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Spores of ectomycorrhizal fungi as inoculants for *Nothofagus pumilio* and exotic conifer seedlings in Patagonia, Argentina: their activity and conservation

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Abstract Ectomycorrhizal (EM) fungi incorporation in nursery production is necessary for afforestation with exotic tree species and native forests restoration programs. The fact that spore use as EM inocula is inexpensive and effective, makes it an option more realistic than vegetative inoculum production for many regions around the world. To determine spore activity from EM species selected as inoculants for *Nothofagus pumilio* and exotic conifers planted in Patagonia, two conservation methods were applied (i.e., dried fruit bodies and spore slurries), and their change over time was assessed (over 8–9 months). Spore activity decreased significantly with time for both native and exotic EM species. Conservation methods showed no significant differences for *N. pumilio* EM species considered together or for exotic conifer EM species. However, spore activity of different EM species behaved differently with conservation method and over time when considered separately. Taxa which better kept spore activity over time were *Austropaxillus statuum* and *Setchelliogaster fragilis* for *N. pumilio*, and *Inocybe kauffmanii* for exotic conifers. However, considering together fruit body spore density and spore activity, the species *Hallingea purpurea* and *Cortinarius* sp. also appeared as suitable for *N. pumilio* and *Rhizopogon roseolus*, *R. villosulus*, *Suillus luteus* and *S. lakei* for exotic conifers. Spore density found in EM fungal fructifications were also established in order to estimate fructification weights necessary to apply a reference dose of active spores in inoculation programs.

Keywords Ectomycorrhiza · Spore activity · *Austropaxillus* · *Setchelliogaster* · *Suillus* · *Rhizopogon*

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Introduction

Lenga [*Nothofagus pumilio* (Poepp. & Endl.) Krasser] forests in the Patagonian Andes of Argentina, as many other tree species in their natural environment around the world, have undergone degradation processes caused by wildfires occurrence (Estadística de Incendios Forestales 1999–2012; Veblen et al. 2003), extreme sheepherding (Vázquez 2002; Veblen et al. 1996), land use change and inappropriate forest management applied for logging. All these situations are detrimental to the conditions necessary for natural regeneration establishment. For this reason, forest restoration programs have been implemented which required the use of high quality seedlings that may survive under adverse conditions. On the other hand, afforestation with fast-growing conifers such as Ponderosa pine [*Pinus ponderosa* (Dougl.) ex Laws.], Radiata pine (*Pinus radiata* D.Don) and Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] in Patagonia, has been carried out in order to produce timber (Salgado Salomon et al. 2011).

The species involved in these processes in Patagonia, (*Nothofagus* spp. and exotic conifers), form obligate associations with ectomycorrhizal fungi (EM) (Smith and Read 2009). Mycorrhizal fungi help plants to increase nutrients and water capture, thus improving their establishment and growth, as well as contributing with soil structure restoration and nutrient-cycle re-establishment (Jasper et al. 1987). Since these conifers are established on grasslands with no EM species (Fontenla et al. 1998), and because altered native forests lose their EM inocula due to degradation, afforestation and restoration activities aim to introduce and/or to re-establish them (Pera and Parladé 2005; Valenzuela Flores 1993). The simplest and generally more appropriate time to incorporate the EM inocula is during plant production at the nursery.

Inoculation with EM should be done through the application of either vegetative inoculum (mycelium) or spores, as the addition of soil or duff with EM propagules has shown low mycorrhization percentages (Barroetaveña and Rajchenberg 2003), has a high risk of pathogen introduction, and does not allow for more appropriate EM species selection (Castellano and Molina 1989). Vegetative inoculum has been reported as being more convenient (Brundrett et al. 1996, 2005; Kuek 1994; Trappe 1977), but its production is difficult and expensive to be carried out. For these reasons, spores are frequently used for inoculation programs, as they do not need a pure cultivation phase as mycelia, which require expensive infrastructure and qualified personnel for their production (Brundrett et al. 2005; Rincón et al. 2007). In addition to this, spores volume is lower, they tolerate storage periods, are very abundant in fungal fruiting bodies and are easy to apply. The main disadvantage relies on their reduced availability, being strictly dependent on fruiting bodies formation subjected to seasonal and yearly climatic variations. Spore inoculation is carried out by watering seedlings with a spore suspension prepared by blending fresh or dried mature fruiting bodies (Brundrett et al. 2005; Parladé et al. 1996).

The time of EM inoculation is always complex if high doses of fertilizer are applied (mainly N and P), as symbiosis establishment is inhibited (Chakravarty and Chatarpaul 1990; Gagnon et al. 1988; Martínez et al. 2007). Spore inoculation before sowing has been frequently used for exotic conifer production (Marx et al. 1984; Theodorou 1984). However, if periods of high nutrient concentration due to ferti-irrigation are to be avoided, good results could be obtained if inoculation is applied during the hardening phase (Martínez et al. 2007). This delay implies the need for inocula storing at least in regions like Patagonia, which has marked seasonality of EM fungal fruiting bodies production. Spore viability (defined by active plus dormant spores) has been reported as negatively affected by time of storage, while reduction intensity varies according to the species

(Miller et al. 1993; Torres and Honrubia 1994). Therefore, it is necessary to establish techniques that allow for maximizing spore viability under storage conditions and to know the differences in species behaviour.

The objectives of this work were: (a) to evaluate the change of spore activity from selected EM species for *N. pumilio* and exotic conifers over time, using two conservation methods and, (b) to estimate the weight of fruit bodies from selected EM species for *N. pumilio* and exotic conifers necessary to inoculate a reference dose of active spores.

Methodology

Selection of EM species

Selection of candidate EM species to be used as spore inoculum considered the following criteria (Barroetaveña 2004): (a) collection facility, determined by size and fruiting habit, (b) fruiting abundance, determined by finding frequency, and size of collections, (c) fruiting period throughout the year, (d) spore density (spores per unit of fruiting body weight, considering complete fruit bodies for hypogeous species, and only the pileus for epigeous or sub-hypogeous species), and (e) tree species reported as hosts (Table 1).

The EM candidate species to inoculate lenga were defined from preliminary surveys (Bassani 2010) of reported EM species (Gamundi and Horak 1993; Garrido 1988; Horak 1980; Singer 1969; Valenzuela Flores 1993):

Austropaxillus statuum (Speg.) Bresinsky & Jarosch [Bas.: *Agaricus statuum*. Syn.: *Paxillus statuum* (Speg.) E. Horak].
Setchelliogaster fragili s (Zeller & C.W. Dodge) E. Horak (Bas.: *Hymenogaster fragilis* Zeller & C.W. Dodge)
Hallingea purpurea (Zeller & C.W. Dodge) Castellano (Bas.: *Hysterangium purpureum* Zeller & C.W. Dodge)
Cortinarius xiphidipus M.M. Moser & E. Horak
Cortinarius sp. (Bas. ***Thaxterogaster)***
The EM candidate species to inoculate exotic conifers were those proposed by Barroetaveña et al. (2005, 2006):
Suillus luteus (L.) Roussel (Bas.: *Boletus luteus* L.)
Suillus lakei (Murrill) A.H. Sm. & Thiers (Bas.: *Boletus lakei* Murrill)
Rhizopogon roseolus (Corda) Th. Fr. (Bas.: *Splanchnomyces roseolus* Corda)
Rhizopogon villosulus Zeller
Tricholoma muricatum Shanks
Hebeloma mesophaeum (Pers) Quél. (Bas.: *Agaricus fastibilis* var. *mesophaeus* Pers.)
Inocybe kauffmanii A.H. Sm.

Inocula preparation and storage

Collections of EM native species were obtained from a lenga forest located within the "Huemules" area (Esquel, Chubut, Argentina: 42°50′S and 71°27′W) in 2 forays during fall 2008, while those associated with exotic conifers were obtained from a mixed Douglas fir, ponderosa pine and radiata pine plantation located at the "Río Azul" footbridge area (Lago Puelo, Chubut, Argentina: 42°04′S and 71°38′W), during the same season.

Table 1 Features of EM fungi selected as potential inoculants for *Nothofagus pumilio* (*N. pumilio*) and exotic conifers (*E. conifers*)

	Species	Ease of collection	Fruiting abundance	Fruiting period (months)	Associated tree species	Spores/gr fresh fruitbody ($\times 10^6$)[a]
N.pumilio	*Austropaxillus statuum*	Easy (epigeous)	High	2	1, 2, 3, 4, 5, 6	2.18
	Setchelliogaster fragilis	Easy (sub-hypogeous)	Medium	3	1, 2, 3, 4, 5	9.69
	Hallingea purpurea	Hard (hypogeous)	Medium to low	3	1, 3, 6	61.44
	Cortinarius xiphidipus	Easy (epigeous)	Low	3	1, 2, 3, 4, 5, 6	1.28
	Cortinarius sp.	Hard (hypogeous)	Low	1	1	22.34
E. conifers	*Rhizopogon villosulus*	Hard (hypogeous)	Medium	3	8	247.5
	Rhizopogon roseolus	Hard (hypogeous)	Medium	3	7	561.5
	Suillus lakei	Easy (epigeous)	High	3	8	7.52
	Suillus luteus	Easy (epigeous)	High	3	7	10.15
	Hebeloma mesophaeum	Easy (epigeous)	High	2	7, 8	1.97
	Inocybe kauffmanii	Easy (epigeous)	Medium	3	7, 8	5.32

References: (1) *N. pumilio*, (2) *N. antarctica*, (3) *N. dombeyi*, (4) *N. obliqua*, (5) *N. alpina*, (6) *N. betuloides*, (7) *Pinus* spp., (8) *P. menziesii*

[a] For epigeous species the stem was not included in weight of fruitbodies

All collections were separated into two groups in order to apply two conservation techniques: (a) suspensions, and (b) drying. Fractions of fruiting bodies of each collection were separated into two groups to reduce variation in spore amount due to different sizes and maturity stages. For epigeous and sub-hypogeous species, only the pileus was used; for hypogeous fungi whole fruiting bodies were used. All material was carefully cleaned with distilled water using a soft brush.

Suspensions were prepared with fresh and clean fruiting bodies; a given weight for each collection was ground and homogenized with a mixer in a variable volume of distilled water, a multiple of the weight used, to obtain suspensions with similar densities. Three replicates were prepared for each species (corresponding to 3 different collections), and stored in a refrigerator at 4 °C during the study. For drying, the specimens of the same three collections of each species were taken to an oven at 40 °C for 48 h. After doing this, they were kept in paper bags and stored under the same conditions as suspensions.

Spore activity measurement

Spore activity was assessed by staining with Blue Tetrazolium Bromide Thiazolyl (An and Hendrix 1988; An et al. 1998; Walley and Germida 1995), which indicates respiratory activity. All selected species were assessed under both conservation methods at 6 different times: 30, 60, 90, 120, 160 and 270 days for *N. pumilio* EM, and 30, 60, 90, 120, 140 and 240 days for exotic conifer EM. The last three measuring times were defined considering the decrease of nutrients (N and P) in the ferti-irrigation regime used by the forest nursery at INTA Trevelin Experimental Station (Trevelin, Chubut, Argentina: 43°06′S and 71°33′W) for *N. Pumilio*, and by the forest nursery at Universidad Nacional de la Patagonia S.J. Bosco (Esquel, Chubut, Argentina: 42°54′S and 71°19′W) for exotic conifers.

Determination of active spores by means of Blue Tetrazolium Bromide Thiazolyl staining

At each assessment, equal parts of Blue Tetrazolium Bromide Thiazolyl (prepared 5 % in distilled water) and 0.5 ml of spore suspension were mixed in a test tube. For the suspensions, the aliquot was taken directly from the bottles, while for dry collections suspensions were prepared spontaneously by grinding collections in a mortar and re-hydrating them with distilled water. Then, the tubes were covered and held in a culture chamber for 48–72 h at 28 °C (±26–29 °C). The evaluation was performed three times for each sample by placing a drop on a slide under a light microscope at 1,000×, counting 300 spores per sample. Red stained spores were considered active, blue stained ones were considered non-active and non stained spores (dormant) were a priori considered as not active. Percentages of each type were registered.

Experimental design and statistical analysis

To determine significant differences in spore activity due to storage time and conservation method, a factorial design with 2 factors: storage time (with 6 levels) and conservation method (2 levels), was applied. Different fungal species were used as replicates. A statistical analysis was performed using repeated measures ANOVA (time being the repeated measure factor) with a significance level of 0.05, using SPSS for Windows v. 17.0. The value taken for each time and species was the average of 3 repetitions performed. It was

confirmed that the data fulfilled the assumptions of normality and homoscedasticity using the Shapiro–Wilk method and Levene's test (Steel and Torrie 1988), both with a significance level of 0.05.

Differences in spore activity between species after 30 days storage (time chosen to indicate immediate use of inocula), and after 270 days (time chosen to indicate utilization during hardening) were detected with one way ANOVA and Tukey's test for multiple comparisons (in this case, the three suspensions for each species were used as repetitions) with a 0.05 significance level. When the normality and/or homoscedasticity assumptions were not met, data transformations were tested and, as a last resort, nonparametric analyses (Kruskal–Wallis test) were performed.

Spore density in the fruiting bodies and calculations for inoculations

Three counts of total spores for each of the 3 suspensions of each species were performed using a hemacytometer under a light microscope with 100 × magnification. Knowing the dilution factor of the suspensions, the concentration (spores/ml) was calculated and then transformed to spore density (spores/g of fresh fruit body) using the following formula:

$$\text{spores/g} = \frac{(\text{spores/ml}) \times (\text{prepared volume (ml)})}{(\text{fresh weigth utilized (gr)})}$$

The fresh weight (g) of each species necessary to inoculate 1,000 seedlings was calculated considering a reference dose of 10^6 active spores, as proved to be the most recommendable to be used with different conifer species and *Eucalyptus* spp. (Chen et al. 2006; Parladé et al. 1996; Pera and Parladé 2005; Rincón et al. 2007). Calculations considered active spores after 30, 120 and 270 days of storage, with the following formula:

$$\text{necessary fresh weigth} = \frac{\left(1 \times 10^6 \text{ active spores}\right) \times 1{,}000 \text{ seedlings}}{(\text{active spores/g})}$$

where: $\text{active spores/g} = \frac{(\text{spores/g}) \times \text{activity } \%}{100\,\%}$

Results

Spores of EM species for both *N. pumilio* and exotic conifers showed significant decrease in their respiratory activity with storage time ($p < 0.0001$ repeated measures ANOVA), showing after 30 days significantly higher spores activity compared to the following evaluation times ($p < 0.05$ for *N. pumilio* EM species and $p < 0.0001$ for exotic conifer EM species). Spores activity between the other evaluations times also showed significant differences ($p < 0.05$), indicating that storage time negatively affected spore activity. The average spore activity for *N. pumilio* EM species was 83.2 % after 30 days and 19.11 % after 270 days for suspensions, and 82.6 % and 18.9 % for dried materials, respectively (Fig. 1a), while for the exotic conifer EM species was 72.8 % after 30 days and 5.87 % after 240 days for suspensions, and 66.8 % and 6.92 % for dried materials, respectively (Fig. 1b). *Tricholoma muricatum* was excluded from statistical analysis as it presented 0 % activity from the beginning of the evaluation. Conservation methods applied did not show significant differences ($p = 0.219$ for *N. pumilio* EM species, and $p = 0.067$ for exotic conifer EM species).

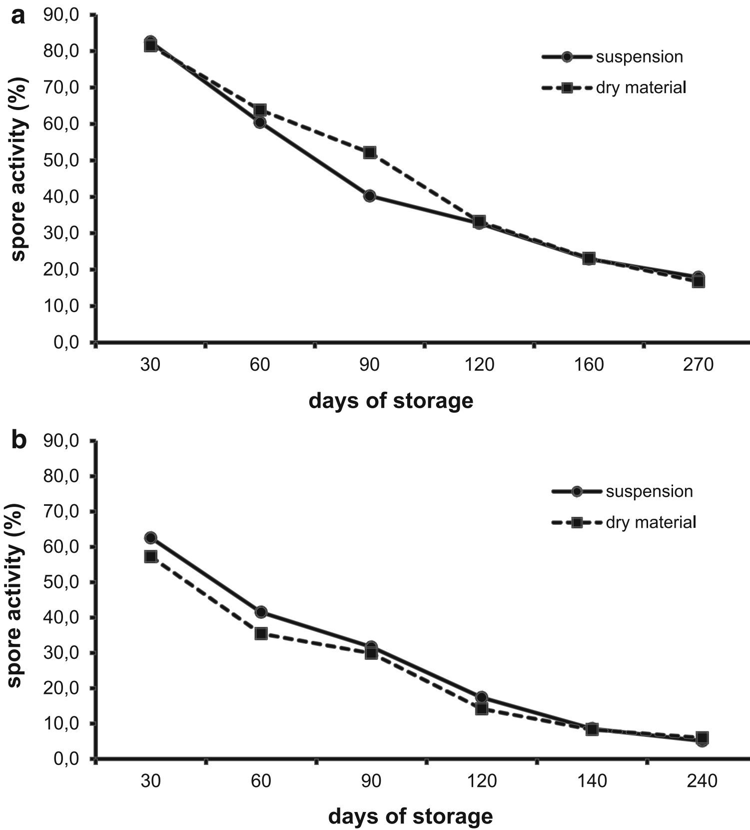

Fig. 1 Evolution of mean spore activity with storage time under both conservation methods, **a** for *N. pumilio* EM species and **b** for exotic conifer EM species

Comparison of spore activity among several EM fungi suitable for *N. pumilio* mycorrhizal infection after 30 and 270 storage days

For suspensions after 30 days, significant differences were found only between *Cortinarius* sp. (mean = 91.9 %), with the highest value, and *Hallingea purpurea* (mean = 60.8 %) with the lowest value ($p = 0.017$ Kruskal–Wallis non-parametric ANOVA), (Fig. 2a). Under dry storage, significant differences were also detected after 30 days ($p < 0.001$ one way ANOVA), *Austropaxillus statuum* (mean = 93.3 %) and *Cortinarius* sp. (mean = 93.1 %) showing significantly higher activity, means being 11.03–31.7 % higher than the others. *Hallingea purpurea* (mean = 61.6 %) showed the lowest spore activity (Fig. 2b).

Spores activity among species for suspension after 270 days showed significant differences ($p = 0.002$ one way ANOVA), *Cortinarius* sp. (mean = 25.9 %) again being the species with significantly higher values compared to *H. purpurea* and *A. statuum* (Fig. 2c). Other species, with means from 11.57 to 19.18 %, did not differ significantly from each other. The dry material also showed significant differences between species after 270 days

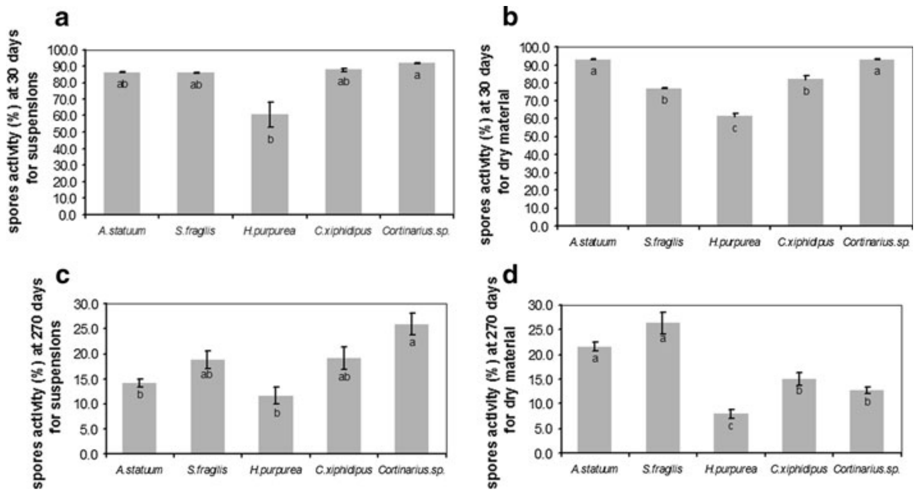

Fig. 2 Mean spore activity with ±1 standard error for *N. pumilio* EM species preserved in suspension (**a**, **c**) or dry (**b**, **d**), after 30 (**a**, **b**) and 270 (**c**, **d**) days of storage. *Different letters* indicate significant differences with $p < 0.05$ from Tukey's test for 3b, 3d and 3c, and from Kruskal–Wallis test for 3a

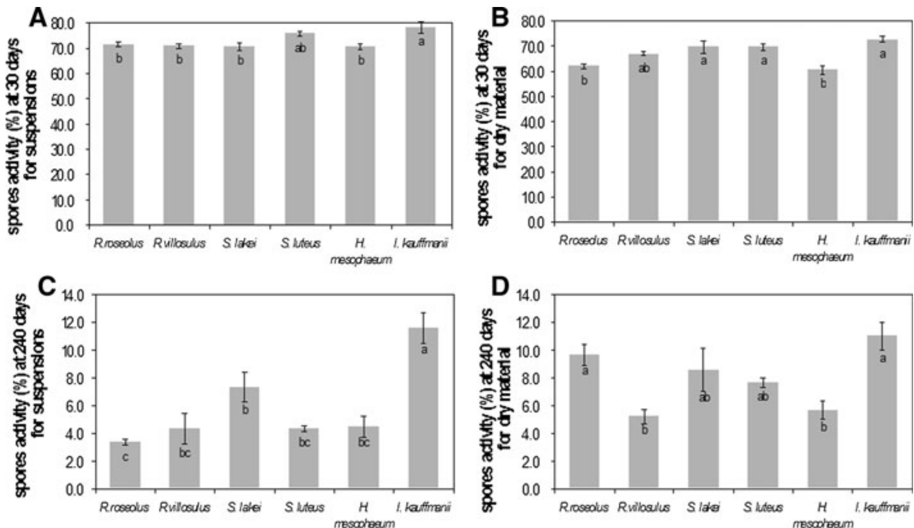

Fig. 3 Mean spore activity with ±1 standard error for exotic conifer EM species preserved in suspension (**a**, **c**) or dry (**b**, **d**) after 30 (**a**, **b**) and 240 (**c**, **d**) days of storage. *Different letters* indicate significant differences with $p < 0.05$, Tukey's test

of storage ($p < 0.001$ one way ANOVA), but in this case *Setchelliogaster fragilis* (mean = 26.33 %) and *A. statuum* (mean = 21.57 %) showed the highest activity. Among the remaining species, *Cortinarius xiphidipus* (mean = 15 %) had a significantly higher value than *H. purpurea* (mean = 7.97 %) (Fig. 2d).

Comparison of spore activity among several EM fungi suitable for exotic conifers mycorrhizal infection after 30 and 270 days of storage

Differences in spore activity among species preserved in suspensions at 30 days were significant ($p = 0.006$ one way ANOVA), *I. kauffmanii* (mean = 78.3 %) showing significantly higher activity as compared to all but *S. luteus*, while the remaining species (with means between 75.7 and 70.3 %) did not differ significantly from each other (Fig. 3a). Under dry storage, significant differences were detected ($p < 0.001$ one way ANOVA). Values for *I. kauffmanii* (mean = 72.6 %), *S. luteus* (mean = 69.6 %) and *S. lakei* (mean = 69.6 %) were significantly higher than those for *Rhizopogon roseolus* (mean = 61.7 %) and *Hebeloma mesophaeum* (mean = 60.3 %) (Fig. 3b). Differences at 270 days were significant for both storage methods ($p \leq 0.005$ one way ANOVA). When preserved in suspension *I. kauffmanii* (mean = 11.6 %) was again the species with the highest activity. The other four species, with means between 3.3 (*R. roseolus*) and 7.3 % (*S. lakei*), did not differ among themselves, except for *R. roseolus* with *S. lakei* (Fig. 3c). Under dry storage conditions, *I. kauffmanii* (mean = 11 %) was again the species with the highest spore activity, significantly higher than *R. villosulus* and *H. mesophaeum* (mean = 5.2 and 5.7 %, respectively). *R. roseolus* (mean = 9.7 %) was significantly higher than *R. villosulus*, while the other species, with averages from 5.2 to 8.6 %, did not differ from each other (Fig. 3d).

Spore density in *N. pumilio* EM species and calculations for inoculations

Data of fruiting body spore density for each *N. pumilio* EM species are presented in Table 1, while fresh weight of fruit bodies necessary to inoculate 1,000 seedlings after different storage times are presented in Table 2. *Hallingea purpurea* was the one with the highest spore density, requiring a lower weight of fruit bodies for inoculation, even though after 270 days of storage it had the strongest activity decrease compared to the other species. It was followed by *Cortinarius* sp. and *S. fragilis*, even though they had 3 and 6 times less spores per gram. *Austropaxillus statuum* and *C. xiphidipus* had 28 and 48 times less spores per gram than *H. purpurea*. If spore density and loss of spore activity over time is considered, *H. purpurea* required the lowest weight of fruit bodies, followed by *Cortinarius* sp. and *S. fragilis* at all testing times.

Table 2 Fresh weight of fruiting bodies needed to inoculate 1,000 seedlings with a reference dose of 10^6 active spores, at 30, 120 and 270 days of storage for *Nothofagus pumilio* EM species

Species	Fresh weight (gr) at 30 days	Fresh weight (gr) at 120 days	Fresh weight (gr) at 270 days
Austropaxillus statuum	533[a]/493[b]	1,344/1179	3,260/2,128
Setchelliogaster fragilis	120/134	372/293	549/392
Hallingea purpurea	26.8/26.4	78.3/76.4	140/203
Cortinarius xiphidipus	891/952	2,587/2,339	4,090/5,208
Cortinarius sp.	48.7/48.1	88.1/120	173/350

[a] Calculation using spore activity for suspensions

[b] Calculation using spore activity for dry materials

Table 3 Fresh weight of fruiting bodies needed to inoculate 1,000 seedlings with a reference dose of 10^6 active spores after 30, 120 and 240 days of storage for exotic conifer EM species

Species	Fresh weight (gr) at 30 days	Fresh weight (gr) at 120 days	Fresh weight (gr) at 240 days
Rhizopogon villosulus	5.71[a]/6.04[b]	24.9/27.7	94.0/77.7
Rhizopogon roseolus	2.49/2.89	11.0/15.4	54.0/29.2
Suillus lakei	188/191	570/586	1,821/1,546
Suillus luteus	130/142	448/632	2,291/1,247
Hebeloma mesophaeum	722/843	4,932/6,196	11,550/8,910
Inocybe kauffmanii	240/259	583/754	1,619/1,707

[a] Calculation using spore activity for suspensions

[b] Calculation using spore activity for dry materials

Spore density in exotic conifer EM species and calculations for inoculations

Data of fruiting body spore density for each exotic conifer EM species are presented in Table 1, while the fresh weight necessary to inoculate 1,000 seedling after different storage times are presented in Table 3. The species with the highest spore density were *R. roseolus* and *R. villosulus* (though having 2 times less spores/gram than *R. roseolus*), followed by *S. luteus* and *S. lakei* (having 55 and 75 times less spores/gram than *R. roseolus* respectively). *Inocybe kauffmanii* and *H. mesophaeum* presented the lowest spore density (105 and 285 times less spores/gram than *R. roseolus*, respectively). If considered together spore density and loss of spore activity over time, *R. roseolus* and *R. villosulus* required the lowest fruit body weight at all testing times, followed by both *Suillus* species.

Discussion

Spore use as EM inocula for seedling production, either for afforestation with exotic tree species or for native forests restoration programs, is a more realistic option *vis à vis* the use of axenic mycelium in regions as Patagonia. The scale of these activities in many parts of the world hardly warrants the existence of a company committed to produce axenic inocula. Therefore, the spore storage possibilities as well as the amount of fruiting bodies needed for inoculation programs constitute basic information necessary to promote their use in nurseries.

There are few studies addressing EM spore viability (Miller et al. 1993; Torres and Honrubia 1994). Tetrazolium salt, widely used to determine proportions of active hyphae (Hamel et al. 1990; Schubert et al. 1987; Sylvia 1987, 1990) endogonaceous spores viability (An and Hendrix 1988; An et al. 1998; Walley and Germida 1995) was found to be optimal for the assessment of spore activity since wall-color changes allow for correctly distinguishing live from dead spores. It was noted, however, that, over time, different species responded differently to staining, a fact that could be due to differences in spore wall thickness and coloration. Another staining technique such as fluorescent staining with diacetate (Torres and Honrubia 1994) allows for distinguishing between active and dormant spores, but it is more expensive, requires the use of an epifluorescent microscope, and

does not add valuable information for nursery inoculation planning, as only active spores are considered when defining inoculum dose. Moreover, a recent paper (Barroetaveña et al. 2012) is in agreement with results presented in this paper, showing that inoculation with spores after approximately 30 days of storage failed to form mycorrhizas with *T. muricatum*, but it was successful with *S. luteus*, *R. roseolus* and *H. mesophaeum*.

This study shows that spores activity decreased significantly over time for both native and exotic EM, in agreement with results from Torres and Honrubia (1994) for EM species of *Pinus* spp. from Spain. Thus, spore inocula maximize its performance when used as fresh as possible. When inoculation has to be done at a time different from that at fruit body collection, it is necessary to select the species that best preserve spore activity and to calculate the amount of fruiting bodies needed.

Conservation methods showed no significant differences, for *N. pumilio* EM species considered together or for exotic conifer EM species considered together. These methods were chosen because they had been reported as being effective, simple to implement, and inexpensive (Brundrett et al. 1996). Collections kept through drying did not present any problem of putrefaction or physical impairment in any case, but suspensions did show these drawbacks occasionally. Therefore, it is unclear when any of these preservation methods based on spore behavior with storage should be chosen, but the fact that dry material needs less room, requires less processing for storage and never presents putrefaction should be considered.

However, spore activity of different EM species behaved differently with conservation method and over time when considered separately. In suspension, *Cortinarius* sp. always showed high activity values, 90 % higher than the activity after 30 days reported by Torres and Honrubia (1994) for a *Cortinarius* sp. that completely lost activity after 180 days of storage. However, dried *Cortinarius* sp. had high values together with *A. statuum* after 30 days, losing significant activity after 270 days if compared to *S. fragilis* and *A. statuum*. This hypogeous *Cortinarius* could not be determined at species level with the keys available in the region (Gamundí and Horak 1993; Garrido 1988; Horak 1980; Singer 1969; Valenzuela Flores 1993), so it might be a new species that will be the subject of a separate study. Both *S. fragilis* and *A. statuum* are readily collected species, are present in average abundance, have good spore densities for their fruiting body size, and frequently occur all along the fall season. Also, they are species recorded throughout the range of *Nothofagus* distribution in Argentina, and associated with most *Nothofagus* species (Castellano and Muchovej 1996; Gamundí and Horak 1993; Horak 1980; Singer 1969; Valenzuela Flores 1993). Garrido (1988), following tests of mycorrhizal synthesis, quoted *A. statuum* as a typical pioneer symbiont to all species of *Nothofagus* in southern Chile, and recommended it as an inoculant for reforestation of badly eroded sites in that country. Among the exotic conifer EM species, *I. kauffmanii* consistently showed significantly higher spore activity than the other taxa under both preservation methods. This species showed 75.4 % more activity than the *Inocybe* sp. reported by Torres and Honrrubia (1994) after 30 days of storage, but its activity decreased with time while for the Spanish species activity increased up to 28.9 % after 180 days of storage. *Suillus luteus* and *S. lakei* had significantly higher activity after 30 days kept in dried state, but decreased considerably after 270 days, whereas *R. roseolus* closely mimicked the occurrences of *I. kauffmanii*. Comparing our values with those reported by Torres and Honrubia (1994), activity after 30 days storage of our *R. roseolus* collections were 67.4 % higher, while our values after 240 days were very similar to theirs after 180 days. Miller et al. (1993) presented highly variable activity measurements for two other *Rhizopogon* species. Our *Suillus* species showed values similar to *Suillus brevipes* (Peck) Kuntze (Miller et al. 1993) but much

higher compared with those presented for other *Suillus* species (between 0.1 and 38.5 % activity) by Torres and Honrubia (1994) after 30 days of storage; after 180 days only *S. granulatus* (L.) Roussel maintained activity (Torres and Honrubia 1994), within the range of our data. These results indicate that: (a) the selection of species for EM inoculation should take into account the time of inoculum application and, (b) the best conservation method depends on the species of fungus to work with. In addition, exotic conifer EM species showed, in general, greater loss of activity than species with *N. pumilio* EM. Other consideration when selecting EM species for exotic conifers, especially when plantations are to be established close to native *Nothofagus* forest, is that species with broad host range, as *H. mesophaeum* and *I. kauffmanii* which present some angiosperm hosts, have potential as weeds, competing with native EM fungi from *Nothofagus*. Some taxa such as *Hallingea* for *Nothofagus* spp., *Tricholoma* for *Pinus* (Barroetaveña et al. 2012; Torres and Honrubia 1994) and *Amanita muscaria* (L.) Lam. (Miller et al. 1993) lose activity in a very short time, and are not good for this type of inoculation, except when used immediately or when their very high spore density justify storing them.

The weight of fresh material needed for inoculation programs with these species, as calculated from the spore counts, can be collected during a typical fall (not extremely dry). Certainly, the later the inocula are used the greater the amount of material needed. Generally, the hypogeous EM taxa require less amount of fruiting bodies due to their high spore density, and it is also very practical to prepare inoculum from them as the whole fruit body can be used, with no need to separate either the stem or context. However, the difficulty in finding them (i.e., about 10 cm of ground need to be removed) and their sometimes small fruiting yield reduce their suitability. Exotic EM species are more abundant and easy to find and collect because of the proximity of plantations to most forest nurseries in Patagonia and also due to the relatively low number of EM taxa present (Barroetaveña et al. 2005).

While these results indicate that the use of inocula with less storage time maximizes their performance, inoculation time must adapt to the general management of the nursery and the ferti-irrigation dose and schedule. In Patagonia, the most promising option is to inoculate seedlings from March to May, when most EM species fruit, and when ferti-irrigation doses have been diminished as it is the hardening period. Thus, seedlings still have one or 2 months in the nursery to establish the symbiosis before reaching the field. Another option already mentioned is to inoculate in the field when planting in fall time, when the spores have high activity levels. This avoids the negative impact of ferti-irrigation, but may have the disadvantage that plants without the symbiosis established could suffer post-planting stress, especially in places with low rainfall or nutrient availability, or in years of extreme climatic conditions.

Conclusions

The percentage of active spores decreases very rapidly in both storage conditions, so it will be difficult to keep this kind of inoculum from one year to another. This inoculation method is suitable if inoculation takes place soon after spore harvesting. Ectomycorhizal species for *N. pumilio* that better keep spore activity over time were *Austropaxillus statuum* and *Setchelliogaster fragilis*, preserved under both storage methods. But, considering together spore density and loss of spore activity over time, *Hallingea purpurea* required the lowest fruit body weight, followed by *Cortinarius* sp., and *S. fragilis* at all testing times. Taking into consideration fruit body abundance and facility of collection in the analysis,

A. statuum should be considered as a very good option. Validation of EM in terms of species persistence and ability to improve seedling development need to be tested in the field, since little is known regarding how the mycorrhizal symbiosis varies according to growth stages of *Nothofagus* and to the conditions of the site to be reforested.

Following the same criteria among the exotic conifers EM species, *I. kauffmanii* was the one that best kept spore activity over time under both storage methods, followed by both *Suillus* species. But, considering together spore density and loss of spore activity over time, *R. roseolus* and *R. villosulus* required the lowest fruit body weight, followed by both *Suillus* taxa. It should be noted that both *S. luteus* and *S. lakei*, commonly known as "pine mushroom", are edible species that generate income to the local economy, so seedlings to be inoculated should carry spores of these species in order to ensure that plantations will have these resources in the future.

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