeline named EGRETTA (Pierart et al., 2018), using public tools and personal scripts. Sequence quality was analysed (pairing, primer trimming, chimera removal) with an optimized protocol based on MOTHUR software packages (Schloss et al., 2009). Dereplication and 97% OTU clustering (Operational Taxonomic Unit) were carried out with an enhanced protocol based on Uparse (Edgar 2013). Sequences were trimmed on ITS1 with HMM approaches (Bengtsson-Palme et al. 2013). Finally, taxonomic assignation and statistical analysis were obtained using personal

scripts. The pipeline used a specific enhanced data mined base which contains fungal sequences (Ascomycota, Basidiomycota, and Glomeromycota) and contaminants (Streptophyta, Nematoda, and protists). The ecology of the fungal OTUs obtained after the taxonomic assignation were determined after a literature survey. Alpha-diversity metrics of assigned endophytes were estimated by calculating the Effective Number of Species index of general order q=1 ($Jost_1$ index) based on Hill numbers (Chao et al., 2010). The fungal community composition was obtained by Weighted Unifrac matrix distance calculation (Lozupone et al. 2011) and displayed in a Principal coordinate analysis.

3. Results

3.1 Fungal associations in the seeds and seedlings

Fungal associations were found in both the seeds and seedling roots of the two *Aechmea* species. Endophytic fungi (i.e., mycosymbionts that colonize the internal tissues of plants without causing damage) were more abundant than pathogens and other fungi (e.g., mycorrhiza, saprotrophs) in all conditions (Fig. 1). Species richness of endophytes ($Jost_1$ index) between T1, T2, and T3 were less diverse for *A. mertensii* than for *A. aquilega* (Fig. 2). For both bromeliad species, the fungus species richness was higher in nutrient-rich substrates (with T1>>T2 and T3≥T2) than nutrient-poor substrates. As expected, the application of Thiabendazole fungicide significantly reduced root fungal colonisation for both bromeliad species (Fig. 3). However, unexpectedly, all seedlings of T4 conditions (poor substrate treated with fungicide) had no root development during the experiment, unlike T2 (poor substrate without fungicide).

According to taxonomic assignation (Table 1 and Supplementary Material Table S1), *A. aquilega* and *A. mertensii* were characterised by similar root-associated fungal endophytes (mainly Ascomycota) in all plant conditions. A total 684 OTUs corresponding to 21 genera and 77 species were assigned for both *Aechmea* species. Taxa found in seeds were also in a broad majority in the corresponding seedlings. The most frequent genera found in both seeds and seedlings were *Trichoderma* spp. and *Fusarium* spp., corresponding to 20 taxa and 261 OTUs (Table 1). Taxonomic

assignation bias were removed with WUnifrac approaches to show phylogenetic distance of endophyte genes contained in T1, T2, and T3 treatments (Fig. 4). A good separation between the two *Aechmea* species were seen on the two first coordinates of the Principal Coordinate Analysis (54.8%). This was also valid when adding a third coordinate (that explained 74.6% of the variance) with d(AMT1,AMT2)=0.567 and d(AAT1,AAT2)=1.018 (three-dimentional Euclidean distances). The phylogenetic distances of endophyte genes between *A*. *aquilega* and *A. mertensii* were closest in T1 conditions (rich substrate without fungicide) while in T3 conditions (fungicide-treated poor substrate) they were very distant (d(AAT1,AMT1)=0.424; d(AAT1,AAT3)=0.972; d(AMT1,AMT3)=1.265).

3.2 Seed germination and seedling survival

Substrate, fungicide, and their interactions had significant effects on seed germination and on final germination percentages for *A. aquilega* and *A. mertensii* (Fig. 5, Table 2 and 3). The final germination percentages were at 20 d for *A. aquilega* and 15 d for *A. mertensii* (Fig. 5A, B). For *A. aquilega*, the final germination was significantly lower for treatment with poor substrate and fungicide (T4: 56.14 ± 8.81% *vs*. T1: 99.33 ± 0.89%, T2: 96.66 ± 2.67% and T3:92.01 ± 3.97%, Tukey's HSD tests, P <0.0001) while T1, T2, and T3 did not differ (Fig. 5A). However, for *A. mertensii*, the final germination was significantly lower for treatments with fungicide (80 ± 6.86% and 10 ± 6.18% for T3 and T4, respectively) compared to those without fungicide (94 ± 2.88% and 99 ± 1.43% for T1 and T2, respectively, Fig. 5B). If the substrate did not account for treatment without fungicide (T1 *vs.* T2, Tukey's HSD tests, P =0.582), it differentiated germination proportion for T3 and T4 (Tukey's HSD tests, P <0.0001).

From 15 to 60 d after the start of germination, the risk of mortality was significantly higher when the seedlings of the two *Aechmea* species lacked fungal associations (i.e., T3 and T4 fungicide treatments) compared to those with fungal associations (Fig. 6A, B, Table 4). With fungal associations, substrate did not affect survival of either bromeliad species.

3.3 Seedling performance

While *A. aquilega* seedlings were significantly smaller with lower density of stomata than *A. mertensii*, sampling date, substrate, and fungicide had significant effects on most of the measured traits for the two species (Table 5, Supplementary Material Figs. S1, S2). The interaction between substrate and fungicide was highly significant for most of the variables, indicating that poor soil condition increased differences in growth and resource uptake traits between treatments. Seedlings of both *Aechmea* species were significantly larger (i.e., higher LL_{max}, TLA, LDM) when growing in rich substrate with fungal associations compared to seedlings growing on either poor substrate or with low fungal associations (Tukey's HSD tests, $P<0.001$ for all cases). Biomass partitioning between leaves and roots depended on the substrate quality with significantly higher investment in roots on poor substrate (with the exception of plants from T4, Table 5, and Supplementary Figs. S1, S2). The fungicide treatment lead to a significant decrease in leaf dimension and biomass, and root biomass for both species, but not in the L:R ratio except for *A. mertensii* (Tukey's HSD tests, $P<0.001$, Table 5). Seedlings growing with low fungal associations had significantly higher LMA and stomatal density, and significantly lower LWC compared to seedlings associated with endophytic fungi (Tukey's HSD tests, $P<0.001$, Supplementary Figs. S1, S2).

4. Discussion

4.1 Evidence of fungal associations in seeds and seedlings of the two *Aechema* species

Currently, an increasing number of studies underscore the presence of non-mycorrhizal fungal root-associates in various plant species from different habitats and biomes (e.g., U'Ren et al., 2009; Parsa et al., 2016; Novotna et al., 2018), suggesting that fungal endophytes are far more abundant than previously thought. This study is the first report of endophytic fungi in seeds and seedling roots of bromeliads. Currently, endophytic fungi in bromeliads remain poorly characterized because previous studies had focused on the occurrence of fungi at adult stages based on microscopic approaches (Allen et al., 1993; Rabatin et al., 1993; Rowe and Pringle, 2005; Grippa et al., 2007; Lugo et al.,

2009), and because the proportion of OTUs identified (in this study *ca*. 50%) was not assigned to a species in a public database (see also Bayman and Otero, 2006; Oliveira et al., 2014). We must also be aware that DNA remains from dormant, inactive or dead cells, so OTU composition in bromeliad seedling roots did not necessarily reflect the active endophytic community.

A large proportion of endophytic non-mycorrhizal fungi (from 50% to 70%) were found in both the seeds and seedling roots of the two *Aechmea* species. Ascomycota was the most OTU-rich as in orchids (e.g., Yuan, Chen, & Yang, 2009; Novotna et al., 2018). While the specific richness of endophytes was less diverse for *A. mertensii* than for *A. aquilega*, no difference in community composition was observed despite the maternal origin of the seeds and seedlings. The community composition of fungal endophytes in seedling roots reflects that of the substrate, suggesting that host seedlings shape the fungal endophytic community (see Peršoh, 2015 for review). There is growing evidence that root-associated fungal endophytes provide underestimated beneficial effects to host plants. In our study, the dominant endophytic fungi were found for both *Aechmea* species in the genera *Fusarium* and *Trichoderma* with five and 15 species, respectively.

Fusarium spp., mostly known as pathogenic fungi in temperate environments were reported as non-pathogenic endophytic fungi in tropical plants such as in banana (Mendoza and Sikora, 2009; Costa et al., 2012) or in orchids (Yuan et al., 2009; Tan et al., 2012). Indeed, endophytic fungi may stay quiescent for their whole life before becoming mutualist or antagonist, depending on the environmental conditions and on the ontogenetic stage of the plant (Sieber, 2007). The non-pathogenic *Fusarium* spp. have the ability to stimulate seed germination and seedling establishment in orchids (Bayman and Otero, 2006), are known to have strong biocontrol activities, and enhance plant growth and reproduction (Harman et al., 2004). *Fusarium* spp. have also been described, together with *Trichoderma* spp. as frequent endophytes of banana (Sikora et al., 2008; Xia et al., 2011). *Trichoderma* spp. are cosmopolitan fungi commonly found as saprotrophs in soils, and have also been reported as endophytic root symbionts (Harman et al., 2004), and as an orchid endophyte (Bayman and Otero, 2006; Yuan et al., 2009; Novotna et al., 2018). Root colonization by *Trichoderma*

spp. frequently enhances root growth and development, plant productivity, resistance to abiotic stresses, and the uptake and use of nutrients (Harman et al., 2004).

Interestingly, all endophytic fungal OTUs found in the seeds were also found in the seedling roots suggesting a vertical transmission of fungal endophytes. Numerous examples of vertical transmission can be found in grasses and forbs (Schardl et al., 2004; Rudgers et al., 2009; Hodgson et al., 2014) but until now this has not been characterised in bromeliads. If vertical transmission does occur, the question arises as to how the endophytes arrive in the seeds.

4.2 Effects of fungal associations and soil quality on germination and seedling emergence

Most studies have found that epiphyte seeds germinate in natural conditions at rates between 0% and 10%. In controlled conditions seed germination is high, suggesting that germination is strongly affected by heterogeneous biotic and abiotic conditions at the microhabitat scale (e.g., Winkler et al., 2005; Mondragón and Calvo-Irabien, 2006; Toledo-Aceves and Wolf, 2008; Goode and Allen, 2009; Mondragón et al., 2015). In our study, seed germination ranged from 10% to 100% depending on the treatment. When fungicide was applied in the nutrient-rich substrate, final germination was not reduced or only by *ca*. 10% for *A. aquilega* and *A. mertensii*, respectively, compared to treatments without fungicide (i.e., germination up to 100%). In contrast, in nutrient-poor substrate, fungicide reduced the germination by 40% and up to 90% for *A. aquilega* and *A. mertensii*, respectively. These results suggest that (1) the seeds of *A. mertensii* relied more on fungal associations for germination compared to *A. aquilega*, only if growing on a nutrient-rich substrate, and that (2) soil quality has a greater influence on germination compared to fungicide.

Other studies on vegetable, fruits and vegetables, showed positive effects of Thiabendazole fungicide on germination (e.g., Kaiser and Hannan, 1987; Campo et al., 2009; Poletine and Zanotto, 2012; Cardoso et al., 2015, 2016). Likewise, seed germination, emergence, and growth of wheat seedlings were not affected by chemical treatment with the Thiabendazole fungicide (Garcia Júnior et al., 2008). However, in very high doses, (Kaiser and Hannan, 1987) found that this fungicide causes

phytotoxic effects in lentil seedlings. In the present study, fungicide did not cause phytotoxic effects on seeds because (1) we did not find any negative effects of the fungicide on seedling foliage by spraying Thiabendazole once a week for 4 weeks on the remaining non-treated seedlings, and (2) germination proportions were significantly more impacted by the soil quality than the fungicide treatment.

4.3 Effects of fungal association and soil quality on seedling survival and performance

While Thiabendazole had a low effect on final germination (if considering nutrient-rich substrate), this fungicide had a strong negative effect on the seedling survival, with significantly higher mortality on fungicide-treated seedlings compared to non-treated ones. These data are consistent with the study of Bayman et al. (2002) which showed that the inhibition of fungi reduced survival of young orchids. Additionally, because the substrate quality, and the interaction between substrate and fungicide, did not affect seedling survival, our results showed that endophytic fungi are an important biotic factor for survival for seedlings of both *Aechmea*.

Fungicide and substrate had significant effects on most of the measured traits related to plant growth and to resource uptake, and the interaction between fungicide and substrate had a significant effect for half of these traits. Thus, we point out that fungal endophytes in *A. aquilega* and *A. mertensii* were also fundamental for growth. The fungicide treatment reduced the quantity of fungal endophytes in seedling roots of the two *Aechmea* species. Since these two species do not have leaf-absorbing trichomes at the seedling stage, water and nutrients must be assimilated by the roots, as indicated by *A. mertensii* growing better in rich compared to poor soil conditions (Leroy et al., 2017). On nutrient-rich substrate, seedlings with few fungal endophytes had grew less well than seedlings with more fungal endophytes, indicating that fungi may enhance nutrient and water supply, which benefits the plants. On nutrient-poor substrate, the growth of the seedlings was reduced and the fungicide further diminished their growth. If considering seedlings in nutrient-poor substrate with fungal endophytes (T2) and seedlings in nutrient-rich substrate with low fungal

associations (T3), values of the different traits were comparable, supporting the hypothesis that fungal endophytes enhance nutrient supply. Traits such as low growth (LL_{max}, total leaf area, leaf and root dry mass), low water content, low L:R ratio, and high LMA and stomatal density indicated that the seedlings suffered reduced water and nutrient supply likely because of the lack of fungal endophytes and/or nutrient-poor substrate.

Biomass partitioning between leaves and roots differed more based on the quality of the substrate than on the quantity of fungal endophytes. Seedlings in low nutrient conditions allocated more biomass to the roots than the shoots, likely to maximize the uptake of limited resources (Weiner, 2004; Zhang et al., 2011). Moreover, high values of LMA indicated that the seedlings were grown in nutrient-poor environment (Wright et al., 2002; Khaled et al., 2005) and corresponded in our study to either treatment with fungal endophytes and nutrient-poor substrate or treatment with low fungal endophytes and nutrient-rich substrate. Water and nutrient uptake may have decreased in the fungicide treatment, because the hydric status of the seedlings was reduced with lower LWC and higher SD compared to seedlings with fungal endophytes. It is likely that seedlings with few fungal endophytes experienced water stress. Fungal endophytes have potential to enhance their host's growth, tolerance to abiotic stress, or resistance to pests and pathogens (Wani et al., 2015). In orchid seedlings, Yoder et al. (2000) showed that endomycorrhizal fungi facilitate the absorption of water. Finally, it has been shown that water deficit leads to an increase in stomatal density in wheat (Yang and Wang, 2001) which was also observed in this study.

4.4 Concluding remarks

We report two entirely novel facts about bromeliads. Firstly, we showed a large proportion of endophytic non-mycorrhizal fungi in both seeds and seedling roots of two *Aechmea* species. Secondly, our results suggested that these endophytic fungi are capable of vertical transmission. Moreover, we found that: (1) roots are the main site of water and nutrient acquisition as the seedlings of these two *Aechmea* species do not have leaf-absorbing trichomes; (2) the substrate

quality modulates the germination and performance of seedlings; and (3) beneficial fungal associations were not so essential for germination but contribute highly to the survival and growth of the seedlings. Altogether our results highlight the key role of fungal endophyte associations in bromeliad growth and nutrition, suggesting that establishment of epiphytic bromeliads is more complex than previously thought. We furnish new insights towards our understanding of vascular epiphytic seedling ecology and bromeliad population ecology. Finally, since fungal endophytes might be crucial at the seedling stage for epiphytic bromeliad growth, these are an additional biotic factor to consider in future germination and seedling establishment studies.

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Figure captions

Figure 1. Percentages of OTU abundance in seeds and 15-d-old seedlings of *Aechmea aquilega* and *A. mertensii* in nutrient-rich soil (T1), nutrient-poor soil (T2) and nutrient-rich soil + fungicide (T3). The category "others" groups arbuscular endomycorrhiza and ectomycorrhiza, yeast, saprotrophic and xylophagous taxa.

Figure 2. Specific richness of endophytic communities measured by $Jost_1$ index (effective number of species) based on Hill numbers in 15-d-old seedlings of *Aechmea aquilega* (light grey) and *A. mertensii* (dark grey) in nutrient-rich soil (T1), nutrient-poor soil (T2) and nutrient-rich soil + fungicide (T3).

Figure 3. Variation in root colonization (%) by fungi of *Aechmea aquilega* (light grey) and *A. mertensii* (dark grey) according to the treatments. (T1) white-sand + compost (i.e., nutrient-rich substrate), (T2) white-sand (i.e., nutrient-poor substrate) and (T3) sterilised white-sand + sterilised compost + fungicide (i.e., nutrient-rich substrate without fungal association). Different letters indicate significant differences (non-parametric Mann-Whitney's test, $P<0.05$) between treatments.

Figure 4. Principal coordinate analysis with Weighted Unifrac matrix distances of endophytic community in 15-d-old seedlings of *Aechmea aquilega* (light grey) and *A. mertensii* (dark grey) in nutrient-rich soil (T1), nutrient-poor soil (T2) and nutrient-rich soil + fungicide (T3).

Figure 5. Cumulative germination percentages of seeds under different substrates and fungicide conditions for (A) *Aechmea aquilega* and (B) *A. mertensii*. T1: no fungicide and nutrient-rich substrate, T2: no fungicide and nutrient-poor substrate, T3: fungicide and nutrient-rich substrate and T4: fungicide and nutrient-poor substrate. The different letters indicate significant differences (*post hoc* Tukey's test, $p<0.001$) in the final germination percentage.

Figure 6. Seedling survival based on Kaplan-Meier estimates under 2 substrates (nutrient-rich and nutrient-poor substrate) and 2 fungicides (with and without fungal association) treatments for (A) *Aechmea aquilega* and (B) *A. mertensii*. T1: no fungicide and nutrient-rich substrate, T2: no fungicide and nutrient-poor substrate, T3: fungicide and nutrient-rich substrate and T4: fungicide and nutrient-poor substrate. The symbol '+' corresponds to left and right censured data.

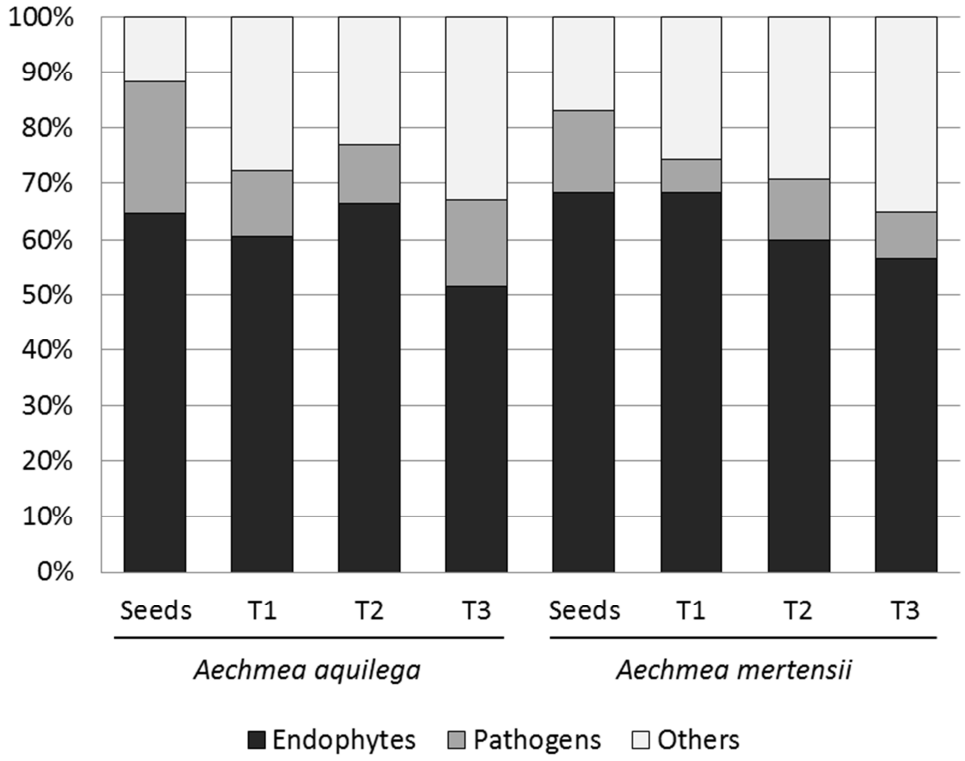
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Seeds
T1
T2
T3
Seeds
T1
T2
T3
Aechmea aquilega
Aechmea mertensii
Endophytes
Pathogens
Others

Figure 1

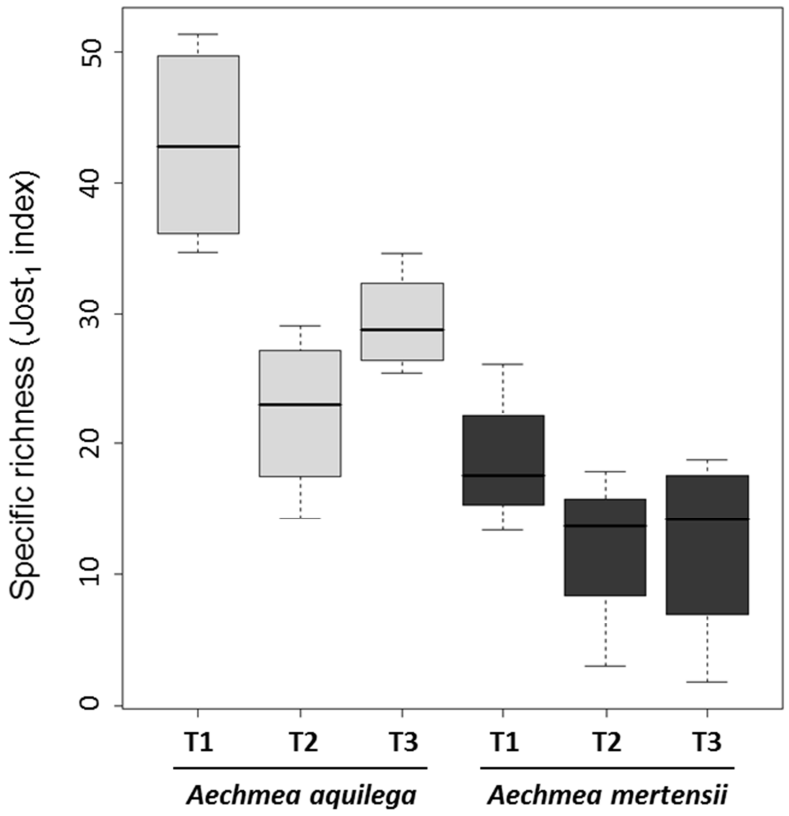
Specific richness (Jost$_1$ index)
0
10
20
30
40
50
T1
T2
T3
T1
T2
T3
Aechmea aquilega
Aechmea mertensii

Figure 2

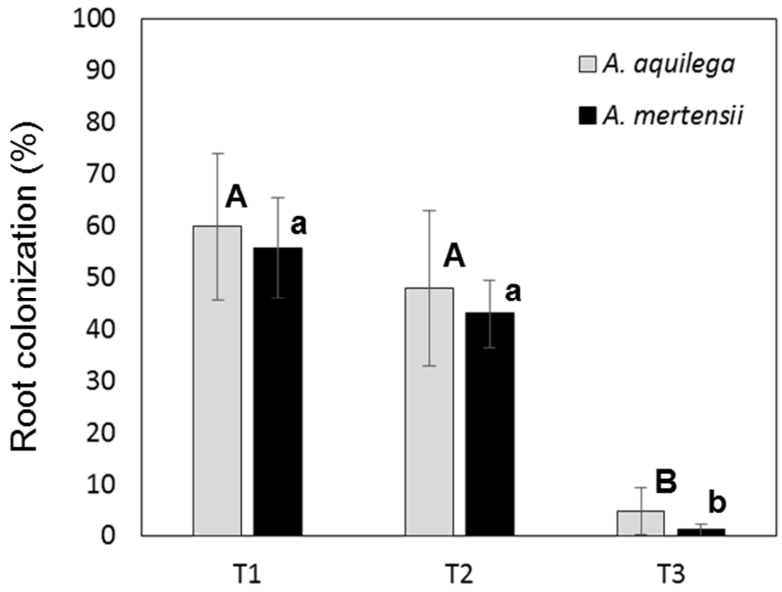
Root colonization (%)
100
90
80
70
60
50
40
30
20
10
0
A. aquilega
A. mertensii
A
a
A
a
B
b
T1
T2
T3

Figure 3

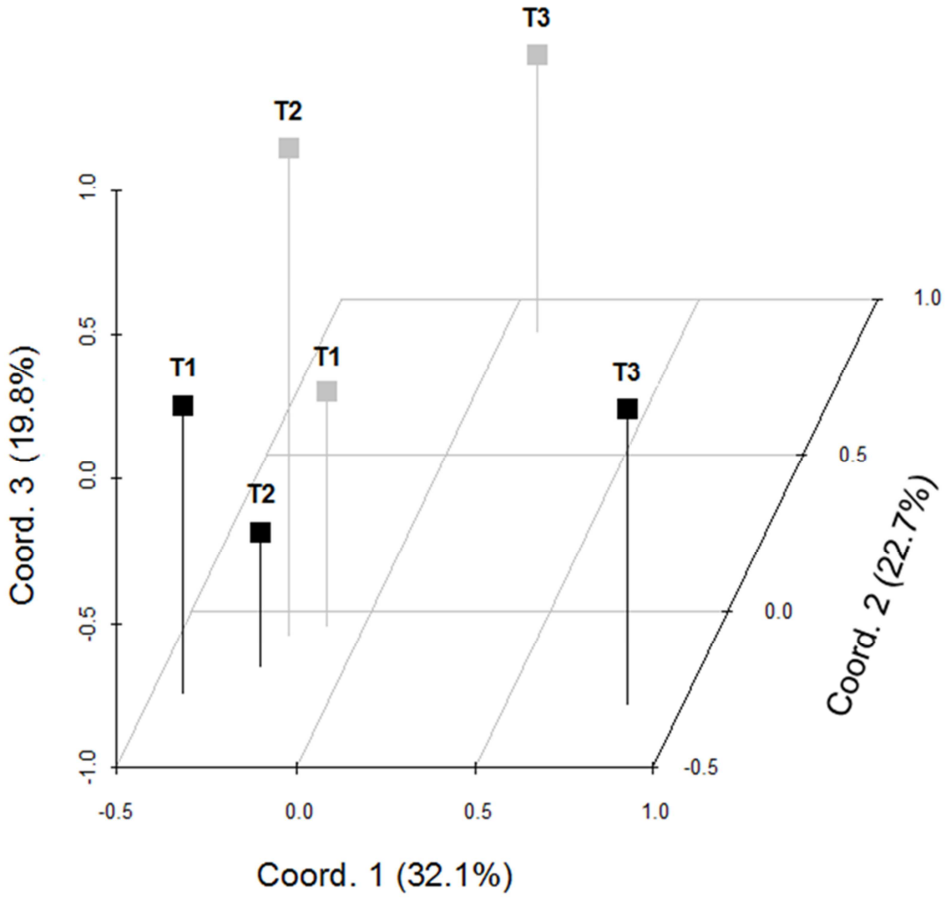
T3
T2
T1
T1
T3
T2
1.0
0.5
0.0
-0.5
-1.0
Coord. 3 (19.8%)
-0.5
0.0
0.5
1.0
Coord. 1 (32.1%)
1.0
0.5
0.0
-0.5
Coord. 2 (22.7%)

Figure 4

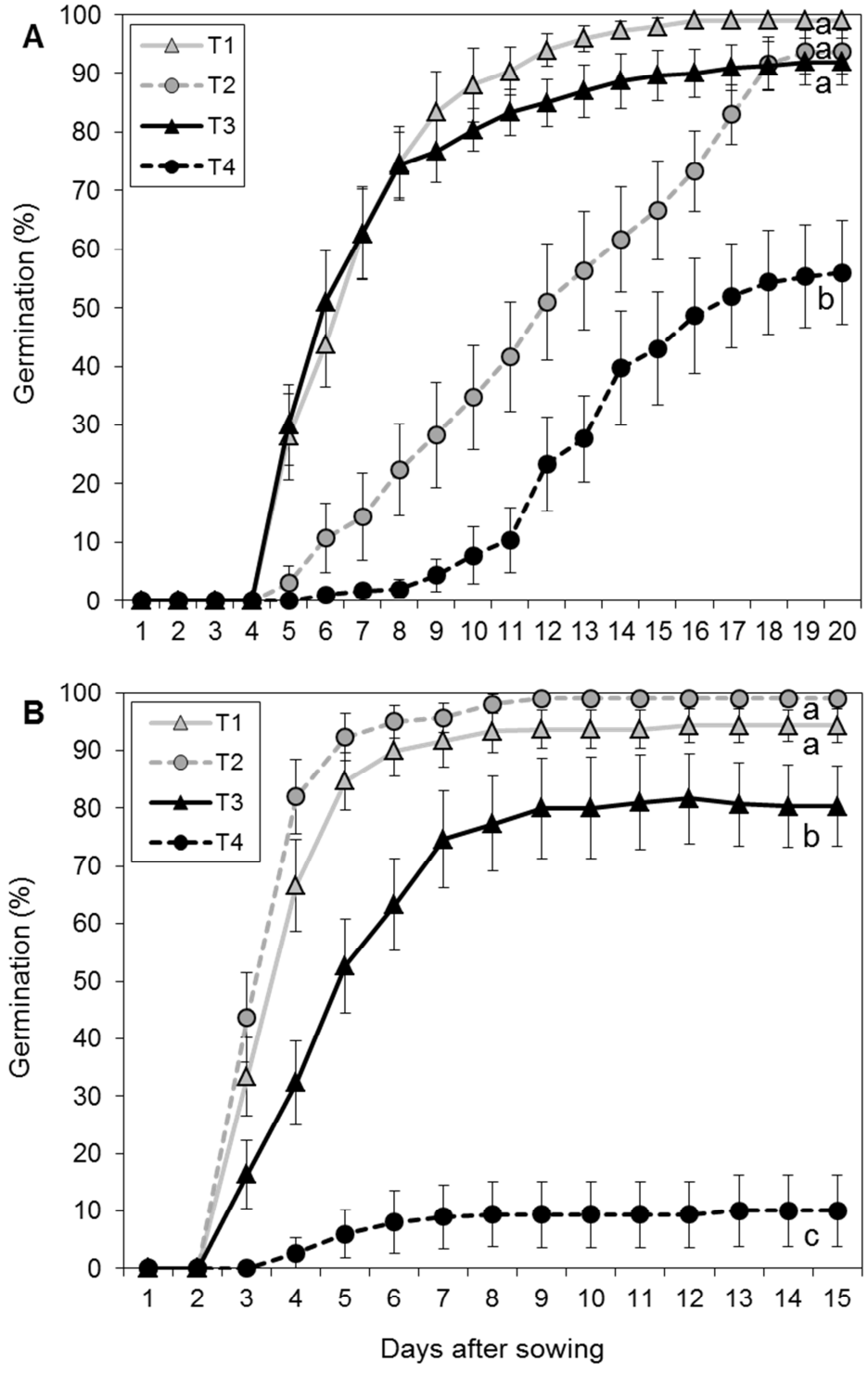
A
T1
T2
T3
T4
Germination (%)
a
a
a
b
B
T1
T2
T3
T4
Germination (%)
a
a
b
c
Days after sowing

Figure 5

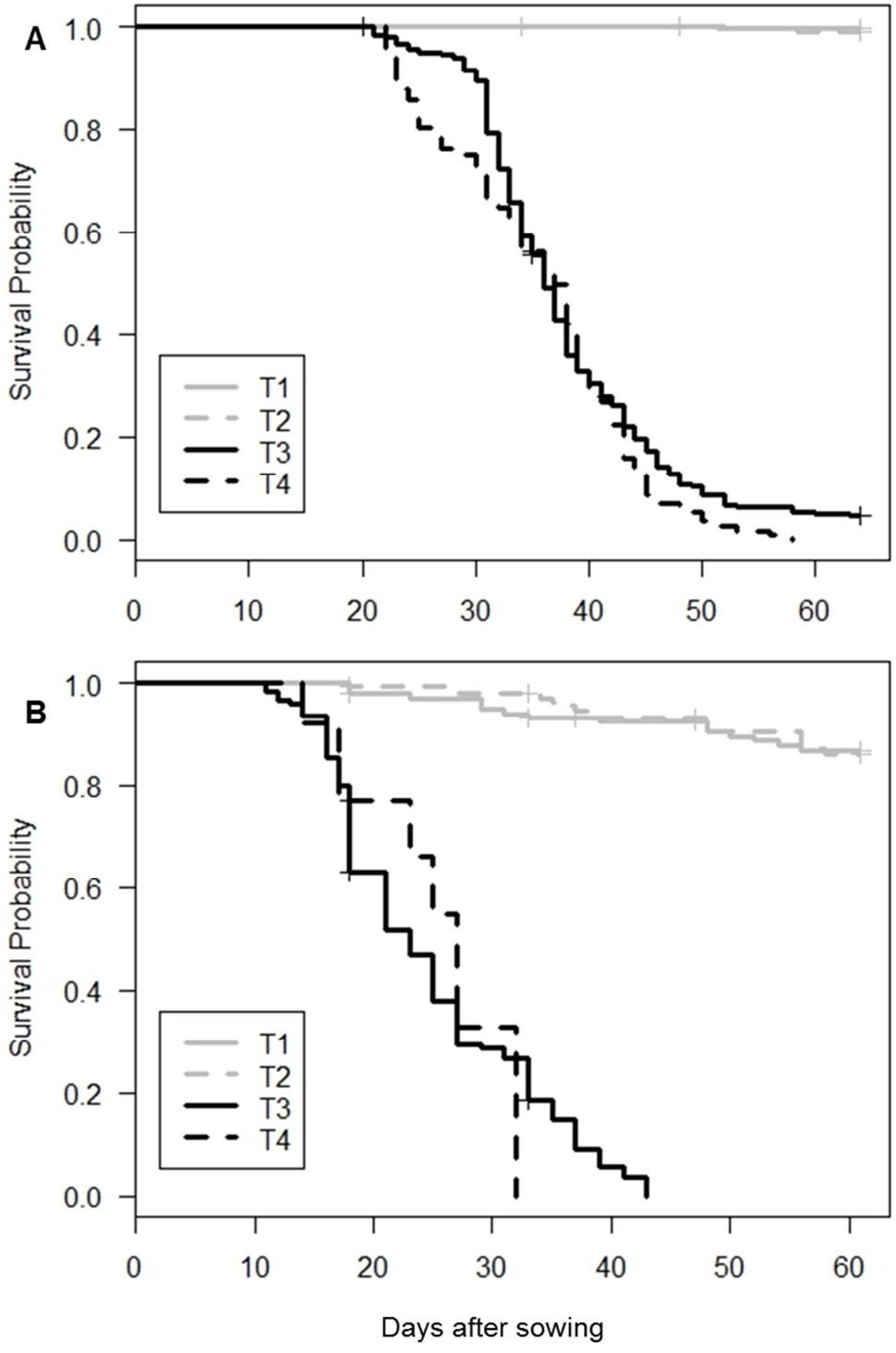
A
B
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
T1
T2
T3
T4
Days after sowing

Figure 6

Table 1. Relative abundance of endophytic fungal taxa in the seeds and 15-d-old seedlings of *Aechmea aquilega* and *A. mertensii* according to nutrient-rich (T1), nutrient-poor substrate (T2) and nutrient-rich plus fungicide (T3). For details of species names see Supplementary Table S1.

	OTU Abundance (%)							
	Aechmea aquilega				***Aechmea mertensii***			
Fungal taxa	**seeds**	**T1**	**T2**	**T3**	**seeds**	**T1**	**T2**	**T3**
Acidomyces sp. (1 taxon)		1.0			3.9			8.8
Acremonium spp. (5 taxa)	6.7	5.8	3	5.2	5.3	1.8	6.3	5.3
Aspergillus spp. (6 taxa)		6.7	7.5	5.2	3.9	6	10	14
Beauveria sp. (2 taxa)			1.5		1.3	2.3	2.5	
Bipolaris spp. (3 taxa)	6.7	2.9	6			0.5	2.5	
Candida sp. (2 taxa)	6.7	2.9	4.5	3.4	7.9	2.8	3.8	7
Chaetomium sp. (2 taxa)	13.3	4.8	4.5	5.2	10.5	6	2.5	5.3
Chaetosphaeria sp. (2 taxa)		1.9	1.5	6.9	1.3		3.8	0
Cladophialophora sp. (2 taxa)		4.8	1.5	5.2	5.3	3.7	7.5	5.3
Colletotrichum spp. (7 taxa)		1.9	1.5	3.4	2.6	4.1	2.5	5.3
Curvularia sp. (2 taxa)		2.9	3			1.8	2.5	3.5
Cyphellophora sp. (1 taxon)		1	1.5			1.8	2.5	
Endomelanconiopsis sp. (2 taxa)			1.5		1.3	0.9	1.3	
Fusarium spp. (5 Taxa)	33.3	22.1	17.9	20.7	35.5	24.9	17.5	19.3
Lasiodiplodia sp. (2 taxa)		4.8	3	3.4	2.6	2.3	2.5	
Penicillium spp. (6 taxa)		9.6	7.5	1.7	3.9	12.9	13.8	
Pestalotiopsis spp. (3 taxa)		1.9	3	3.4	6.6	1.8	5	3.5
Phomopsis sp. (2 taxa)		1	4.5		2.6	0.5		
Scedosporium spp. (4 taxa)		4.8	7.5	3.4	1.3	7.8	1.3	1.8
Trichoderma spp. (15 taxa)	40	20.2	10.4	32.8	9.2	13.8	12.5	15.8
Trichosporon spp. (3 taxa)		4.8	11.9	3.4		5.5	6.3	7
Total OTU amount	**16**	**110**	**69**	**60**	**80**	**220**	**84**	**60**
Total taxa amount	**6**	**44**	**35**	**36**	**26**	**46**	**39**	**25**

Table 2. Summary of logistic regression analysis showing effects of time, substrate, fungicide, and their interactions on germination of *Aechmea aquilega* and *A. mertensii.*

Treatment	estimate	SE	Z	*P*
Aechmea aquilega				
(intercept)	-6.64	0.08	-75.28	**<0.0001**
Time	0.39	0.005	77.36	**<0.0001**
Substrate: rich	3.57	0.06	54.14	**<0.0001**
Fungicide: no	1.77	0.05	30.86	**<0.0001**
substrate: rich x fungicide: no	-1.39	0.07	-17.99	**<0.0001**
Aechmea mertensii				
(intercept)	-7.77	0.13	-58.25	**<0.0001**
Time	0.56	0.01	53.27	**<0.0001**
Substrate: rich	4.31	0.09	44.01	**<0.0001**
Fungicide: no	5.99	0.11	53.84	**<0.0001**
substrate: rich x fungicide: no	-4.77	0.12	-39.44	**<0.0001**

Bold characters indicate that the P-value is significant.

Table 3. ANOVA summarizing effects of substrate and fungicide and their interactions on the final germination proportion at 15 d for *Aechmea mertensii* and 20 d for *A. aquilega*. Plant identity was taken into account as a random effect to control maternal effect.

Treatment	df	SS	Mean square	F	*P*
Aechmea aquilega					
Substrate	1	180.01	180.01	60.53	**<0.0001**
Fungicide	1	245.01	245.01	82.38	**<0.0001**
Substrate x Fungicide	1	115.20	115.20	38.74	**<0.0001**
Residuals	75	226.02	2.97		
Aechmea mertensii					
Substrate	1	515.11	515.11	174.47	**<0.0001**
Fungicide	1	1178.11	1178.11	398.88	**<0.0001**
Substrate x Fungicide	1	667.01	667.01	225.74	**<0.0001**
Residuals	75	229.65	3.02		

Bold characters indicate that the P-value is significant.

Table 4. Summary of Cox proportional hazard analysis showing the effects of substrate, fungicide and their interactions on seedling survival for *Aechmea aquilega* and *A. mertensii*. For the substrate environment, rich soil is the reference level; for the fungicide, without fungicide is the reference level; and for the drought, without drought is the reference level.

Treatment	Coefficient β	SE of β	Z	P	Hazard ratio
Aechmea aquilega					
Substrate: rich	-0.20	0.11	-1.80	0.07	0.81
Fungicide: without	-6.20	0.71	-8.66	**<0.0001**	0.002
Substrate: rich x Fungicide: without	-0.54	1.22	-0.43	0.66	0.58
Aechmea mertensii					
Substrate: rich	-0.04	0.33	-0.12	0.904	0.96
Fungicide: without	-3.84	0.45	-8.54	**<0.0001**	0.02
Substrate: rich x Fungicide: without	0.07	0.48	0.11	0.916	1.05

Bold characters indicate that the P-value is significant.

Table 5. ANOVA table summarizing the effects of Species and Date (D), substrate (S) and fungicide (F) and their interactions on ten leaf and root traits reflecting seedling performance of *Aechmea aquilega* and *A. mertensii*. Seed origin was taken into account as a random effect to control maternal effect. LLmax: longest leaf length, LA: leaf area, LDM: leaf dry mass, RDM: root dry mass, LRratio: leaf to root ration, LMA: leaf mass area, LWC: leaf water content and SD: stomatal density.

		LLmax	**TLA**	**LDM**	**RDM**	**LRratio**	**LMA**	**LWC**	**SD**
Species	$F_{1,290}$	145.6	145.6	111.2	50.7	16.29	0.05	2.49	211.9
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.819	0.11	**<0.0001**
Aechmea aquilega									
Date	$F_{1,151}$	209.47	111.57	258.98	194.87	0.86	11.72	16.89	54.51
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.353	**<0.0001**	**<0.0001**	**<0.0001**
Substrate	$F_{1,151}$	1009.37	301.33	298.11	78.82	318.12	186.87	141.17	6.94
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0094**
Fungicide	$F_{1,151}$	898.95	569.22	879.43	441.08	65.20	24.15	192.01	40.78
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
D x S	$F_{1,151}$	15.87	9.49	1.21	58.56	1.31	4.89	5.03	3.37
	P	**<0.0001**	**0.0024**	0.272	**<0.0001**	0.253	**0.028**	**0.026**	0.068
D x F	$F_{1,151}$	21.01	38.33	23.46	211.74	53.01	6.09	0.62	10.82
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.014**	0.429	**0.0012**
S x F	$F_{1,151}$	78.56	4.07	22.92	4.05	67.10	16.78	16.96	0.80
	P	**<0.0001**	**0.045**	**<0.0001**	**0.045**	**<0.0001**	**<0.0001**	**<0.0001**	0.372
D x S x F	$F_{1,151}$	0.32	3.91	7.59	67.96	56.20	29.12	36.62	0.83
	P	0.568	**0.049**	**0.006**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.361
Aechmea mertensii									
Date	$F_{1,124}$	59.51	40.26	127.78	52.56	1.61	113.13	24.78	1.01
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.208	**<0.0001**	**<0.0001**	0.315
Substrate	$F_{1,124}$	151.52	97.81	65.06	8.63	127.22	32.16	3.48	0.008
	P	**<0.0001**	**<0.0001**	**<0.0001**	**0.004**	**<0.0001**	**<0.0001**	0.064	0.929
Fungicide	$F_{1,124}$	255.08	311.93	216.98	181.73	0.78	34.75	163.01	246.35
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.379	**<0.0001**	**<0.0001**	**<0.0001**
D x S	$F_{1,124}$	0.33	0.66	5.70	0.01	0.03	26.73	16.89	1.68
	P	0.563	0.417	**0.018**	0.897	0.863	**<0.0001**	**<0.0001**	0.197
D x F	$F_{1,124}$	0.44	4.49	1.02	3.18	1.58	0.35	0.10	0.06
	P	0.507	**0.036**	0.312	0.077	0.211	0.551	0.743	0.801
S x F	$F_{1,124}$	13.85	0.34	2.60	38.77	29.98	26.93	1.28	15.40
	P	**<0.0001**	0.561	0.108	**<0.0001**	**<0.0001**	**<0.0001**	0.259	**<0.0001**
D x S x F	$F_{1,124}$	3.75	3.70	0.06	0.01	0.01	3.91	7.62	2.32
	P	0.055	0.056	0.801	0.0898	0.896	0.051	**0.006**	0.129

Bold characters indicate that the P-values are significant.