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Site and seasonal influences on the fungal community on leaves and stems of *Pinus* and *Quercus* seedlings in forest nurseries

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Fungal assemblages present in nursery seedlings were surveyed, aimed to evaluate influences of site, season, host and plant part. Four sampling sites were selected; three of them were sampled twice, in spring and autumn. Needles/leaves and stems were collected from five host species: *Quercus ilex, Q. pyrenaica, Pinus pinea, P. nigra,* and *P. sylvestris* and surface-sterilized with sodium hypochlorite. A total of 1557 fungal strains, pertaining to 59 'species' or morphological types, were recovered. The dominant species, with higher isolation frequencies were *Alternaria alternata* complex, *Cladosporium cladosporioides, Phoma* sp., *Clonostachys rosea, Ulocladium* sp., *Trichothecium roseum, Cytospora leucosperma,* and *Rhizopus* sp. Median values of fungal species per observation differed significantly among the sites. The diversity of fungal species was significantly higher in the spring, and isolation frequencies for most of the dominant species were also dependent on the season, however the number of isolates per sampling was not influenced by the season. The results showed that the most frequent species (*Alternaria alternata* complex and *Cladosporium cladosporioides*) were associated with autumn observations, whereas higher frequencies of saprobic fungi (*Aspergillus niger, Ulocladium* sp. and *Cytospora* sp.) were found in spring.

Keywords: Fungal endophytes, site influences, seasonal influences, forest nurseries.

As part of the Communitarian Agrarian Policity in the European Union, producers have been encouraged to cease cultivation in marginal, non productive lands, replacing former crops by tree plantations. Reforestation of these areas, mainly aimed to reduce erosion and to increase environmental benefits, has led to a reactivation of forest plant production and to an increase of forest nurseries to meet the raising demand. The survival and early growth of the reforested plants depends to a great extent on the physiological quality of the

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seedlings produced in the nurseries, so optimal phytosanitary conditions in the seedlings are required. The study of fungi associated with nursery seedlings has traditionally been focused on seeds (Punam & Mehrotra 1999, Sateesh & Bath 1999, Prabha & Bohra 1999, Garbagnoli & Irigoyen 1999), cones (Mercier & al. 1991, Mercier 1993, Lilja & al. 1995) and rhizosphere (Kraft & al. 2000, Gupta & al. 1999, Annesi & Motta 1994, Camporota & Perrin 1994). Other studies have been also centred on treatments to reduce fungal attacks (Sunita 1999, Asiegbu & al. 1999, Lori & al. 1999).

However, the fungal community of aerial plant tissues (needles, leaves and stems) has been barely studied in nursery seedlings, even though these mycota could influence the development of other diseases such as damping off, one of the most important and widely distributed forest nursery diseases (Nef & Perrin 1999, Sesan & Taut 1998, Pedersen & al. 1999, Sutherland & al. 1990, Perrin & Camporota 1998, Kozlowski & Métraux 1998).

In this work we have analysed the influence of different factors (site, season, host and plant tissue) on the fungal communities of *Pinus* and *Quercus* seedlings in the North Plateau region in Spain. Samples of leaves/needles and stems were taken from seedlings growing at four different nurseries. To study the seasonal factor, samples were taken from three nurseries in the spring and in the autumn.

Material and methods

Sampling

Fungal species associated with *Quercus ilex* L., *Q. pyrenaica* Willd., *Pinus pinea* L., *P. nigra* Arn. and *P. sylvestris* L. were studied in each nursery (*Q. ilex* and *P. sylvestris* could not be sampled in spring in one of the nurseries). Samples were collected in dry weather at four different forest nurseries in the North Plateau in Spain (Castilla y León Autonomous Community). (Fig. 1).

Similar samplings were carried out in the spring and the autumn in 3 of the 4 nurseries, to allow for an analysis of the differences between fungal populations in both seasons. In each sampling, a tray with 35 symptomatic seedlings randomly chosen in the nursery was taken for each host. Seedlings were carried to the laboratory and processed within 24 hours after collection. A total of 8 samples (replications) of leaves/needles and 4 samples of stems were studied for each host in each sampling occasion. Nine (4 to 5 mm length) fragments of plant material were included for each replication. For statistical purposes, every combination of site, season, host species and plant part was considered to be an 'observa-

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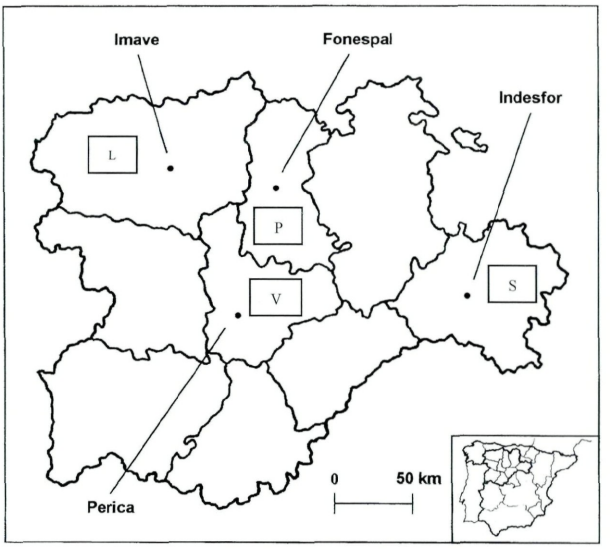

Fig. 1. Geographic location of the four sample sites. Imave (León); Fonespal (Palencia); Perica (Valladolid) and Indesfor (Soria).

tion'. Thus, the study comprised a total of 66 'observations' made from a total of 396 samples/replications (33 stem observations from 132 replications and 33 needle/leaf observations from 264 replications).

Fungal isolation and identification

The fungal isolation procedures were similar to those employed by other authors (Bettucci & al. 1999, Bettucci & Alonso 1997). Agar medium PCA was used to favour the sporulation of the different species, making the identification of strains easier. Plant tissues were aseptically dissected, surface-sterilised by immersion in 2% sodium hypochlorite for 7 min and rinsed several times in sterile distilled water. Dry fragments were plated directly onto PCA agar medium in 9 mm sterile Petri and incubated for 2 weeks at 25°C to stimulate sporulation (Evans & Reeder 2001). After incubation, fungal isolations were studied and grouped into morphological species by using a stereomicroscope and analysing the shape and colour of the colonies, and the main characteristics of fungal structures. Identification of *Alternaria* species is extremely difficult, thus *A. alternata* isolates found in this study have been relativated to *A. alternata* complex. Doubtful samples were confirmed in The Estación Fitopatológica 'do Areeiro' (Pontevedra, Spain).

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Statistical data analysis

Univariate analysis

Data were analysed with different statistical tests. The statistical package SPSS+ (SPSS Inc., Illinois, USA) was used for Kruskal-Wallis, Wilcoxon Mann and Whitney and χ^2 tests. The two first were used to compare the medians of number of species per observation among sampling sites and seasons respectively. The χ^2 goodness-of-fit test was applied to the analyses of the isolation frequencies for the dominant species with respect to the sampling site and to the season.

Multivariate analysis

The 396 samples were obtained from 66 different observations according to nursery, season, host and part plant tissue. For each of the 66 observations, each fungal species was classed into 3 distinct categories based on its isolation frequencies as follows: 0 absence (0%); 1 (1–50%) and 2 (>50%). The complete fungal assemblages obtained in the 7 samplings were compared and the categorical variables (site, season, host, part) were subjected to Multiple Correspondence Analysis (MCA).

The technique of MCA is based on a singular value decomposition of the Burt's table (the equivalent of a covariance matrix for categorical data) (Gifi 1990; Gil & al. 2002). This technique is used to analyse multivariate data and to display their dispersion in a plot.

A Cluster analysis was realised using the MCA scores for every observation as input variables. The Square Euclidean Distance was used in order to compute the distance matrix between observations. Classification was carried out using an agglomerate algorithm based on Ward's method (Everitt 1980). The number of groups was judged visually based on the resulting dendrogram. Once the groups of observations were defined, a non-parametric statistical test (χ^2 contingency analysis) was applied to detect differences in the fungal frequency categories for each group.

Finally a M-L χ^2 Test ($P<0,05$) was used to detect relationships in the nursery, season, host and plant part variables within and among the previously established groups.

Results and Discussion

Diversity of fungal assemblages

A total of 1557 fungal isolates, accounting for 59 'species' or morphological types, were obtained from the 396 needle/leaf and

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stem samples. A range of 17–35 different species were recorded in each sampling. The highest number of isolates (268) was recovered from samples of the Imave nursery (Leon) in the spring of 2000, although the number of fungal species was higher (35) in Fonespal (Palencia) (Tab. 1). The Kruskal-Wallis test revealed significant differences ($P < 0.05$) among the medians of species per observation in each sampling.

Tab. 1. – Number of fungal isolations and species recovered from the seven samplings.

Sampling	Total species	Total isolates	Medians of species/observation
1	22	268	11
2	35	226	10
3	21	149	9
4	18	256	8
5	19	242	8
6	17	232	9
7	20	184	8

Samplings: 1. Imave (spring); 2. Fonespal (spring); 3. Perica (spring); 4. Imave (autumn); 5. Perica (autumn); 6. Indesfor (autumn); 7. Indesfor (spring).

Table 2, shows the isolation frequencies of the most abundant fungal species recovered. All of these species occurred in at least two samplings, and a total of 7 species were isolated from all samplings. Number of species were higher in the spring, although number of isolates seemed not to be affected by the season (Mann & Whitney test, $P < 0.05$).

Tab. 2. – Relative isolation frequencies of the main fungal species recovered from seven samplings in nursery seedlings.

Species / Sampling	1	2	3	4	5	6	7	TOTAL
Alternaria alternata complex	73,3	76,7	68,8	95,0	93,3	96,7	87,5	84,8
Aspergillus niger Tiegh.	26,7	0,0	4,2	0,0	0,0	0,0	0,0	4,5
Cladosporium cladosporioides (Fresen.) G. A. de Vries	50,0	38,3	18,8	70,0	86,7	75,0	52,1	57,1
Cytospora sp.	18,3	0,0	6,3	0,0	0,0	0,0	0,0	3,5
Cytospora leucosperma (Pers.: Fr.) Fr.	25,0	3,3	16,7	25,0	6,7	0,0	4,2	11,6
Fusarium sp.	5,0	5,0	4,2	3,3	5,0	0,0	12,5	4,8
Gonatobotrys sp.	0,0	3,3	0,0	6,7	8,3	10,0	4,2	4,8
Clonostachys rosea (Link: Fr.) Schroers et al.	38,3	1,7	29,2	21,7	20,0	18,3	8,3	19,7
Pestalotia hartigii Tubeuf	0,0	8,3	2,1	0,0	8,3	6,7	4,2	4,3
Pestalotiopsis funerea (Desm.) Steyaert	8,3	25,0	6,3	5,0	0,0	0,0	0,0	6,6
Phoma sp.	40,0	60,0	45,8	70,0	51,7	53,3	75,0	56,3
Rhizopus sp.	5,0	0,0	8,3	13,3	10,0	11,7	2,1	7,3
Sclerophoma sp.	6,7	10,0	2,1	5,0	3,3	15,0	0,0	6,3
Unidentified Deuteromycete C	10,0	11,7	10,4	3,3	3,3	3,3	10,4	7,3
Trichothecium roseum (Pers.) Link	23,3	10,0	18,8	10,0	26,7	6,7	0,0	13,9
Ulocladium sp.	38,3	10,0	22,9	15,0	5,0	8,3	29,2	17,9

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Alternaria alternata complex was the most frequent endophyte found in this study in accordance with previous studies on other forest hosts (Bettucci & Alonso 1997). It occurred in all the plates from all samplings, apart from being the most frequent in all the cases. This species has been already reported from forest nursery seedlings (Soldevilla 1995) and its possible pathogenic role has been pointed out by several authors (Mittal & Wang 1987, Chand & al. 2000, Su & al. 1998, Gao & al. 1999).

Isolation frequencies for most of the dominant species were dependent on the season. Thus, *A. alternata* complex and *Cladosporium cladosporioides* were isolated preferentially in the autumn, and differences between isolation frequencies in both seasons was statistically significant for both species (χ^2 test, $P<0.05$). However, in an earlier study analysing the endophytic fungal assemblages in *Quercus ilex*, *A. alternata* complex was found higher in spring (Collado & al., 1999). In our study, *A. alternata* complex isolation frequency was significantly higher in *Pinus* than in *Quercus* seedlings. Those plant genera have extremely different chemistry which is bound to affect fungal colonization. Also, *A. alternata* complex isolation frequency was higher in leaves/needles than in stems (data not shown). The effect of environmental exposure has been analysed on other hosts, showing the importance of leaf shape in relation to fungal dispersion (Petrini & Carroll 1981). Furthermore, the continental climate conditions in our study sites, with sudden cold temperatures early in the autumn season, may produce small damages in the plant tissues favouring these fungal infections.

Other species such a *Ulocladium* sp., were significantly more frequent in the spring, and even *Aspergillus niger* and *Cytospora leucosperma* were only recovered in this season. Isolation frequencies for the latter species were higher in *Quercus* seedlings and on leaf tissues. This species was also found as part of the fungal community associated to *Quercus* trees in Spain (Muñoz & al., 1996). Higher exposition of *Quercus* leaf shape could explain the abundance of this fungus in the spring, when the greater irrigation in the nurseries may favour the dispersion of fungal spores (Collado & al., 1999).

Finally, only the occurrence of *Clonostachys rosea* seemed not affected by the season nor it was particularly associated to any plant part. These results are in accordance with previous reports on this species from other forest nurseries, where it was isolated from *P. pinaster* seedlings and from the nursery atmosphere (Soldevilla 1995)

The results obtained in the present analysis show that the seasonal factor determines the composition of the fungal assemblages in forest nursery seedlings and this has also been observed for other hosts species in the field, such as *Quercus ilex* (Collado & al., 1999),

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Pseudotsuga menziesii (Carroll & Carroll 1978) and *Euterpe oleracea* (Rodrigues 1994).

Combined influence of site, season, host and plant part on mycota

The Burt's table for the 66 observations (Tab. 3) shows that most of the sampled observations (84.8%) were associated with category 2 (isolation frequency higher than 50%) of *A. alternata* complex, while in 15.2% of the observations category 1 (1–50% isolation frequency) of *A. alternaria* was obtained. Thus this fungal species was recovered in all 66 observations. High occurrence of this species in seedlings was also found in the South Plateau region, under quite similar climate conditions (Soldevilla 1995) but was rarely recovered from Cantabric nurseries, where high relative humidity conditions are prevalent throughout the year. In addition, *Cladosporium cladosporioides* and *Phoma* sp. frequency patterns were similar; They were absent from 12.1% and 6% of the observations respectively, both of them were recovered 40.9% as category 2 and 46.9% and 53% respectively as category 1. The frequency patterns for the rest of the species was very similar, generally with category 0 (absence) percentage being the higher.

Tab. 3. – Diagonal values obtained from Burt's table used for the Multiple Correspondendce Analysis (MCA) of observations.

	A. alternata C.			*Aspergillus niger*			*C. cladosporioides*			*Cytospora sp.*			*C. leucosperma*		
Categories	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
0	–	–	–	59	0	0	8	0	0	58	0	0	48	0	0
1	–	10	0	0	6	0	0	31	0	0	7	0	0	15	0
2	–	0	56	0	0	1	0	0	27	0	0	1	0	0	3

	Fusarium sp.			*Gonatobotrys sp.*			*C. rosea*			*P. hartigii*			*P. funerea*		
Categories	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
0	51	0	0	51	0	–	36	0	0	57	0	–	53	0	0
1	0	14	0	0	15	–	0	22	0	0	9	–	0	12	0
2	0	0	1	–	–	–	0	0	8	–	–	–	0	0	1

	Phoma sp.			*Rhizopus sp.*			*Sclerophoma sp.*			*Deuteromycete C*			*T. roseum*			*Ulocladium sp.*		
Categories	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
0	4	0	0	46	0	–	52	0	–	43	0	–	32	0	0	26	0	0
1	0	35	0	0	20	–	0	14	–	0	23	–	0	33	0	0	38	0
2	0	0	27	–	–	–	–	–	–	–	–	–	0	0	1	0	0	2

Categories based on isolation frequencies: 0 (0%), 1 (1–50%); 2 (>50%)

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The MCA analysis revealed that the categories related to the most frequent species, such as *A. alternata* complex and *Cladosporium cladosporioides*, were found to be the most important variables for the first MCA axis (Fig. 2a). Thus, category 1 for these species appeared in the right side of the diagram whereas category 2 observations were located in the left side. Considering that both fungi were more frequently observed in the autumn, this may indicate a seasonal orientation for the first MCA axis, from left (autumn) to right (spring). On the contrary, for the saprotrophic *Ulocladium* sp., *Cytospora* sp. and *Aspergillus niger*, an increasing abundance trend is observed from the left to the right side of the graph. Frequencies for these species were higher in the spring, the last appearing only in this season. Again, season could explain, at least partially, the situation of these categories related to the first MCA axis. Thus, categories 1 and 2 of *Aspergillus niger* and *Cytospora* sp, appeared located together on the right side of the diagram, where only spring observations were found.

In Figure 2b, the MCA ordination diagram for observations is presented (only the first two axes were retained). Observations located on the right side of this diagram have been sampled in the spring, while a predominance of autumn observations can be observed when moving to the left side of the diagram. This result is in accordance with the previous univariate analysis and confirms that seasonal differences can be observed in the first MCA axis.

Classification of the 66 observations into different groups was based on the MCA scores. Since the diagram for the first two MCA axes is directly interpretable, we used only these two axes for performance clustering, in spite of the loss of some variability contained in other axes. The Cluster analysis showed that three groups observations can be distinguished, following the aforementioned trends of the MCA results (Fig. 3a). The most numerous is group 3 with 50 observations, followed by group 1 with 12 observations and by group 2 with 4 observations. The same groups have been represented on the ordination diagram (Fig. 3b), in order to show their main characteristics, which can be summarised as follows (Tab. 4):

Group 1: Observations in this group are composed by higher isolations frequencies of *Ulocladium* sp, *Aspergillus niger and Cytospora* sp.; Category 2 observations of these species are always included in this group.

Fig. 2. Multiple Correspondence Analysis (MCA): ordination of isolation frequencies for fungal species (a) and for observations (b). Only the first two ordination axes were retained.

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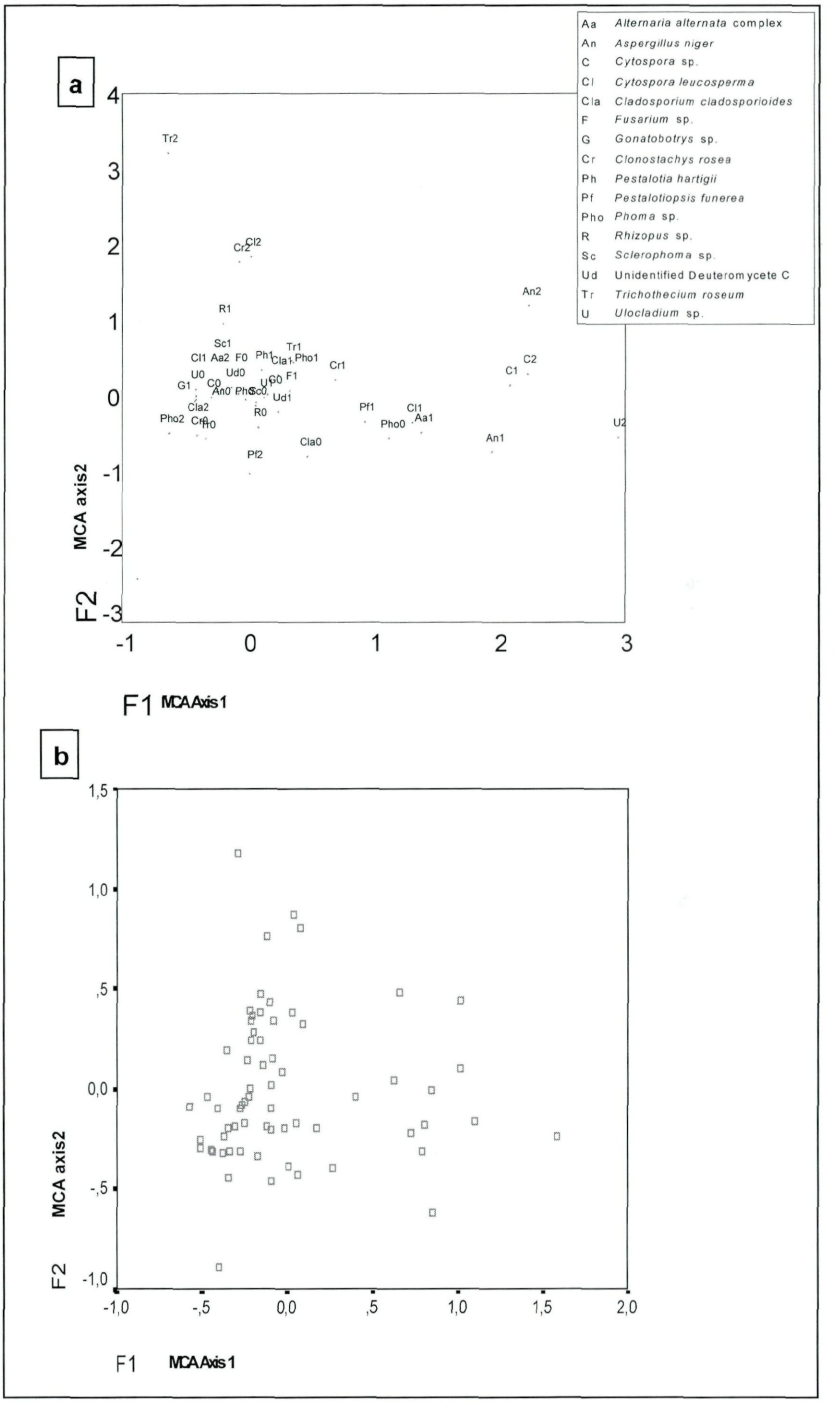

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Group 2: This set includes 100% of sampled observations with category 2 for *Trichothecium roseum* and a higher percentage of *C. leucosperma* category 2 than in the other groups. Besides, all the observations of *Clonostachys rosea* in this group are of category 2.

Group 3: This set is characterized by higher *A. alternata* complex, *Cladosporium cladosporioides* and *Phoma* sp. isolation frequencies in comparison with other fungal species.

Tab. 4. – Relative frequencies of the different categories for the fungal species in the observation groups from the Cluster Analysis.

	A. alternata			*C. Aspergillus niger*			*C. cladosporioides*			*Cytospora sp.*			*C. leucosperma*		
Group-Category	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
1	0	58	42	58	33	8	25	75	0	42	50	8	25	75	0
2	0	0	100	100	0	0	0	75	25	100	0	0	50	0	50
3	0	6	49	96	4	0	10	38	52	98	2	0	86	12	2

	Fusarium sp.			*Gonatobotrys sp.*			*C. rosea*			*P. hartigii*			*P. funerea*		
Group-Category	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
1	67	33	0	92	8	0	25	67	8	83	17	0	42	58	0
2	75	25	0	75	25	0	0	0	100	75	25	0	100	0	0
3	80	18	2	74	26	0	66	28	6	88	12	0	88	10	12

	Phoma sp.			*Rhizopus sp.*			*Sclerophoma sp.*			*Deuteromycete C*			*T. roseum*			*Ulocladium sp.*		
Group-Category	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
1	17	75	8	83	17	0	83	17	0	42	58	0	17	83	0	25	58	17
2	0	100	0	25	75	0	75	25	0	75	25	0	0	75	25	25	75	0
3	4	44	52	70	30	0	78	22	0	70	30	0	60	40	0	44	56	0

Thus, isolation frequencies of fungal species were different among the three established groups. In addition, two different variables (site and season) were significantly different among the three groups. The first group included only spring observations, and 67% of all observations within this group were taken from Imave nursery (Tab. 5). On the other hand, autumn and Indesfor nursery observations were more numerous in the third group (M-L χ^2 Test, $P < 0.05$; Tab. 5). These results are in accordance with the above univariate

Fig. 3. Clustering of isolation frequencies of the fungal species into three groups. – a. Dendrogram. – b. Representation of the observation groups over the MCA ordination diagram.

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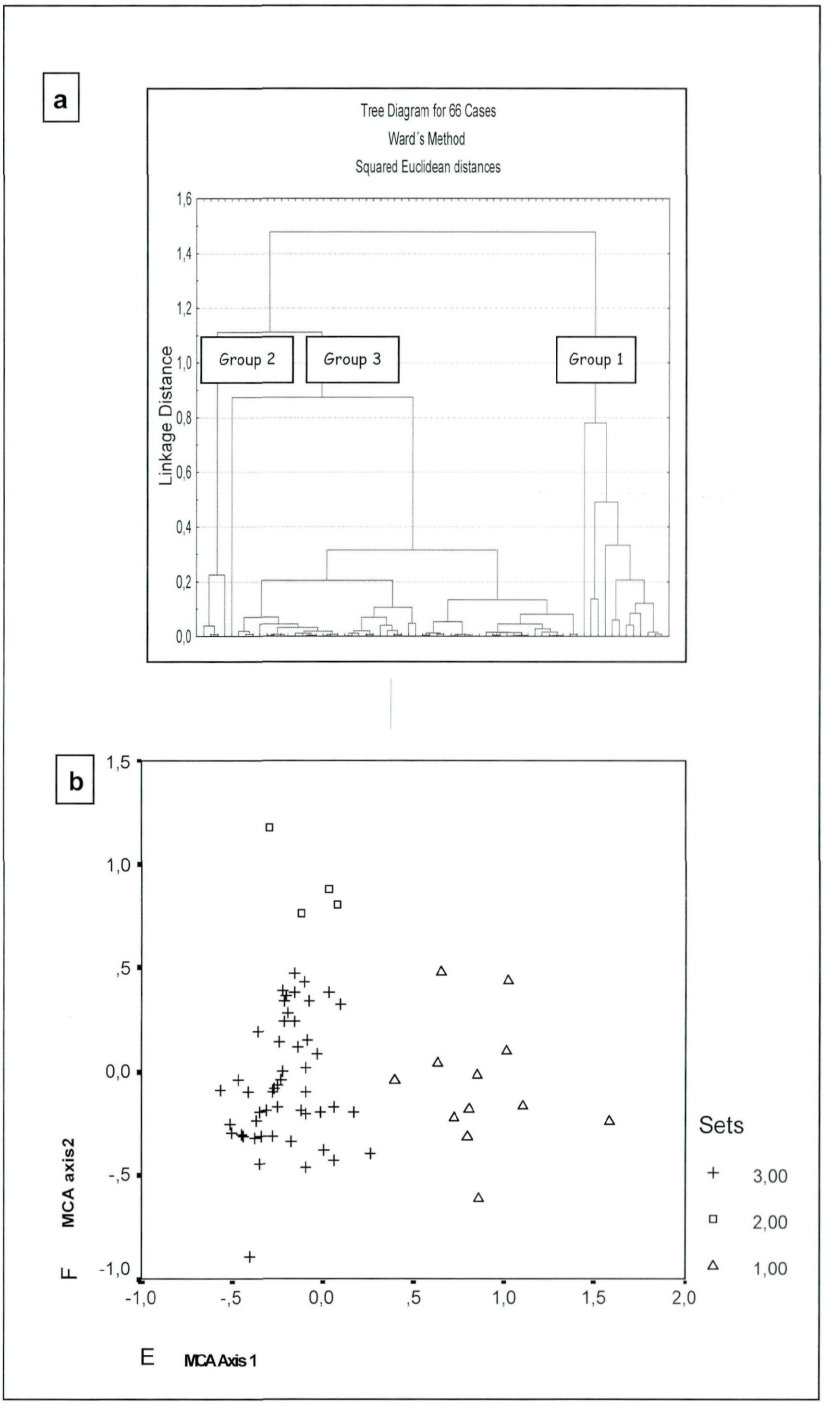

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results, so the first group was found in the right side of the diagram corresponding to spring and Imave observations.

Tab. 5. – Relative frequencies of the variables in the observation groups from the Cluster Analysis.

			Nursery			Season		
Group	Fonespal	Imave*	Perica	Indesfor*	P<0,05	Spring*	Autumm*	P<0,05
1	0,08	0,67	0,25	0,00	**<	1,00	0,00	**<
2	0,00	0,25	0,50	0,25	>	0,50	0,50	>
3	0,20	0,20	0,26	0,34	**<	0,44	0,56	**<

	Host			Part		
Group	Pinus	Quercus	P>0,05	Stems	Leaves	P>0,05
1	0,50	0,50	>	0,42	0,58	>
2	0,25	0,75	>	0,50	0,50	>
3	0,66	0,34	>	0,52	0,48	>

** significant differences among variables within groups (M-L χ^2 test; P<0,05).
* significant differences among roups within a variable (M-L χ^2 test; P<0,05).

The analysis of fungal isolations in nursery seedlings is most often reduced to a listing of the main species found, sometimes including isolation frequencies without further analysis to fully describe the existing variation. The use of MCA, though purely descriptive, allows objective grouping of observations based on multiple characteristics. In our case, variability for the most important species was related to the season and to the site. Therefore, geographical factors (nursery location, Fig. 1) may affect the composition of the mycota recovered, provided that the nurseries sampled are distant or environmentally different. This result should be attributed to the fungi being subject to different selection pressures in each ecological niche, as pointed out in other studies (Collado & al., 1999). This may lead to the establishment of fairly specific fungal populations at each site, as reported for other host species (Petrini & al., 1992). The influence of season was also clear and this could be likely due to the seedlings being grown uncovered in a relatively flat area, with high exposure to the environment.

On the other hand, influences on fungal communities related to plant part have not been observed, contrarily to the results obtained by Bettucci & Alonso (1997), who found that tissue-specific preferences characterised the endophytic fungal communities in *Eucalyptus* young plants. However, in that case the study was carried out on field plants with older tissues, where different water potentials may significantly affect the fungal assemblages (Chapela & Boddy 1988, Bisinger & Sieber 1994). In our case, the younger tissues studied could explain that no significant plant part related differences were found. Similarly, differences in fungal communities have not

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been observed between both host plant genera, what could be due to the generalist and unspecific character of most of the fungal species found (Nef & Perrin 1999, Soldevilla 1995, Old & al., 1991)

In summary, the results here presented reveal the effect of the sampled nurseries and of the season in the isolation frequencies of the fungal communities obtained from *Quercus* and *Pinus* seedlings; in that sense, the most frequent species (*Alternaria alternata* complex and *Cladosporium cladosporioides*) were associated with autumn observations, whereas the higher frequencies for saprophytic fungi (*Aspergillus niger, Ulocladium* sp. and *Cytospora* sp.) were found in spring. On the other hand, no differences in fungal communities were found related to plant part or to plant genus.

The knowledge of the influence of ecological factors on fungal communities in forest nurseries is essential to prevent diseases and therefore to avoid introducing pathogenic fungal species in reforested areas.

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