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Effect of carbon and nitrogen sources, pH and temperature on in vitro culture of several isolates of *Amanita caesarea* (Scop.:Fr.) Pers.

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Abstract Several isolates were obtained from sporocarps of *Amanita caesarea* (Scop.: Fr.) Pers. associated with *Quercus suber* and *Castanea sativa* coming from the southwest of Spain. Culture conditions were optimized for these isolates. The largest radial growth was obtained at pH 6–7, and optimal growth temperature was 24–28°C depending on the isolate. Albumin bovine and nitrate produced the largest patch size diameters, but the greatest mycelium dry weight yields were obtained with ammonium. Mannitol produced the largest radial growth, and mannitol and glucose yielded the biggest mycelium dry weights. Although variations in growth behaviour between isolates were observed, only one internal spacer sequence–restriction fragment length polymorphism type was obtained.

Keywords *Amanita caesarea* · Ectomycorrhiza · In vitro culture · ITS sequences

Introduction

Amanita caesarea (Scop.: Fr.) Pers. is a delicious edible species known as Caesar's mushroom (Yun and Hall 2004). It forms ectomycorrhizas with several host plants, including *Pinus strobus*, *Pinus virginiana*, *Castanea sativa*, *Castanopsis carlesii*, *Castanopsis hystrix*, *Fagus sylvatica*, *Quercus baronii*, *Quercus faginea*, *Quercus liaotungensis*, *Quercus lusitanica*, *Quercus petraea*, *Quercus pubescens*, *Quercus pyrenaica*, *Quercus robur* and *Quercus suber* (González et al. 2002; Meotto et al. 1997; Trappe 1962; Yang et al. 1999). In the southwest of Spain *Q. suber* and *C. sativa* are the main hosts of *A. caesarea*. The cork tree wood are very extensive and show social and economical relevance in our region by the exploitation of virgin cork and the utility of their acorns as fodder for the Iberian pigs. *A. caesarea* is a thermophilic species which, in our climatic conditions, appears early in autumn, from October to middle November when it is simultaneously temperate and rainy. Occasionally, if it rains in spring, there is a sporocarp outbreak in spring–summer. *A. caesarea* generally grows in different soil texture with slightly acidic pH close to 6–6.5. Superficial tilling favours its fructification as what frequently occurs in chestnut tree orchards. In spite of the forestry interest of many of *A. caesarea* mycorrhizal plants, there are few field studies (Meotto et al. 1999) and there are no cultural conditions specified for *A. caesarea* (Yang et al. 1999).

Different ecological and physiological factors affect the growth of mycorrhizal fungi and also mycorrhiza synthesis and function in nature (Bowen 1994). Physical characteristics and nutrients of soils, especially carbon and nitrogen sources, greatly influence the establishment and growth of ectomycorrhizal fungi in the field and also in controlled conditions in the laboratory (France and Reid 1983; Lilleskov et al. 2002). Different authors have analysed the influence of nitrogen (Baar et al. 1997; Chalot and Brun 1998; Dickie et al. 1998; Finlay et al. 1992; Rangel-Castro et al. 2002; Sarjala 1999), carbon (Ferry and Das 1968; Lamb 1974; Palmer and Hacskaylo 1970), phosphorus (Sawyer et al. 2003a), temperature (Hacskaylo et al. 1965; Sánchez et al. 2001), pH (Hung and Trappe 1983) and water stress (Machado and Bragança 1994; Mexal and Reid 1973) on the growth of ectomycorrhizal fungi. In this work, we studied the effect of different carbon and nitrogen sources, pH and temperature on the growth of four different strains of *A. caesarea* to improve growth in pure culture and to check differences among them. Since *A. caesarea* is a very difficult species to culture in vitro, we performed nuclear rDNA restriction fragment length polymorphism (RFLP) analysis to confirm identity of the mycelium grown on Petri dish and also to check if there are intraspecific variations.

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Materials and methods

Sporocarp isolation and culture conditions

Sporocarps were collected in the southwest of Spain in autumn 1999. Mycelial isolates were obtained by propagating pieces of the fruit-body caps on Modified Melin Norkrans medium (MMN; Marx 1969) and subcultured every 6 months (Molina and Palmer 1982). Strains used in this work were CT1 and CT3 isolated in the province of Huelva from *C. sativa* and CT4 and ZL1 isolated in the province of Sevilla from *Q. suber*. Culture medium for characterization studies was a modified MMN (named MMN*) containing per liter of the following: 20 g glucose, 3 g malt extract, 1 g yeast extract, 0.05 g $CaCl_2$, 0.025 g NaCl, 0.25 g $(NH_4)_2HPO_4$, 0.012 g $FeCl_3$, 0.003 g thiamine, 1 g KH_2PO_4, 0.150 g $MgSO_4 \cdot 7H_2O$, 20 g agar, pH 6.0. Small Petri dishes (5.5 cm in diameter) were used. Agar plugs of 5-mm diameter cut from the actively growing margin of subcultures were put at the centre of Petri dishes and used as starting inoculum in the experiments. All cultures were sealed with parafilm and incubated in the dark at 24°C. Mycelial dry weight and/or colony growth diameter were measured. To measure dry weight, Petri dish agar media were covered with a cellophane sheet (no. 1650963, Bio-Rad), over which, the fungal mycelium was grown (Guerin-Laguette et al. 2000). Mycelium was separated from cellophane by immersion in distilled water and weighed after drying at 85°C during 12 h. Seven replicates were used in each experiment. pH experiments were performed on MMN* (containing mannitol instead of glucose). The pH values (5, 6, 7 and 7.5) were adjusted with KOH or HCl prior to autoclaving and were verified after sterilization. Colony diameters were measured after 40 days in the dark at 24°C. To study the influence of temperature on *A. caesarea* growth, inoculated MMN* (mannitol) was incubated during 40 days in the dark at 20, 24 and 28°C. Growth was estimated as colony diameters. Albumin bovine (fraction V), $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ were assayed as nitrogen sources. Nitrogen sources (116 mg N/l) were added to MMN* (mannitol) minus $(NH_4)_2HPO_4$. Two controls with no nitrogen source were included. Colony diameters and mycelial dry weights were measured after 40 days at 24°C. The following carbon sources (20 g/l) were added to MMN* (without glucose) solid medium: starch, glucose, mannitol, fructose, xylose and sucrose. Controls with no carbon source were included. Strains were grown in the dark during 40 days at 24°C. Colony diameter and mycelial dry weight were measured.

PCR-RFLP analyses

DNA extraction, polymerase chain reaction (PCR) and RFLP analyses were performed according to Gardes and Bruns (1996) using sporocarps and fresh mycelia grown on MMN* agar medium covered with a cellophane sheet. The internal spacer sequence (ITS) region of the rDNA was amplified using the primer pair ITS-1f and ITS-4b. RFLP patterns were generated with *Alu*I, *Rsa*I and *Hin*fI, and the restriction fragments were separated by electrophoresis on the 2% agarose gels. Horizontal electrophoresis was carried out in 0.5× Tris–acetate–EDTA buffer, and gels were stained with ethidium bromide.

Table 1 Growth of *A. caesarea* strains at different pHs[a]

Strain	Colony diameter (cm)				
	pH				
	5	6	7	7.5	LSD
CT1	2.26 a	2.32 a	1.71 b	1.67 b	0.315
CT3	2.60 ab	2.76 ab	2.87 a	2.54 b	0.325
CT4	2.47 b	2.77 b	3.74 a	2.50 b	0.550
ZL1	3.73 ab	4.07 a	3.34 b	2.50 b	0.443

LSD Least significant difference
[a]Values are means of seven replicates. Means followed by the same letter across rows are not significantly different at $P<0.05$. Strains were grown on MMN* (mannitol) for 40 days at 24°C

Statistical analyses

Statistical analyses were performed using Statistix software (version 1.0, NH Analytical Software, USA). Analysis of variance was used to analyse the experimental data. If the test was significant (expressed at the $P<0.05$ level) and more than two treatments were involved, the Duncan test was used for mean comparisons.

Results and discussion

Since there were no previous data on in vitro growth of *A. caesarea*, we used MMN medium (Marx 1969) as a starting point in this study. A modified MMN medium containing 2% carbon source, instead of 1%, and supplemented with 1 g/l of yeast extract (MMN*) was used in all subsequent studies. The largest fungal colony diameters were obtained at pH 6 for isolates CT1 and ZL1 and pH 7 for CT3 and CT4. All the isolates were able to grow at pH 5 and 7.5 (Table 1). Temperature studies

Table 2 Growth of *A. caesarea* strains at different temperatures[a]

Strain	Colony diameter (cm)			
	Temperature			
	20°C	24°C	28°C	LSD
CT1	1.21 b	1.95 a	1.81 a	0.239
CT3	1.44 c	2.34 b	2.70 a	0.250
CT4	1.60 b	2.91 a	3.29 a	0.651
ZL1	1.88 b	2.93 a	2.60 a	0.664

LSD Least significant difference
[a]Values are means of seven replicates. Means followed by the same letter across rows are not significantly different at $P<0.05$. Strains were grown on MMN* (mannitol) (pH 6) for 40 days

Table 3 Growth of *A. caesarea* strains on different nitrogen sources[a]

Strain	Parameter	Nitrogen source				
		Control	$(NH_4)_2HPO_4$	$Ca(NO_3)_2$	Albumin bovine	LSD
CT1	Colony diameter (cm)	2.66 b	2.23 c	3.10 a	3.21 a	0.247
	Dry weight (mg)	6.50 d	37.97 a	15.29 b	11.34 c	2.234
CT3	Colony diameter (cm)	2.24 b	2.20 b	2.30 b	2.72 a	0.161
	Dry weight (mg)	8.19 b	36.24 a	9.40 b	7.80 b	1.689
CT4	Colony diameter (cm)	2.22 b	2.18 b	3.15 a	2.75 ab	0.606
	Dry weight (mg)	6.58 c	16.67 a	8.67 b	7.77 bc	1.364
ZL1	Colony diameter (cm)	2.45 b	2.51 b	3.63 a	3.37 a	0.532
	Dry weight (mg)	7.33 c	30.56 a	13.39 b	6.66 c	4.425

LSD Least significant difference
[a]Strains were grown for 40 days at 24°C. Values are means of seven replicates. Means followed by the same letter across rows are not significantly different at $P<0.05$

indicated that growth, estimated as colony diameter, was better at 24 and 28°C than at 20°C for all the strains (Table 2). These results could probably be related to the thermophilic behaviour *A. caesarea* showed in the field, where changes in the mycelium conducting to sporocarp formation require coincidence of rainfall with high temperatures, which generally occurs early in autumn. In the pH and temperature studies, where the same carbon and nitrogen sources were used, mycelial morphology and growth density were similar for each isolate, so growth was only measured as colony diameter. In nitrogen source studies, the results indicated that albumin bovine and NO_3^- produced the largest colony diameters but the best mycelium dry weight yields were obtained with NH_4^- (Table 3). These results agree with those reported by Sawyer et al. (2003b), who showed lower biomass yields on NO_3^- and bovine serum albumin than on NH_4^- for isolates of several Australian *Amanita* species. In general, mannitol produced the largest colony diameters, and the biggest biomass yields were obtained on glucose and mannitol (Table 4). Starch, sucrose and fructose were used less efficiently, and xylose inhibited the growth of all the strains at the concentration tested. Inclusion of yeast extract was not essential for growth in contrast to that reported other genera such as *Lactarius* (Oort 1981). To summarise, requirements to obtain optimal growth of *A. caesarea* seem to be 24–28°C, pH 6–7, mannitol and glucose as carbon sources and ammoniun as a source of nitrogen. The comparison of ITS-RFLP patterns from sporocarps and in-vitro-grown mycelium showed the identity of the isolates (Fig. 1). Although only one ITS-RFLP type was observed for the four strains with *Rsa*I, *Alu*I and *Hin*fI restriction enzymes, differences were observed in culture growth in that the strain CT1 did not develop on fructose and CT4 used mannitol lesser efficiently than the other strains. Variation in growth behaviour between isolates of the same species has also been reported by Anderson et al. (1999), Finlay et al. (1992) and Keller (1996). *Q. suber* and *C. sativa*, which are two important *A. caesarea* hosts, are abundantly produced each year in nurseries for forest regeneration purposes. Thus, the knowledge of some in vitro growth requirements of *A. caesarea* is a first step towards inoculum production for field or nursery applications to increase the sporocarp production and ecological benefits to trees.

Table 4 Growth of *A. caesarea* strains on different carbon sources[a]

Strain	Parameter	Carbon source								
		C1[b]	C2[c]	Starch	Glucose	Fructose	Mannitol	Sucrose	Xylose	LSD
CT1	Colony diameter (cm)	1.64 c	1.47 c	1.64 c	2.06 b	0 d	2.85 a	1.66 c	0 d	0.201
	Dry weight (mg)	2.69 de	4.01 cd	4.57 cd	56.23 a	0 e	36.83 b	7.70 c	0 e	3.772
CT3	Colony diameter (cm)	1.44 d	1.81 c	2.07 b	1.45 d	0.71 e	3.26 a	2.19 b	0 f	0.252
	Dry weight (mg)	4.03 e	5.04 e	5.68 de	32.60 b	22.37 c	46.70 a	8.04 d	0 f	2.880
CT4	Colony diameter (cm)	1.42 b	1.52 ab	1.62 ab	1.00 c	0.78 c	1.72 a	1.60 ab	0 d	0.239
	Dry weight (mg)	2.48 d	3.20 d	3.62 d	14.66 a	12.05 b	6.46 c	6.10 c	0 e	2.327
ZL1	Colony diameter (cm)	2.79 a	2.27 b	1.86 bc	1.11 de	0.79 b	2.27 b	1.39 cd	0 f	0.475
	Dry weight (mg)	2.74 c	3.23 c	4.70 bc	21.30 a	12.24 b	26.66 a	8.19 bc	0 c	8.318

LSD Least significant difference
[a]Values are means of seven replicates. Means followed by the same letter across rows are not significantly different at $P<0.05$. Strains were grown for 40 days at 24°C
[b]MMN* minus glucose and yeast extract
[c]MMN* minus glucose

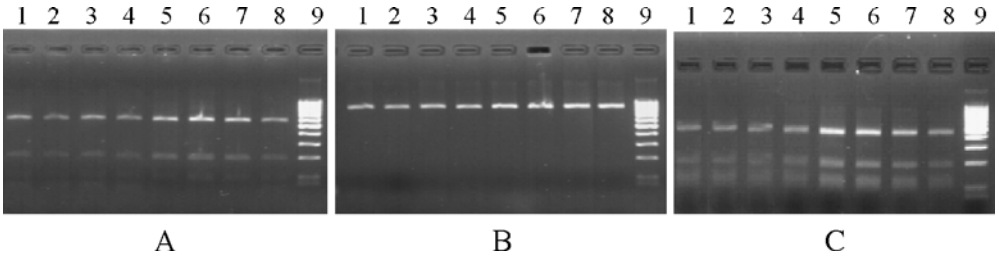

Fig. 1 RFLP patterns of PCR-amplified ITS of sporocarp and fresh mycelium grown on MMN* of several isolates of *A. caesarea*. Lanes *1–4* sporocarp of CT1, CT3, CT4 and ZL1, respectively. Lanes *5–8* in-vitro-grown mycelium of CT1, CT3, CT4 and ZL1, respectively. Lane *9* 100-bp ladder. *A Alu*I, *B Rsa*I, *C Hin*fI

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