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Article

Distribución vertical de hongos en hojas de tres especies de pinos en Nuevo León, México

Vertical distribution of fungi on leaves of three pine species from *Nuevo León*, Mexico

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Resumen

Se estudió la distribución vertical de hongos en siete tipos de hojas de *Pinus arizonica*, *P. cembroides* y *P. pseudostrobus* mediante los métodos de aislamiento en medio de cultivo en caja de Petri (método indirecto) y con el uso de cámaras húmedas (método directo) durante un año. Por el método de cultivo se obtuvieron 57 taxones, 56 se adscriben al Phyla Ascomycota y solo uno (*Gymnopus androsaceus*) a Basidiomycota; *Lophodermium australe, Pestalotia stevensonii* y *Rhizosphaera kalkhoffii* fueron las especies más abundantes; mientras que *Cladosporium cladosporioides, Alternaria alternata y Ceuthospora* sp resultaron las más frecuentes. Con la cámara húmeda se identificaron 28 taxones; *Lophodermium australe, Preussia* sp. y *Pestalotia stevensonii* fueron más abundantes*; Preussia* sp. y *Coniochaeta ligniaria* fueron los hongos más frecuentes. Para los dos métodos, *P. arizonica* presentó la mayor diversidad de hongos, seguida de *P. pseudostrobus* y *P. cembroides*. Se observaron patrones definidos de hongos en cada una de las especies de pinos y para los tipos de hojas estudiados, de estos últimos, las muertas en el suelo y las grises presentaron la diversidad fúngica más alta. Conforme avanza su grado de descomposición, el número de especies de hongos disminuye.

Palabras clave: Ascomycota, Basidiomycota, diversidad fúngica, *Pinus arizonica* Engelm., *Pinus cembroides* Zucc., *Pinus pseudostrobus* Lindl.

Abstract

The vertical distribution of fungi in seven types of pine leaves of *Pinus arizonica*, *P. cembroides*, and *P. pseudostrobus* was studied during one year by the method of isolation in culture medium in Petri dishes (indirect method) and by the use of moist chambers (direct method). 57 taxa of fungi were identified by the culture medium method, 56 taxa are adscribed to the Phyla Ascomycota and only one species (*Gymnopus androsaceus*) belongs to the Phyla Basidiomycota; *Lophodermium australe, Pestalotia stevensonii* and *Rhizosphaera kalkhoffii* were the most abundant species; *Cladosporium cladosporioides, Alternaria alternata* and *Ceuthospora sp.* were the most frequent taxa. By the moist chamber method 28 taxa were determined; *Lophodermium australe, Preussia* sp., and *Pestalotia stevensonii* were the most abundant species; *Preussia* sp., and *Coniochaeta ligniaria* were the most frequent species. Well defined patterns of fungi were observed in both methods for the three pine species and for the studied leave types. *Pinus arizonica*, registered the bigger fungal diversity followed by *P. pseudostrobus* and *P. cembroides*. As the leaf degradation rate grows, the number of species of fungi decrease.

Key words: Ascomycota, Basidiomycota, fungal diversity, *Pinus arizonica* Engelm., *Pinus cembroides* Zucc., *Pinus pseudostrobus* Lindl.

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Introduction

The succession patterns in the communities of fungi in various trees of temperate climates and tropical tree species have been studied by several researchers: Frankland (1998) discusses the nature and the mechanisms of succession of fungi on the soil and in litterfall; Sieber-Canavesi and Sieber (1993) conclude that the edaphic conditions and management practices in *Abies alba* Mill. have an impact on succession, and they classify leaf fungi into three groups: 1) endophytes from the living leaves; 2) endophytic colonizers and survivors from senescent and dead attached leaves; 3) fungi that colonize dead leaves only. Tokumasu *et al.* (1994) separated the fungi in *Pinus sylvestris* L. into two groups: those of hanging and recently fallen leaves, and those of leaf litter; they concluded that the frequency of the fungi diminishes with the advance of decay.

Rosenbrock *et al.* (1995) observed a reduction of the microbial mass in *Alnus glutinosa* (L.) Gaertn. as decay progressed; Sridhara and Ahmad (1993) studied the succession of fungi in the litter from four tree species in a tropical forest of India.

Endophytic fungi produce no symptoms in their hosts; they are important components of the plant mycobiome; they interact and overlap in function with mycorrhizal, pathogenic, epiphytic, saprobic and other important fungal groups that colonize vegetal tissues (Porras-Alfaro and Byman, 2001).

Given the ecological and, in some cases, economic importance of endophytic fungi, there are numerous studies on plant species, including pines; some prominent species are *Pinus nigra* J.F.Arnold (Kowalski and Zych, 2002); *P. halepensis* Mill. (Botella and Diez, 2011); *P. densiflora* Siebold & Zucc. and *P. koraiensis* Siebold & Zucc. (Yoo and Eom, 2012); *P. thunbergii* Parl. (Kim *et al.,* 2012); *P. wallichiana* A.B.Jacks. (Qadri *et al.,* 2014); *P. massoniana* Lamb. (Yuan and Chen; 2014); *P. sylvestris* L. (Millberg *et al*., 2015); *P. taeda* L. (Oono *et al.*, 2015), and *P. monticola* Douglas ex D.Don (Bullington and Larkin, 2015).

Literature cites various studies on inventories of fungi in the pine and broadleaf litter (Kendrick, 1958; Sankaran, 1994; Parungao *et al.* 2002). As well as research assessing

the abundance and diversity of fungi on litterfall (Bills and Polishook, 1994; Prakash *et al.,* 2015; U'Ren and Arnold, 2016).

Literfall plays a prominent role in forest ecosystems because it provides energy, coal, nitrogenand nutrients. The forest productivity is directly related to the degree of decomposition of organic matter (Rahman *et al.*, 2013). For these reasons, numerous authors have conducted research on the role of fungi and the degrees of decomposition in various tree species (Rihani *et al*., 1995; Sankaran; 1993; Osono, 2011).

In Mexico, the studies by Heredia (1987, 1994) and Arias and Heredia (2014) register fungi associated to tree species in tropical and montane mesophytic forests. However, there is no history of fungi associated to pine litterfall; therefore, the purpose of this study was to determine the vertical distribution patterns of fungi present on the leaves of three *Pinus* species of *Nuevo León*.

Materials and Methods

Study area

Three sampling plots were established as follows, one for each pine species:

1. School Forest, *Iturbide Nuevo León*; pine forest with *Pinus pseudostrobus* Lindl., other species identified within the plot are *Quercus canbyi* Trel., *Amelanchier denticulata* (Kunth) K. Koch, *Arbutus xalapensis* Kunth, and *Prunus serotina* Ehrh. 24º42'51" N 099º51'67" W; 1 630 masl.

2. *Pablillo, Galeana, Nuevo León;* pine forest with *Pinus arizonica* var. *stormiae* Mart. 24º36'51" N 100º00'16" W; 2 000 masl.

3. *Pablillo, Galeana, Nuevo León*; pine forest with *Pinus cembroides* Zucc. 24º37'11" N 100º00'38 W; 2 000 masl.

Leaf sampling

Ten fascicles of the following leaf types were collected at random, on a fortnightly basis during one year, in the selected plots: green leaves, senescent leaves, dead leaves still attached to the tree, "hanging" dead leaves *(i.e.* those that have not reached the topsoil and are suspended over the vegetation), topsoil leaves that have not yet begun to decay (L), leaves of the topsoil middle layer with some signs of decay (F1), topsoil leaves of the lower layer with signs of advanced decay (F2).

The samples were utilized for the isolation of fungi through the methods described below.

Isolation of leaf fungi in a culture medium

All the collected leaves were measured in the laboratory, and three leaves were selected at random from each sample and processed for the isolation of the fungi through a modification of the protocol proposed by Kowalski and Zych (2002). The leaf surfaces were sterilized with ethanol at 96 % during one minute. Next, they were treated with sodium hypochlorite at 3 % during 10 minutes. Finally, they were submerged in ethanol at 70 % during 30 seconds. The leaves thus treated were cut transversally using a sterilized knife (disinfected by the flame of a burner). The number of cuts varied according to the size of the leaves. The sections were placed —using tweezers (sterilized by the flame of a burner)— in a Petri dish with 2.5 % Malt Extract Agar and incubated at 22 °C, in a Shellab L120 incubator during the time required for the colonies to become visible to the naked eye.

All the colonies thus obtained were counted and identified by means of routine mycological techniques, through direct observation of preparations mounted on lactic acid, in glycerine-alcohol-water (GAW), or in distilled water, under a compound microscope. The works by Sutton (1980), Minter (1981), Dennis (1981), Ellis (1971, 1976), Arx (1981) and Nag Raj (1993), and the website of the Index Fungorum (2018) (http://www.indexfungorum.org/) were consulted in order to corroborate the names of the species and their authors.

Subcultures of all the morphospecies present in the dishes were made in order to obtain vouchers of the studied fungi, which were deposited in the strain collection of the *Herbario Micológico de la Facultad de Ciencias Forestales (CFNL)* (School of Forest Sciences Mycological Herbarium) (CFNL), as well as of those whose species could not be determined because no morphological structures allowing their identification were available at the time.

Leaf fungi in moist chambers

A fascicle of leaves was selected at random from each sample collected fortnightly at the sample site for each leaf type, and it was placed in a moist chamber, which consisted in a Petri dish with sterilized Kraft paper humidified with sterile distilled water. These dishes were kept moist during a month, after which they were placed in an Acros™ ART14JKX freezer at 5 °C during one week, in order to prevent an infestation by mites. The samples were then examined under a Karl Zeiss™ Axiostar Plus microscope, as by the routine mycological techniques (Hawksworth, 1974). The species were identified based on specialized literature (Sutton, 1980; Minter, 1981; Dennis, 1981; Ellis, 1971, 1976; Arx, 1981; Nag Raj, 1993).

The information was organized by collection and by leaf type, on an Excel 2013 spreadsheet, in order to estimate the richness, abundance, and frequency of the fungi associated to the leaves. Richness corresponded to the number of fungal species observed (total, per leaf type, per pine species); absolute abundance (Aba), to the number of individuals per species, in relation to the total number of individuals present in the study area (ni). Absolute frequency was calculated based on the number of points with the species, divided by the total number of sampled points; the fungal pattern in each of the studied leaves was compared by using a cluster analysis, with the PAST 3 statistical software (Hammer *et al.*, 2001).

Results and Discussion

Fungal diversity obtained with the method of culture in a Petri dish

26 leaf samplings were carried out for each of the species considered. 1 638 samples were processed for the three pine species.

The analysis included a total of 23 332 fungal colonies (Table 1).

Table 1. Studied colonies by pine species.

Pine species	Number of colonies
Pinus arizonica Engelm.	10 146
Pinus cembroides Zucc.	1 636
Pinus pseudostrobus Lindl.	11 550

Moreover, 1 953 subcultures were obtained, from which 57 fungal taxa were determined; 56 of these are adscribed to the Phyla Ascomycota, and only one (*Gymnopus androsaceus*), to the Phyla Basidiomycota. According to Voříšková and Baldrian (2013), Ascomycota was the most abundant in green and senescent leaves, with 88.5 % and 99.5 % of amplifications, respectively. These findings agree with the present study, although the above authors used a molecular method to determine the fungal species.

Haňáčková *et al*. (2015) utilized a culture method and a molecular method, and they concluded that both produced similar dominant species, and that in the early stage of decay, ascomycetes are predominant, and basidiomycetes occur only occasionally; in this, they also agree with the results documented here.

40 fungal taxa were registered for *Pinus arizonica*, 28 for *P. cembroides* and 32 for *P. pseudostrobus*. The complete list is shown in detail in Table 2. The most abundant species were *Lophodermium austral* Dearn., Pestalotia *stevensonii Peck and*

Rhizosphaera kalkhoffii Bubák, while *Cladosporium cladosporioides* (Fresen.) G.A. de Vries*, Alternaria alternata* (Fr.) Keissl. and *Ceuthospora* sp. were the most frequent. According to Yuan and Chen (2014), *Lophodermium* taxa are frequent pioneers on leaves and play an important role in litter decay.

Table 2. Fungal taxa registered per *Pinus* species.

Species	*Pinus arizonica*	*Pinus cembroides*	*Pinus pseudostrobus*
Alternaria alternata (Fr.) Keissl.	X	X	X
Aspergillus sp.	X	X	
Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud	X		X
Ceuthospora sp.	X	X	X
Cladosporium cladosporioides (Fresen.) G.A. de Vries	X	X	X
Conoplea elegantula (Cooke) M.B. Ellis	X	X	X
Epicoccum nigrum Link	X	X	X
Fusarium lateritium Nees	X	X	
Gymnopus androsaceus (L.) Della Magg. & Trassin.	X		
Leptostroma sp.	X		
Lophodermium australe Dearn.			X
Mycoglaena sp.	X		
Nigrospora oryzae (Berk. & Broome) Petch	X		
Penicillium sp.	X	X	X
Pestalotia stevensonii Peck		X	
Pestalotiopsis funerea (Desm.) Steyaert	X		X
Phialophora sp.	X	X	X
Phoma sp.	X		X
Preussia sp.	X	X	

Pseudopithomyces chartarum (Berk. & M.A. Curtis) Jin F. Li, Ariyaw. & K.D. Hyde	X		
Rhizosphaera kalkhoffii Bubák		X	
Sarea resinae (Fr.) Kuntze			X
Scolecobasidium sp.			X
Sphaeropsis sapinea (Fr.) Dyko& B. Sutton	X		X
Torula herbarum (Pers.) Link			X
Trichoderma harzianum Rifai			X
Tritarachium sp.	X		
Ulocladium sp.			X
Unidentified 01	X	X	X
Unidentified 02	X		
Unidentified 03	X		X
Unidentified 04	X	X	X
Unidentified 05	X	X	X
Unidentified 06		X	
Unidentified 07	X		X
Unidentified 08	X		X
Unidentified 09	X		X
Unidentified 10	X		X
Unidentified 11			X
Unidentified 13		X	X
Unidentified 14	X	X	X
Unidentified 15	X	X	
Unidentified 16		X	
Unidentified 17		X	X
Unidentified 18		X	X

Unidentified 19	X	X		
Unidentified 20	X			
Unidentified 21	X			
Unidentified 22	X			
Unidentified 23		X		
Unidentified 24	X	X		
Unidentified 25	X	X		
Unidentified 26		X		
Unidentified 27				X
Unidentified 28	X			
Unidentified 29	X	X		
Unidentified 30	X			X

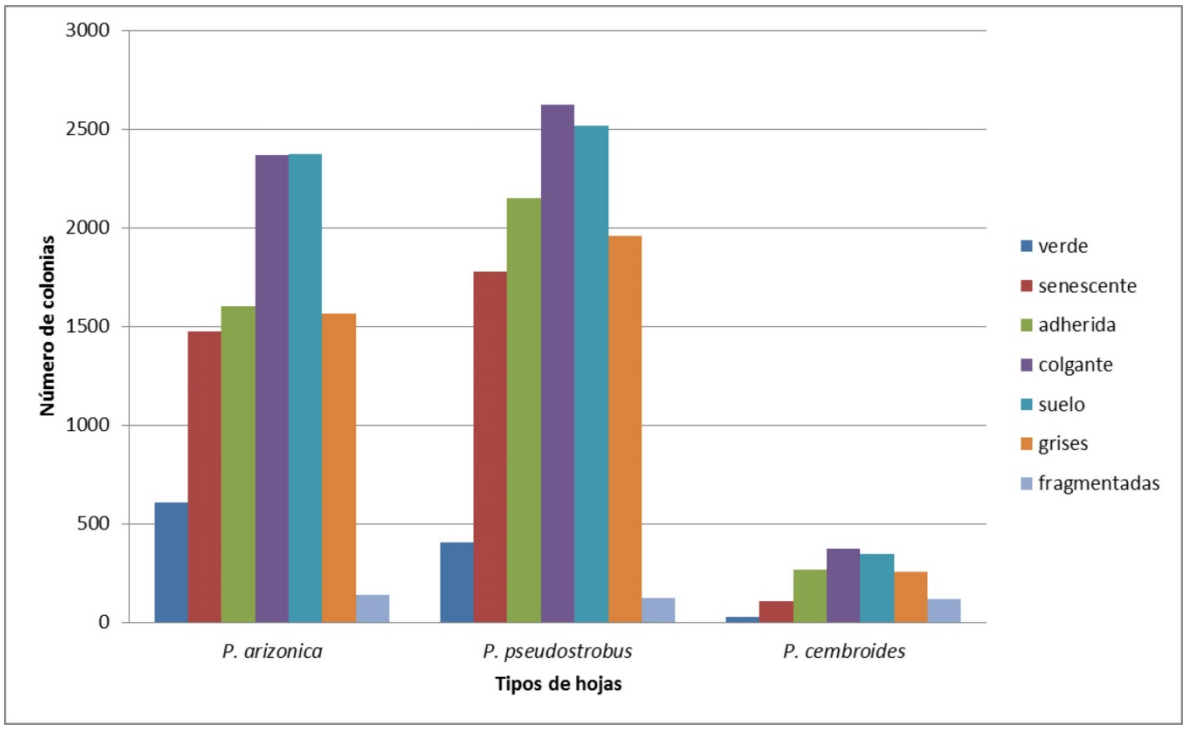
The taxon with the greatest richness of fungi was *P. arizonica*, followed by *P. pseudostrobus* and *P. cembroides*. However, the highest number of colonies was registered in *P. pseudostrobus*, followed by *P. arizonica*. The number of colonies in *P. cembroides*, compared to the other pines, was 10 to 11 times smaller. This may be related to the length of the leaves. The average lengths of the sampled leaves were 173.9 mm for *P. pseudostrobus*, 156.1 mm for *P. arizonica*, and 27 mm for *P. cembroides*. Table 3 shows the minimum, mean and maximum number of fungal species per pine species and leaf type.

Table 3. Minimum, maximum and mean numbers of fungal species.

Leaf type	*P. arizonica*			*P. cembroides*			*P. pseudostrobus*		
	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.
Green	0	1.9	6	0	0.4	3	0	1.7	5
Senescent	1	2.8	5	0	1.5	4	1	2.8	6
Attached	2	3.7	7	1	2.9	5	1	3.7	7
Hanging	2	4.2	9	2	3.4	5	1	4.5	8
Litter	2	5.3	8	2	3.0	6	3	4.8	10
Gray	2	5.1	8	0	2.7	5	3	5.0	8
Fragmented	1	1.7	4	1	2.1	4	1	2.0	4

In all cases, leaf litter and gray leaves exhibited the highest richness. Green leaves —especially those of *P. cembroides*— exhibited the lowest richness. The number of fungal species always diminished with the progress of the decomposition of the leaves. Tokumasu *et al.* (1994) cite similar results and state that hanging leaves and leaf litter exhibited the largest number of isolations.

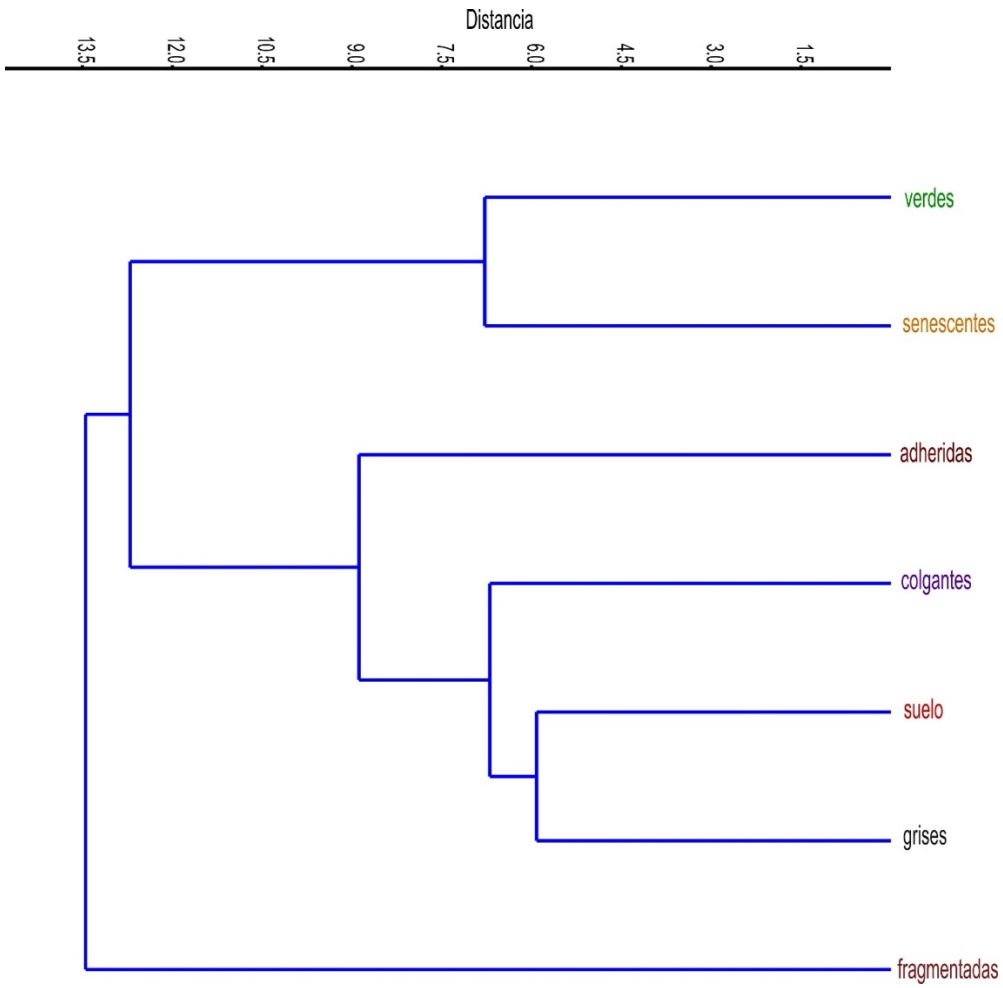
As for the number of colonies, it was highest among the dead hanging leaves and the leaf litter. Fragmented and green leaves were the ones with the lowest number of colonies (Figure 1).



Número de colonias = Number of colonies; *Tipo de hojas* = Leaf type; *Verde* Green; *Senescente* = Senescent; *Adherida* = Attached; *Colgantes* = Hanging dead leaves; *Suelo* = Litter; *Grises* = Grey; *Fragmentadas* = Fragmented.

Figure 1. Number of colonies per leaf type in the studied pine species.

In terms of the fungal richness patterns on the seven leaf types, the cluster analysis (Figure 2) distinguishes three groups: one made up of green and senescent leaves; another, by dead leaves (attached, hanging and litter), and one consisting of fragmented leaves only.



Verde Green; *Senescente* = Senescent; *Adherida* = Attached; *Colgantes* = Hanging dead leaves; *Suelo* = Litter; *Grises* = Grey; *Fragmentadas* = Fragmented.

Figure 2. Cluster analysis of the richness of fungal species in the various leaf types of the three studied pine species

Fungal diversity of litterfall with moist chambers

27 samplings were carried out for *Pinus arizonica* and *P. cembroides* and 26 for *P. pseudostrobus*. A total of 400 samples were analyzed; 28 fungal taxa were identified on the attached leaves, the hanging leaves and the leaf litter. Table 4 shows the complete list of species.

Table 4. List of fungal taxa obtained with the moist chamber method.

Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud
Chaetomium sp.
Chloridium sp.
Coniochaeta ligniaria (Grev.) Cooke
Desmazierella acicola Lib.
Discosia strobilina Lib. ex Sacc.
Fusarium lateritium Nees
Herpotrichia sp.
Lachnum sp.
Leptomelanconium pinicola (Berk. & M. A. Curtis) R. S. Hunt
Leptostroma sp.
Lophodermium austral Dearn.
Gymnopus androsaceus(L.) Della Magg. & Trassin.
Meloderma desmazieri (Duby) Darker
Mytilinidion mytilinellum (Fr.) H. Zogg
Niesslia exilis (Alb. &Schwein.) G. Winter
Oidiodendron griseum Robak
Penicillium sp.
Periconia byssoides Pers.
Pestalotia stevensonii Peck
Pestalotiopsis funerea (Desm.) Steyaert
Phoma sp.
Preussia sp.
Rhinocladiella sp.
Rhizosphaera kalkhoffii Bubák
Scolecobasidium sp.
Septonema sp.
Sphaeropsis sapinea (Fr.) Dyko & B. Sutton

Nine species were recorded for *Pinus cembroides*, of which *Rhizosphaera kalkhoffii*, *Pestalotia stevensonii* and *Preussia* sp. were most commonly found in attached leaves. *Desmazierella*

acicola and *Discosia strobilina* were the most commonly found fungal species on leaf litter (Table 5). Tokumasu *et al.* (1994) observed similar patterns for *Pinus sylvestris* in Europe and noted the presence of *D. acicola* in leaf litter.

Table 5. Absolute frequencies of the isolated fungi.

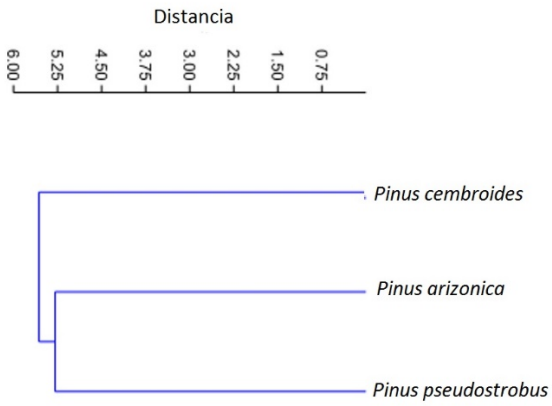
Pinus	***P.cembroides***			***P. pseudostrobus***			***P.arizonica***		
	A	**H**	**L**	**A**	**H**	**L**	**A**	**H**	**L**
Aureobasidium pullulans	5	6							
Chaetomium sp.							1		1
Chloridium sp.							1		1
Coniochaeta ligniaria	1		1	3			7	7	4
Desmazierella acicola	4	5	21			1			
Discosia strobilina		1	12			6			1
Fusarium lateritium							1		
Gymnopus androsaceus				10	9	1	1	1	
Herpotrichia sp.						7			
*Lachnum*sp.							3	1	
Leptomelanconium pinicola							6	5	1
Leptostroma sp.									1
Lophodermium australe				27	25	19			
Meloderma desmazieri				2	3	3			
Mytilinidion mytilinellum						3			
Niesslia exilis				12	9	2	1		1
Oidiodendron griseum				1					1
Penicillium sp.							2	6	4
Periconia byssoides	8	7	3				1		
Pestalotia stevensonii	15	17	12				1		
Pestalotiopsis funerea							3	2	1
Phoma sp.		2							
Preussia sp.	14	19	6	2	6	2	5	2	
Rhinocladiella sp.				3	1		1	1	
Rhizosphaera kalkhoffii	18	14	2						
Scolecobasidium sp.				1					
Septonema sp.									1
Sphaeropsis sapinea				3					

A = Attached leaves; H= Hanging leaves; L= Leaf litter.

14 fungal species were identified in *P. pseudostrobus*: *Lophodermium australe, Niesslia exilis* and *Gymnopus androsaceus* were the most abundant in attached leaves and *Lophodermium australe* and *Discosia strobilina* were the most abundant in the leaf litter.

18 taxa were determined in *P. arizonica*. This pine species exhibited the highest richness; it also had the lowest absolute frequencies.

Well defined patterns were observed for the three pine species (Figure 3). Only *Discosia strobilina* and *Preussia* sp. were common fungi in the studied pine species.



Distancia = Distancia

Figure 3. Cluster analysis of the fungal richness on the leaves of the three pine species.

When comparing the results obtained with the two techniques utilized, the moist chamber (direct method) turned out to be more effective for the determination of the fungal species. Parungao *et al.* (2002) reached to similar conclusions in their study on the fungal diversity in a tropical forest. Although the isolation in a culture medium (indirect method) made it possible to obtain twice as many taxa using the direct method, many of the cultures failed to form reproductive structures; for this reason, the list of unidentified species was long (50.87 %). This problem is discussed by Bills and Polishook (1994) and Polishook *et al.* (1996), who cite 37 to 45 % of the taxa as

unidentifiable. Furthermore, better patterns were defined for each pine species through the moist chamber technique. Many of the taxa identified with this means were not isolated in a culture medium. An example of this was *Desmazierella acicola*, very frequently found in the dead leaves of *Pinus cembroides*, which, according to Ponge (1991) is also a frequent fungus in the leaf litter of *Pinus sylvestris*. 13 species were identified for both taxa; of these, the most abundant was *Lophodermium australe*.

Conclusions

There is a great richness of fungal species associated to *Pinus arizonica*, *P.cembroides* and *P. pseudostrobus* leaves: 57 taxa were obtained with the indirect method, and 28 taxa, with the direct method. The indirect method makes it possible to obtain twice as many taxa than the direct method, but the number of unidentifiable fungal species is high (50 %). In order to estimate the richness of fungal species, more than one method must be used. The richness and abundance of fungi differs according to the pine species and to the leaf type.

The hanging dead leaves and the leaf litter exhibit the largest number of fungal colonies. The fungal richness diminishes as decay advances.

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Contribution by author

The author was in charge of the sampling, the identification of the species, the data analysis, and the drafting of the manuscript.

Conflict of interests

The author declares no conflict of interests.

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