Mycobiology

Research Article

Influence of the Culture Media and the Organic Matter in the Growth of *Paxillus ammoniavirescens* (Contu & Dessi)

Elena Fernández-Miranda Cagigal*,# and Abelardo Casares Sánchez

Department of Biology of Organism and Systems, Area of Plant Physiology, University of Oviedo, 33006 Oviedo, Spain

Abstract The genus *Paxillus* is characterized by the difficulty of species identification, which results in reproducibility problems, as well as the need for large quantities of fungal inoculum. In particular, studies of *Paxillus ammoniavirescens* have reported divergent results in the *in vitro* growth while little is known of its capacity to degrade organic matter. For all the above, and assuming that this variability could be due to an inappropriate culture media, the aim of this study was to analyse growth in different culture media (MMN, MS, and 1/2 MS) and in the case of MMN in presence/absence of two types of organic matter (fresh litter and senescence litter) to probe the saprophytic ability of *P. ammoniavirescens*. We also evaluated the effects of pH changes in the culture media. Growth kinetics was assessed by weekly quantification of the area of growth in solid culture media over 5 wk, calculating the growth curves and inflection points of each culture media. In addition, final biomass after 5 wk in the different culture media was calculated. Results showed that best culture media are MS and 1/2 MS. Moreover, an improvement in growth in culture media containing decomposing fall litter was observed, leading to confirm differences in the culture media of this species with others of the same genus. Further, we established that all growth media suffered a significant acidification after fungal growth.

Keywords *In vitro* culture, Organic matter, *Paxillus ammoniavirescens*, pH

The genus *Paxillus* Fr. (*Agaricomycota*, *Boletales*, *Paxillaceae*) is widespread in the Northern Hemisphere and forms ectomycorrhizal associations with various host plant species, including hardwood and coniferous trees [1, 2], and is common an array of ecosystems including degraded or contaminated habitats. The species *Paxillus involutus* is one of the best studied and a model species for ecological and physiological studies. Several authors have reported a large variability in the morphology and physiology between different isolates of this fungus, which suggests that *P. involutus* encompasses multiple species [3-5, 7].

Phylogenetic analyses of nuclear DNA performed by different authors [2, 8, 9] confirmed the presence of five different species in the *P. involutus* complex: *P. involutus*, *P. ammoniavirescens*, *P. cuprinus*, *P. obscurosporus*, and *P. vernalis*; among which it is extremely difficult to differentiate based on morphological characters or differential ammonia reactions. Misidentification can prompt numerous reproducibility problems and difficulties optimizing culture conditions, as demonstrated by the study of Jargeat *et al.* [2], who observed conflicting behaviours in the same culture medium for *P. ammoniavirescens*, *P. cuprinus*, and *P. involutus*.

Traditionally the most employed culture medium is Melin-Norkrans agar medium (MMN) [10], but numerous species present culturing issues in this medium. Molina and Palmer [11] argue these problems might be due to an inappropriate culture media, as demonstrated with the recalcitrant fungus *Cantharellus cibarius* that only grew in modified fries medium (MFM) [12], a complex and highly defined medium. In the case of *P. ammoniavirescens* very little is known about its growth kinetics, and only two authors [2, 5] analyzed the growth of this species with varying outcomes. Additionally, it has been demonstrated that *P. involutus* in symbiosis with *Pinus sylvestris* seedlings have only a reduced ability to decompose fresh beech litter (*Fagus sylvatica*) [13]. Unfortunately, there is a lack of reporting

Mycobiology 2017 September, **45**(3): 172-177
https://doi.org/10.5941/MYCO.2017.45.3.172
pISSN 1229-8093 • eISSN 2092-9323

*Corresponding author
E-mail: cagigal_11@hotmail.com
#Present address: BioCarbon Engineering, Oxford, UK

Received January 26, 2017
Revised May 11, 2017
Accepted July 3, 2017

on the saprotrophic abilities of *P. ammoniavirescens*.

Assuming that the observed variability could be caused by not using a culture media that provide the essential substances necessary for the optimal growth of *P. ammoniavirescens*, and with the main objective of using *P. ammoniavirescens* in reforestation programs, we studied in detail the growth kinetics of this species in different culture media with/without different types of organic matter, aiming to increase mycelium production. Additionally, we determined the pH variation caused by the fungi growth in different culture media.

MATERIALS AND METHODS

Insolates and culture conditions. Several pure mycelia cultures were obtained from fruit bodies collected in a polluted site of Asturias (North Spain), under *Betula celtiberica* [14]. Basidiome fragments were placed on solid MMN medium in Petri dishes (90 × 15 mm) containing 15 mL medium, and incubated at 23°C in the dark. Cultures are available at the culture collection of the Area of Plant Physiology, Department of Biology of Organism and Systems, University of Oviedo.

PCR and DNA sequencing. DNA was extracted from culture (pool of five isolates) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR was performed following Hedh *et al.* [8] using the primers ITS1 and ITS4 [15] and a further clean up of PCR products (NucleoSpin Gel and PCR Clean-Up kit; Macherey-Nagel, Düren, Germany) was performed before being sequenced using Big Dye Terminator 3 and an ABI 3730xl DNA Analyzer (both from Applied Biosystems, Foster City, CA, USA). The consensus sequence was determined by overlapping sequence data from both DNA strands, using the software Geneious Pro 4.8.2. Sequence is available from the GenBank database under the accession number KP241048.

Alignment and phylogenetic analyses. For species determination, the generated internal transcribed spacer (ITS) sequence was compared with 14 sequences from GenBank (Table 1). Sequences were aligned using MUSCLE (v3.7) [16], and gaps were scored as missing data. The best-fit evolutionary model was determined using the software jModelTest-2.1.4 [17] comparing different nucleotide substitution models, and selected based on the Akaike Information Criterion [18]. Bayesian analyses were performed using MrBayes v3.2.2 [19], with four Markov Chain Monte Carlo tests running simultaneously for 1,000,000 generations under General Time Reversible evolutionary model [20] with the proportion of invariable sites (I). Evolutionary trees were saved every 1,000 generations and the first 25% were discarded as burn-in. For the remaining trees, a majority rule consensus tree was computed to obtain estimations for Bayesian posterior probabilities (PPs). The phylogenetic tree was visualized and edited with FigTree v1.4.1.

Maximum likelihood (ML) genealogies were constructed using MEGA v5.2 [21], under the same evolutionary model used for Bayesian analyses. The bootstrap consensus tree was inferred from 10,000 replicates. In both analyses *Paxillus rubicundulus* was used as outgroup.

Table 1. ITS sequences of the genus *Paxillus* included in the studied

Species	ID GenBank	Location	Host species
P. ammoniavirescens	KF261398[a]	Ramonville-Saint-Agne, France	*Cedrus* sp.
	KF261400[a]	″	*Betula* sp.
	KF261414[a]	Toulouse, France	*Pinus* sp.
	KF261385[a]	Eauze, France	*Alnus* sp.
			Salix sp.
	JN661717[b]	Esterel-Les Espagnols, France	*Quercus suber*
			Populus sp.
	JN661715[b]	″	*Alnus* sp.
	JN661719[b]	Sardinia, Italy	*Populus* sp.
	JN661718[b]	″	ND
	JN661711[b]	Kent, England	ND
P. cuprinus	**KF261379**[a]	Castanet-Tolosan, France	*Betula* sp.
P. involutus	KF261368[a]	Cantaous, France	*Picea* sp.
P. obscurosporus	**EU084665**[c]	Baviera, Germany	*Tilia* sp.
P. vernalis	JN661720[b]	Miami, EEUU	ND
Outgroup			
P. rubicundulus	EU084667[c]	Scotland	ND

All sequences are identified with the ID GenBank, the location and the host. Type specimens in bold.
ITS, internal transcribed spacer; ND, not determined.
[a]Jargeat *et al.* (2014) [2].
[b]Vellinga *et al.* (2012) [9].
[c]Hedh *et al.* (2008) [8].

Effects of culture media and organic matter. For the growth experiments in nine nutritive media, a 10 mm diameter mycelium plug was taken with a cork borer from actively growing mycelia (2 wk on MMN at 23°C). This plug was placed into Petri dishes (90 × 15 mm) containing 20 mL of each medium. The culture media lacking organic matter were MMN, Murashige and Skoog medium (MS) [22], and a modification of the previous media called 1/2 MS, wherein calcium and other macronutrients are halved. To test the effect of organic matter on fungi growth, we used an MMN medium, to which we added two types of organic matter at different concentrations. The first consisting of green leaves collected directly from the tree (green organic matter [G]); and a second, consisting of fallen leaves (brown organic matter [B]). In both cases, leaves were washed and dried at 70°C for 24 hr. Later, they were ground to reach a size lower than 1 mm. In both cases three concentrations were assayed: 0.25, 0.5, and 1 g per plate. For a homogeneous distribution of the organic matter in the culture media, it was autoclaved separately and added in the solidifying media.

Growth was quantified weekly over the course of 5 wk by measuring the area occupied by fungal colonies in the different culture media, using the image analysis program Image-Pro Plus v4.5 [23]. At the end of this period, biomass was measured using dry colony weight. For this purpose, we eliminated the agar and traces of organic matter by heating the colonies in a warm water bath. Thereafter, the fungal material was dryed at 75°C until it reached a constant weight. Ten samples per treatment were evaluated.

pH variation. To determine the pH variations caused by fungal growth in the nine culture media used, the pH was initially adjusted to 5.6 in all media before autoclaving at 120°C for 20 min and 20 mL of each medium was poured into 10 Petri dishes (90 × 15 mm). Once solidified, the pH was measured on two plates (three points per plate, called Control 1) per media to determine how the autoclaving process affected it. In six plates, a 10 mm diameter fungal plug was taken with a cork borer from actively growing mycelia (2 wk on MMN at 23°C), and incubated at 23°C in the dark until the colony had achieved a diameter of 5 cm. pH was measured at three zones on each plate: inside the mycelium, at the border of the mycelium and in an area without growth. In each zone four points were measured. After 5 wk, two more plates of each medium without inoculum (Control 2) were evaluated for changes to the pH.

Statistical and mathematical analysis. All data were analysed by analysis of variance (ANOVA) and *post hoc* Fisher's least significant difference (LSD) tests using SPSS software (SPSS Inc., Chicago, IL, USA). The level of significance was 0.05 for all purposes. The inflection point, which represents the moment when the exponential growth begins to decelerate, was calculated as the second derivative of the linear equation of the growth curves of each medium.

RESULTS AND DISCUSSION

Phylogenetic analyses. Sequence analyses of the ITS-rDNA from the examined samples produced identical

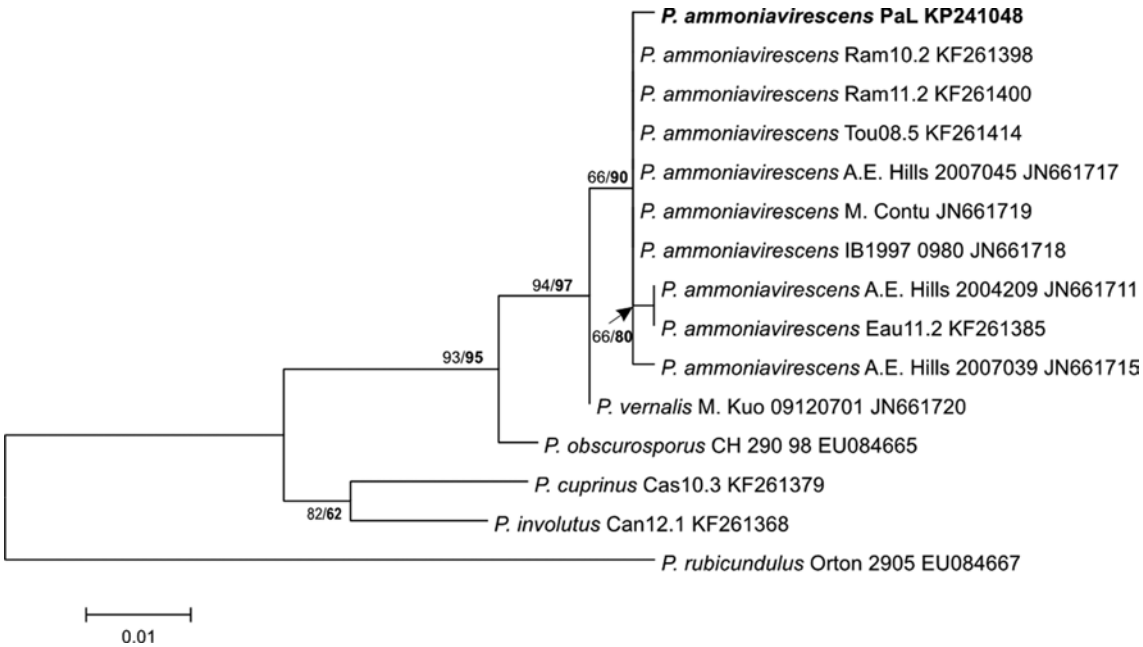

Fig. 1. Maximum likelihood (ML) consensus tree derived from the internal transcribed spacer data set. On each branch, the percentages (%) of 10,000 bootstrapping replicates supported by ML are shown, so are the Bayesian posterior probabilities (PPs) (in bold). The sequence of our isolates is shown in bold. The tree was rooted using a sequence from *Paxillus rubicundulus*. The scale shows the expected number of changes per nucleotide. PPs and bootstrap values under 50 not included.

topology and relationships among clades using ML (66% bootstrap) and Bayesian (90% PPs) tree analyses (Fig. 1), placing our isolate clearly inside the *P. ammoniavirescens* clade, in line with the results obtained by other authors [2, 8, 9].

Effects of culture media and organic matter. We observed significant differences among the various culture media, with MS and 1/2 MS showing higher growth, while 1 g G MMN medium shows the worst growth index (Fig. 2).

Growth kinetics of filamentous fungi is difficult to characterize, especially in solid medium. Erroneously, there is a tendency to interpret their growth in the same manner as with bacteria and yeast. However, growth in these fungi only takes place at the tip of the hypha and not in each individual cell. French and Hebert [24] consider that when a constant and uniform progress of the fungi in solid medium is observed, the fungus has a defined growth, whereas when the growth is not uniform the fungus has an undefined growth, which varies in response to different environmental factors. Analyzing the growth curves, we can observe that in all culture media where the fungi grew, a *lag* phase took place in the first week, except in 1/2 MS medium where it started growing exponentially from the beginning. Aside of this fact, in solid medium *P. ammoniavirescens* seems to have a defined growth, without constant growth which decelerated between the second and fourth week in all the culture media, as was reflected by the inflection points.

Our results demonstrate that MS and 1/2 MS were the best culture media for *P. ammoniavirescens*, given that whitin 3 wk all isolates covered the entire medium; with the lowest inflection points (MS 2.91 and 1/2 MS 2.07) and were also the ones which generated the highest biomass after 5 wk. Both media were very rich in microelements and vitamins, improving on the growth descriptions for malt extract peptone (MP) medium, which seemed to be key for increasing the growth rates on this species. It is also notable that when comparing the results obtained in MMN and MMN supplemented with organic matter, an improvement of growth rates is observed in all media with brown litter, as well as in the 0.25 g G MMN. The other two culture media with fresher litter showed either a decrease (0.5 g G MMN) or an inhibition (1 g G MMN) of growth.

The highest fungi dry weight was recorded in MS and 1/2 MS as culture mediums (Fig. 3). There were no significant differences between MMN and MMN with brown organic matter, although in the 0.5 g B MMN and 1 g B MMN the biomass increased and did not show a significant difference with 1/2 MS, which clearly supports that addition of G organic matter caused a significant decrease of dry weight.

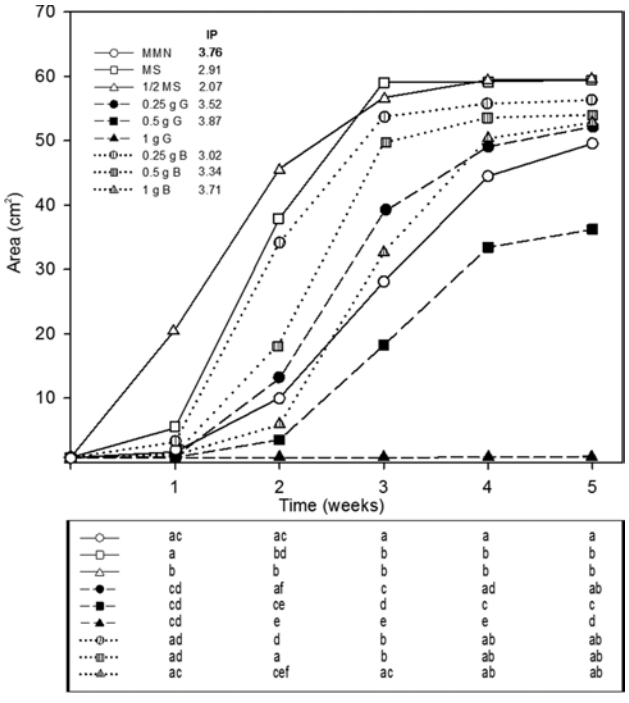

Fig. 2. Growth curves of the colonies of *Paxillus ammoniavirescens* in the nine culture media analyzed. The ordinates axis represents the growth area expressed in cm^2 and the abscissa axis the amount of time in weeks spent in culture. IP indicates the inflection point. Different letters within a single column indicate significant differences between different cultures media weekly for 5 wk ($p \leq 0.05$ ANOVA and *post hoc* least significant difference tests). MMN, Melin-Norkrans agar medium; MS, Murashige and Skoog medium; G, green organic matter; B, brown organic matter plus MMN.

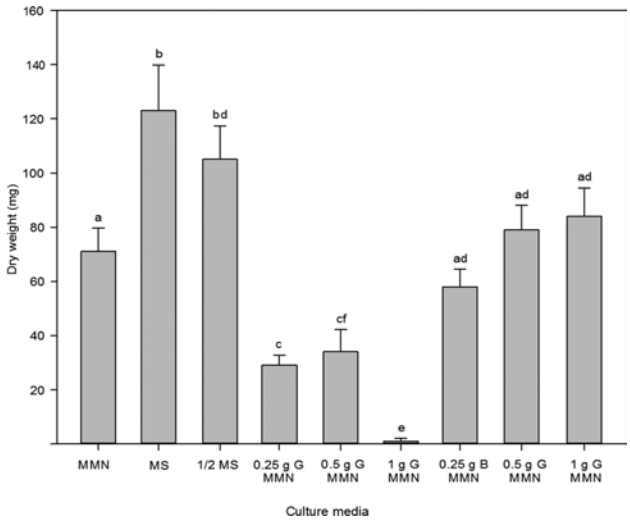

Fig. 3. Means ± standard error of dry weight (mg) of the colonies of *Paxillus ammoniavirescens* after 5 wk of growth in the different culture media. Different letters above bars indicate significant differences between culture media ($p \leq 0.05$ ANOVA and *post hoc* least significant difference tests). MMN, Melin-Norkrans agar medium; MS, Murashige and Skoog medium; G, green organic matter; B, brown organic matter plus MMN.

Table 2. pH of *Paxillus ammoniavirescens* colonies in the different culture media

Medium	Control 1	Control 2	Total	Inside	Border	Without
MMN	5.39 ± 0.06 a	5.44 ± 0.07 a	4.08 ± 0.07 b	4.11 ± 0.11 b	4.16 ± 0.12 b	3.96 ± 0.11 b
MS	5.55 ± 0.02 a	5.51 ± 0.10 a	4.17 ± 0.09 b	4.30 ± 0.13 b	4.12 ± 0.18 b	4.03 ± 0.17 b
1/2 MS	5.59 ± 0.02 a	5.49 ± 0.08 a	4.67 ± 0.07 b	4.66 ± 0.12 b	4.69 ± 0.15 b	4.66 ± 0.12 b
0.25 g G	5.09 ± 0.04 a	5.76 ± 0.05 b	4.41 ± 0.07 c	4.44 ± 0.11 c	4.34 ± 0.13 c	4.42 ± 0.15 c
0.5 g G	5.09 ± 0.06 a	5.52 ± 0.02 b	4.31 ± 0.05 c	4.37 ± 0.11 c	4.20 ± 0.04 c	4.36 ± 0.06 c
1 g G	4.99 ± 0.03 a	5.65 ± 0.06 b	ng	ng	ng	ng
0.25 g B	5.45 ± 0.03 a	5.52 ± 0.02 a	4.34 ± 0.06 bc	4.16 ± 0.06 b	4.36 ± 0.14 bc	4.56 ± 0.10 c
0.5 g B	5.42 ± 0.01 a	5.71 ± 0.02 a	4.49 ± 0.08 bc	4.70 ± 0.15 b	4.32 ± 0.12 c	4.40 ± 0.11 bc
1 g B	5.35 ± 0.02 a	6.58 ± 0.08 b	5.19 ± 0.10 ac	5.47 ± 0.16 a	4.99 ± 0.17 c	5.01 ± 0.18 c

Values are presented as mean ± standard error.
Different notation within a single row indicate significant differences within a single medium.
Control 1, pH of culture media after autoclaving; Control 2, pH after 5 wk ($p \leq 0.05$ ANOVA and *post hoc* least significant difference tests); MMN, Melin-Norkrans agar medium; MS, Murashige and Skoog medium; G, green organic matter; B, brown organic matter plus MMN.

Only two authors [2, 5] have studied the growth of *P. ammoniavirescens* with conflicting results; while Hahn and Agerer [5] observed slow growth (1 cm per month) in yeast malt agar, Jargeat *et al.* [2] reported a faster growth of all isolates on MP medium. This last author hypothesized that this discordance was due to insufficient cultures under analysis. However, the composition of the culture media used was also very different, the MP medium used by Jargeat *et al.* [2] is more complex than the medium used by Hanh and Agerer [5], with numerous microelements and macronutrients of MMN media, which could also explain those divergences in growth rates. Moreover, previous results from our group [23] showed that in a similar medium to that used by Hahn and Agerer [5], the culture medium MEPA [6], a significantly lower growth is observed as compared with other culture media (MMN, BAF Biotin-aneurin-folic acid agar [25], and MFM). In sight of these results, we hypothesise that the differences observed in this species are not only due to genetic variability but could be potentially caused by an inappropriate culture media.

pH variation. Data for Control 1 showed that autoclaving slightly modified the initial pH, as seen by the acidification of the MMN, MS, and 1/2 MS media, which is not attributable to the addition of organic matter (Table 2). Additionally, a significant pH increase is observed after 5 wk in plates without fungus in all culture media with green organic matter and in 1 g B MMN (Control 2).

Our results showed that the addition of green organic matter caused an initial acidification greater than that exhibited by all other culture media. Moreover, in absence of fungi, the pH increased in all culture media with organic matter, with significant differences found in all those containing G organic matter and in 1 g B MMN. Fungal growth produced significant acidification in all media, moreover there were also significant differences among the various areas recorded in all media with B organic matter.

In conclusion, assuming that environmental conditions are stable, pH variations in the culture media, the depletion of some elements or essential substances or even the accumulation of toxic metabolites [24] can affect the growth of *P. ammoniavirescens*. Further, the results above allow us to conclude that culture media MS and 1/2 MS are the most suitable for the growth of *P. ammoniavirescens*. Moreover, *P. ammoniavirescens* was shown to be capable of decomposing fallen leaf litter but not fresh litter. The use of MS and organic matter could potentially help to improve the culture of recalcitrant fungal species.

REFERENCES

1. Wallander H, Söderström B. *Paxillus*. In: Carney JW, Chambers SM, editors. Ectomycorrhizal fungi: key genera in profile. Berlin: Spinger-Verlag; 1999. p. 231-52.
2. Jargeat P, Chaumeton JP, Navaud O, Vizzini A, Gryta H. The *Paxillus involutus* (*Boletales*, *Paxillaceae*) complex in Europe: genetic diversity and morphological description of the new species *Paxillus cuprinus*, typification of *P. involutus* s.s., and synthesis of species boundaries. Fungal Biol 2014;118:12-31.
3. Laiho O. *Paxillus involutus* as a mycorrhizal symbiont of forest trees. Acta For Fenn 1970;106:1-73.
4. Fries N. Intersterility groups in *Paxillus involutus*. Mycotaxon 1985;24:403-10.
5. Hahn C, Agerer R. Studium zum *Paxillus involutus* Formenkreis. Nova Hedwigia 1999;69:241-310.
6. Bormann C, Baier D, Hörr I, Raps C, Berger J, Jung G, Schwarz H. Characterization of a novel, antifungal, chitin-binding protein from *Streptomyces tendae* Tü901 that interferes with growth polarity. J Bacteriol 1999;181:7421-9.
7. Bresinsky A. Observations on mycobiota in Estonia. Folia Cryptogam Est 2006;42:1-9.
8. Hedh J, Samson P, Erland S, Tunlid A. Multiple gene genealogies and species recognition in the ectomycorrhizal fungus *Paxillus involutus*. Mycol Res 2008;112(Pt 5):965-75.
9. Vellinga EC, Blanchard EP, Kelly S, Contu M. *Paxillus albidulus*, *P. ammoniavirescens*, and *P. validus* revisited. Mycotaxon 2012;119:351-9.
10. Marx DH. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infection. I.

Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 1969;59:153-63.
11. Molina R, Palmer JG. Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC, editor. Methods and principles of mycorrhizal research. St. Paul (MN): APS Press; 1982. p. 115-29.
12. Danell E. *Cantharellus cibarius*: mycorrhiza formation and Ecology. Acta Universitatis Upsaliensis, Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 35. Uppsala: Swedish University of Agricultural Sciences; 1994.
13. Colpaert JV, van Tichelen KK. Decomposition, nitrogen and phosphorus mineralization from beech leaf litter colonized by ectomycorrhizal or litter-decomposing basidiomycetes. New Phytol 1996;134:123-32.
14. Fernández R, Bertrand A, Casares A, García R, González A, Tamés RS. Cadmium accumulations and its effect on the *in vitro* growth of woody fleabane and mycorrhized white birch. Environ Pollut 2008;152:522-9.
15. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego (CA): Academic Press; 1990. p. 315-22.
16. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32: 1792-7.
17. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 2012;9:772.
18. Akaike H. A new look at the statistical model identification. IEEE Trans Automat Contr 1974;19:716-23.
19. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 2012;61: 539-42.
20. Tavaré S. Some probabilistic and statistical problems in the analysis of DNA sequences. Lect Math Life Sci 1986;17:57-86.
21. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
22. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 1962;15:473-97.
23. Fernández-Miranda Cagigal E, Alvarado García P, Alonso-Graña López M, Majada Guijo J, Casares Sánchez A. Influencia de las condiciones culturales en el crecimiento de diferentes hongos ectomicorrícicos. Cuad Soc Esp Cienc For 2008;28: 207-11.
24. French ER, Hebert TT. Métodos de investigación fitopatológica. In: de la Cruz M, editor. Serie de Libros y Materiales educativos. Vol. 43. San Jose (CA): Instituto Interamericano de Cooperación para la Agicultura; 1980. p. 47-56.
25. Moser M. Die Gattung *Phlegmacium* (Schleimköpfe). Die Pilze Mitteleuropa, Bd. IV. Bad Heilbrunn: J. Klinkhardt; 1960.