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FUNGAL ENDOPHYTES: ISOLATION AND DIVERSITY AMONG *NICOTIANA TABACUM* (SOLANACEAE) ORGANS

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The diversity of fungal endophytes is poorly known and particularly in the case of *Nicotiana tabacum*, the literature is limited. The present study assessed and compared the diversity and distribution of endophytic fungi between different organs of tobacco plants. We calculated the relative frequency and rates of colonisation and of isolation of endophytic fungi in roots, stems and leaves, as well as the Shannon–Wiener and Simpson diversity indexes. Similarities between assemblages from the studied organs were also analysed. A total of 1588 endophytic fungal strains assigned to 31 morphospecies were isolated. The highest diversity of endophytes was found in leaves, being *Fusarium graminearum* and *Alternaria botrytis* the most common fungal species. This study provides information on the distribution of fungal endophytes inhabiting leaves, stems, and roots of *N. tabacum* and thus can serve as a starting point for increasing our comprehension on the interactions in which these fungi are involved.

Key words: diversity, endophytic fungi, fungal isolation, species richness, tobacco

INTRODUCTION

Nicotiana tabacum L. (tobacco) is a native American herbaceous plant belonging to the Solanaceae family, which is cultivated worldwide as an industrial crop and used as a model system in plant biotechnology. According to FAO, tobacco production worldwide has been estimated in 7.1 million tons, comprising 40 million ha. China, Brazil, India, USA and Argentina are among the main countries where tobacco is produced.

Fungi are considered as endophytes when they inhabit living plant tissues during the whole or part of their life cycle, without causing any disease or symptoms to their host. A wide diversity of fungal endophytes has been documented for all plant organs such as leaves, petioles, reproductive structures, twigs, stems, and roots (Currie *et al.* 2014). It is hypothesised that fungal endophytes, in contrast to known pathogens, generally have greater phenotypic plasticity and thus more options to interact with their host than pathogens. Endophytes gain protection, nutrients and dissemination by the host, whereas the host gains tolerance to both biotic and abiotic stresses. In this sense, there

are studies about the influence of fungal symbionts on plant growth and development, about the protection they provide against predators and pathogens and also on the improved tolerance of hosts to heavy metals (Brundrett 2006, Li *et al.* 2011). According to Aly *et al.* (2010) this tolerance is thought to be achieved by secondary metabolites produced by these fungi, which have attracted the researchers´ attention due to their role in plant biology and physiology. Despite this, studies on endophytic fungi of tropical plants are an unexplored field worldwide (Arnold *et al.* 2001). Mainly, the tropical plants where diversity of fungal endophytes was studied are of medicinal transcendence, such is the case of *Tripterygium wilfordii* Hook F. (Kumar and Hyde 2004), *Vitex negundo* L. (Sunayana *et al.* 2014) and *Brucea javanica* (L.) Merr (Choi *et al.* 2005).

Only a few species of endophytic fungi of a few tropical plants have been explored hitherto, especially of *N. tabacum*. To date, there are few articles regarding tobacco endophytic fungi and bacteria (Duan *et al.* 2013, Jin *et al.* 2017, Liu *et al.* 2015, Mastreta *et al.* 2009, Spur and Welty 1974). Particularly, Spur and Welty (1974) and Jin *et al.* (2017) studied fungal endophytes of the tobacco plant but they focused only on those communities found in leaves and roots, respectively. Endophyte genera isolated in these studies were *Alternaria*, *Boeremia*, *Cladosporium*, *Epicoccum*, *Phoma* and *Pyrenochaeta* among others.

Considering information above, we hypothesised that endophytic fungal communities are similar in composition, diversity and distribution among plant organs, to those in other tropical plants studied. Therefore, the present study aimed at investigating the diversity of fungal endophytes of *N. tabacum* in Province of Jujuy, Argentina and its pattern of distribution among plant organs.

MATERIALS AND METHODS

Collection of plant samples

Four tobacco fields (Field 1: 24° 25′ 8.3″ S, 65° 01′ 26.8″ W, 796 m a.s.l; Field 2: 24° 33′ 37.1″ S, 64° 54′ 36.8″ W, 610 m a.s.l.; Field 3: 24° 29′ 50.8″ S, 64° 59′ 19.6″ W, 584 m a.s.l; Field 4: 24° 29′ 02.6″ S, 64° 58′ 21.7″ W, 706 m a.s.l.) located in Province of Jujuy (Argentina) were sampled in December 2014. The native vegetation of Jujuy is an evergreen/semi evergreen forest with mean temperature of 23 °C and mean annual precipitation of 680 mm.

Patches of 10 × 10 m were delimited at each tobacco field. Ten healthy and asymptomatic plants of *N. tabacum* (variety K394), which grown several metres apart from each other, were collected randomly from the centre and periphery of the patches.

Leaves, stems, and roots from tobacco plants were separated, placed into sterilised plastic containers, and preserved at 4 °C before processing in the laboratory.

Isolation and determination of fungal endophytes

Endophytes were isolated from plant organs using the method described by Vega *et al.* (2008) with modifications. First, the material was washed for 10 minutes under running tap water, then and under laminar flow their surfaces were sterilised by diving in 70% ethanol (v/v) for two minutes, then in 2.6% sodium hypochlorite (v/v) for three minutes, and finally cleaned out with sterile distilled water and dried out on sterile tissue paper. Edges of every plant piece were cut to remove dead tissue resulted from surface sterilisation process. Squares of 10–15 mm were obtained from leaves using a sterile scalpel. Segments of 1 cm long from stems and roots were also cut. Eventually, all plant material (2,160 total pieces, 720 belonging to each of the three organs) was placed in Petri dishes containing Potato Dextrose Agar (PDA, Britania ® S.A., Buenos Aires, Argentina) amended with antibiotics to inhibit bacterial growth. To assess the effectiveness of sterilisation, each organ was pressed onto the agar surface according to Schulz *et al.* (1998). Plates were labelled accordingly and incubated under controlled conditions (24±1 °C, 70±5% RH, dark). During the course of a month, plates were checked periodically.

Fungal colonies that emerged from the edges of the segments were placed on antibiotic-free PDA plates by "hyphal tipping". Malt extract agar dishes were used for those isolates that remained sterile on PDA.

Once pure cultures were obtained, morphotypes were recognised according to shape, texture, and colour of mycelium and growth rates. Fungi were mounted onto slides using lactophenol-cotton blue and sealed with nail polish. Morphology and characteristics of the reproductive structures and spores were analysed based on Domsch *et al.* (2003) and Seifert *et al*. (2011). Those isolates that remained sterile in both culture media were classified as 'mycelia sterilia' according to cultural features.

Patterns of colonisation

The relative frequency of the endophytic fungi in each plant organ was calculated as the number of strains of a given species/ total number of fungi in a particular organ. To establish fungal occurrence, colonisation rate (CR) and isolation rate (IR) were calculated for each organ. CR was defined as the "number of fragments from which one or more endophytic fungi were isolated/total number of analysed fragments", while IR as the "number of fungal endophytes/total of analysed fragments".

Since ANOVA assumptions were not met, the Kruskal–Wallis test and the multiple post-hoc test of Dunn were performed to compare the CR and IR of endophytic fungi between different organs of *N. tabacum*, using InfoStat software version 2004.

Table 1
Morphospecies of endophytic fungi isolated from tobacco roots, stems, and leaves and their respective frequencies

Fungal taxon	Family	Order	Organ		
			Root	Stem	Leaf
Absidia sp.	Cunninghamellaceae	Mucorales	0.14±0.02	–	–
Acremonium sp. 1	*Incertae sedis*	Hypocreales	–	–	0.08±0.003
Acremonium sp. 2	*Incertae sedis*	Hypocreales	0.07±0.005	0.1±0.04	–
Acremonium sp. 3	*Incertae sedis*	Hypocreales	–	–	0.07±0.006
Alternaria alternata	Pleosporaceae	Pleosporales	–	–	0.07±0.001
Alternaria botrytis	Pleosporaceae	Pleosporales	–	–	0.12±0.01
Alternaria sp. 1	Pleosporaceae	Pleosporales	–	0.05±0.008	–
Aspergillus flavus	Trichocomaceae	Eurotiales	0.14±0.09	–	–
Aspergillus niger	Trichocomaceae	Eurotiales	0.09±0.002	–	–
Aspergillus terreus	Trichocomaceae	Eurotiales	0.15±0.02	–	–
Cladosporium sp.	Cladosporiaceae	Capnodiales	–	–	0.05±0.001
Emericella	Trichocomaceae	Eurotiales	–	–	0.06±0.003
Emericelopsis	*Incertae sedis*	Hypocreales	–	0.1±0.02	–
Fusarium graminearum	Nectriaceae	Hypocreales	–	–	0.12±0.01
Fusarium oxysporum	Nectriaceae	Hypocreales	–	0.12±0.02	–
Fusarium solani	Nectriaceae	Hypocreales	–	–	0.08±0.002
Fusarium sp. 1	Nectriaceae	Hypocreales	–	0.04±0.005	–
Fusarium sp. 2	Nectriaceae	Hypocreales	–	–	0.04±0.007
Fusarium tabacinum	Nectriaceae	Hypocreales	0.08±0.001	0.11±0.04	–
Humicola grisea	Chaetomiaceae	Sordariales		0.09±0.002	–

Table 1 (continued)

Fungal taxon	Family	Order	Organ		
			Root	Stem	Leaf
Mucor sp.	Mucoraceae	Mucorales	0.09±0.005	–	–
Nigrospora sp.	Trichosphaeriaceae	Trichosphaeriales	0.06±0.001	–	–
Penicillium sp. 1	Trichocomaceae	Eurotiales	–	0.1±0.03	–
Penicillium sp. 2	Trichocomaceae	Eurotiales	–	0.07±0.001	0.06±0.005
Penicillium sp. 3	Trichocomaceae	Eurotiales	0.1±0.04	–	–
Trichoderma viride	Hypocreceae	Hypocreales	0.09±0.001	–	–
Mycelia esterilia 1	–	–	–	0.05±0.002	–
Mycelia esterilia 2	–	–	–	0.06±0.001	–
Mycelia esterilia 3	–	–	–	0.05±0.004	0.02±0.002
Mycelia esterilia 4	–	–	–	–	0.06±0.001
Mycelia esterilia 5	–	–	–	0.08±0.002	0.07±0.003

Diversity analysis

The total richness of endophytic fungi in each organ was considered as the observed species richness while the expected richness was calculated with Chao 2 and Jack 1, using the software Estimate S (Saucedo-García *et al.* 2014). To compare the community structure of fungal endophytes from roots, stems, and leaves, the alpha and beta components were evaluated. The alpha diversity in each organ was calculated using the diversity indexes of Shannon–Wiener (H′) and Simpson considering 1,000 runs of bootstrapping with replacement, with the software Estimate S (Colwell 2013, Magurran 1988). The diversity of the endophyte community in each organ was compared using the non-parametric Kruskal–Wallis test with the multiple post-hoc test of Dunn, because ANOVA assumptions were not met. Beta diversity was estimated with Jaccard′s similarity index, whose values ranged from zero to one, when there are no shared species between communities or when all species are shared, respectively (Arnold *et al.* 2001). Similarities between the communities of endophytes from roots, stems, and leaves

Table 2
Rates of colonisation (CR) and isolation (IR) of endophytic fungi in tobacco roots, stems and leaves

	CR	IR
Root	0. 24±0.07[a]	0.78±0.25[a]
Stem	0.28±0.08[b]	0.71±0.30[a]
Leave	0.29±0.13[b]	0.68±0.39[a]

Table 3
Shannon–Wiener and Simpson diversity indices of endophytic fungi in tobacco roots, stems and leaves

	Root	Stem	Leaf
Shannon	2.16±0.18[a]	2.41±0.19[b]	2.47±0.21[c]
Simpson	8.27±1.21[a]	10.54±1.58[b]	11.06±1.72[c]

were also explored using a Principal Component Analysis (PCA). Jaccard matrix of similarity and PCA were performed with PAST version 2001 (Hammer *et al.* 2001).

RESULTS

Abundance, diversity, colonisation and isolation rates of fungal endophytes

A total of 1,588 isolates of endophytic fungi assigned to 31 morphospecies in the Ascomycota and Zygomycota were isolated from roots (516), stems (504) and leaves (568). The relative frequencies of isolation and mean values of CR and IR are shown in Tables 1 and 2, respectively. Significant differences were observed in the CR between roots, stems, and leaves (CR: H = 5.92, p = 0.047). Instead, the IR showed no significant differences between organs (IR: H = 0.81, p = 0.6668).

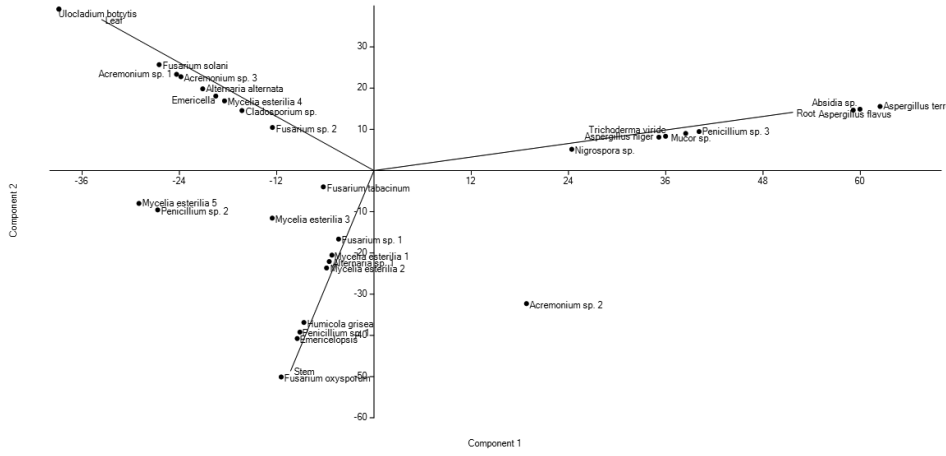

Fig. 1. Principal Component Analysis (PCA) of endophytic fungi in tobacco plants

Diversity analysis

Accumulation curves of the observed species richness, and the estimated richness of Chao 2 and Jack 1 for roots, stems, and leaves are shown in Figure 2. Our results showed that the expected species richness was similar to the observed one, thus our sampling effort can be considered as appropriate.

Significant differences in the different diversity indices were obtained (Shannon: H = 66.92, p < 0.0001; Simpson: H = 58.75, p < 0.0001) (Table 3). The highest diversity values were found in leaves, for the computed indices. Jaccard matrix of similarity (Table 4) showed that the fungal communities present in the three organs are well differentiated. The highest value of similarity was found between stems and leaves (J = 0.173).

Principal Component Analysis showed that 86.64% of the total variability was explained by the two first axes. In summary, 31 fungal morphospecies were analysed, 26 of them were exclusively associated to one specific organ, while only five were associated to more than one (Fig. 1).

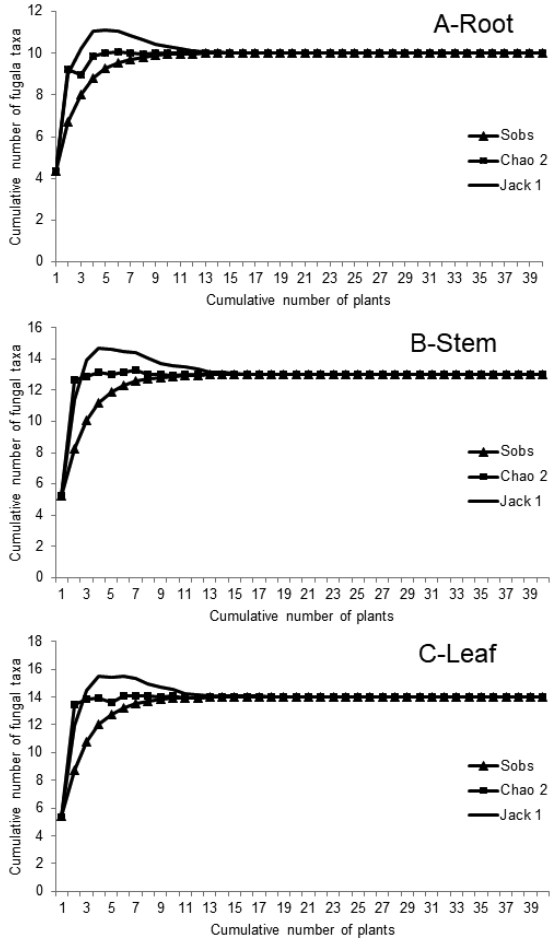

Fig. 2. Species accumulation curves (Sobs), Chao 2 estimated richness (Chao 2) and Jack-knife 1 estimated richness (Jack 1) in roots (A), stems (B) and leaves (C) of tobacco plants

DISCUSSION

In this paper, we studied the assemblages of fungal endophytes from 2,160 fragments of tobacco leaves, stems, and roots and isolated 1,588 strains

Table 4
Jaccard matrix of similarity between tobacco organs

	Root	Stem	Leaf
Root	1	–	–
Stem	0.095	1	–
Leaf	0.043	0.174	1

assigned to 31 morphotaxa. Sunayana *et al.* (2014) isolated 143 fungal endophytes from 1,350 fragments of *Vitex negundo*, and Sun *et al.* (2008) obtained 973 fungal strains from 1,144 fragments of six medicinal plant species. Furthermore, Kumar and Hyde (2004) assessed the fungal endophytes of *T. wilfordii* and reported 343 isolates recovered from 500 samples. Differences in the number of fungal endophytes recovered can be due to differences in the climatic conditions between sampling sites and hosts, which might both influence the degree of fungal colonisation (Collado *et al.* 2000). In addition, depending on the method used to register fungal endophytes, slow-growing species could be underestimated if they appear together with fast growing ones (Zheng *et al.* 2016). Therefore, these results need to be complemented with further studies using other methods or a combination of methods to detect hidden endophytic species.

The mean rates of colonisation and isolation of endophytes from *N. tabacum* were both similar between roots, stems and leaves. Overall, the rates obtained in the present study were lower than the ones obtained by Kumar and Hyde (2004) for *T. wilfordii*. Choi *et al.* (2005) and Zhou (2015) also obtained higher values in *B. javanica* and in *M. cochinchinensis*, respectively. Sun *et al.* (2008), studied the fungal communities of six medicinal plants and reported values of CR between 47.9–63.1%, and of IR between 0.7–0.93. The values registered in the present study are in agreement with the colonisation rates for bark, twigs and leaves registered by Sunayana *et al.* (2014), although these authors found higher values for bark instead of leaves.

The species of fungal endophytes reported herein were previously found in other plants, being the Ascomycetes the predominant fungi (Zhou 2014). Our results showed that the endophytic assemblages of *N. tabacum* included species from cosmopolitan genera, such as *Alternaria*, *Fusarium*, *Humicola*, *Mucor*, and *Nigrospora*, among others. All these genera have previously been isolated as endophytes from tropical regions and have been found colonising leaves, bark, twigs, and stems and many of them are noteworthy since they are potential sources of novel compounds with bioactive activities (Vieira *et al.* 2011, Zhou 2014).

As previously reported by Spur and Welty (1974) also for *N. tabacum* leaves, in this study a diverse assemblage of endophytic fungi was found, with 14 species identified. Concordantly, *Alternaria* spp. and *Cladosporium* spp. were frequently isolated, however, in our study the predominant species in leaves were *F. graminearum* and *A. botrytis*, which were not isolated by the previously referred authors. Furthermore, Jin *et al.* (2017) have also frequently registered *Fusarium* spp. in tobacco plants. Other endophyte genera isolated from tobacco in China were *Alternaria*, *Boeremia*, *Epicoccum*, *Phoma*, *Pyrenochaeta*, *Stagonosporopsis*, while *Pyrenochaeta*, *Rhizopycnis*, and *Setophoma* were isolated particularly from roots (Liu *et al.* 2015).

Spur and Welty (1974) stated "the number and type of endophytic fungi in leaves of *N. tabacum* changed with the growth and development of leaves". Thus, the assemblages described in this study would represent the species present in the particular moment in which the samples were collected.

Results obtained from the diversity analysis indicated that the assemblages present in leaves, stems, and roots of *N. tabacum* are well defined. This has also been observed for other plant species, since the different organs may represent different substrates for endophytes (Espinosa-Garcia and Langenheim 1990), as well as because of the existence of microclimatic differences between organs, as Carroll and Petrini (1983) demonstrated for conifer endophytes. Thereby, both factors should be considered when the endophyte distribution is being described.

Finally, another thing to consider when attempting unravelling fungal-plant interactions is that at least some of them, and mostly depending on environmental conditions, could change from mutualistic to pathogenic. For example, the potential pathogenic nature of some endophytes should be considered, since some of the species recorded in this study are able to attack tobacco leaves, as in the case of *A. alternata* that produces the Brown Spot disease (Jing *et al.* 2018). In addition, *Fusarium* spp. are known as causal agents of a plant disease commonly known as "fusariosis" in tobacco plants, producing generalised wilting and considerable yield losses (Berruezo *et al.* 2018).

CONCLUSION

This study reported the presence of fungal endophytes in leaves, stems, and roots of *N. tabacum.* Some of the recorded species of *Fusarium* and *Alternaria* should be carefully considered due to their potential pathogenic nature. It would also be important to do further investigations on which factors may trigger the shift in the fungal-plant relationship from endophytic to pathogenic, particularly in the case of tobacco plants.

This study increases our knowledge on fungal-plant interactions and represents a starting point of further investigations on the role that native endophytic fungi may play in growth promotion and heavy metals uptake by *N. tabacum*.

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