agronomie (1996) 16, 709-719

Soluble proteins and polypeptide profiles of spores of arbuscular mycorrhizal fungi. Interspecific variability and effects of host (myc+) and non-host (myc−) *Pisum sativum* root exudates

A Samra, E Dumas-Gaudot *, V Gianinazzi-Pearson, S Gianinazzi

Laboratoire de phytoparasitologie Inra-CNRS, CMSE, Inra, BV 1540, 21034 Dijon cedex, France

(Received 30 July 1996; accepted 23 September 1996)

Summary — Total soluble proteins from spores of four species of arbuscular mycorrhizal fungi (*Gigaspora rosea*, *Scutellospora castanea*, *Acaulospora laevis* and *Glomus mosseae*) have been resolved by two-dimensional electrophoresis. Polypeptide profiles were compared to *Gig rosea* chosen arbitrarily as a reference. Major differences were mainly characterized by the disappearance of some polypeptides as compared to *Gig rosea*. Several polypeptides were only detected in *Gig rosea*, *S castanea* and *A laevis*. We also investigated polypeptide profiles from spores of *G mosseae* germinated in water or in root exudates from two host (myc+) genotypes of *Pisum sativum* L: the genotype cv Frisson and a nodulation-defective mutant (P56), and from a non-host (myc-) mycorrhiza-resistant mutant (P2). Although hyphal growth was weakly stimulated by the pea root exudates compared to water, no difference in the polypeptide patterns was observed in extracts for spores germinated in either sterilized water or in the various pea root exudates.

pea genotypes / arbuscular mycorrhizal fungi / polypeptide profiles / spore germination

Résumé — Protéines solubles et profils polypeptidiques de spores de champignons mycorhizogènes arbusculaires : variabilité interspécifique et effet des exudats racinaires de génotypes hôte (myc+) et non-hôte (myc−) de *Pisum sativum*. Les protéines solubles totales des spores de quatre espèces de champignons mycorhizogènes arbusculaires (*Gigaspora rosea*, *Scutellospora castanea*, *Acaulospora laevis* et *Glomus mosseae*) appartenant à l'ordre des glomales ont été analysées par électrophorèse bidimensionnelle. Les profils polypeptidiques ont été comparés à celui de *G rosea*, choisi arbitrairement comme référence. Les modifications les plus importantes se caractérisent par la disparition de quelques polypeptides par rapport à *G rosea*. Plusieurs polypeptides ont été détectés seulement dans *G rosea*, *S castanea* et *A laevis*. L'analyse de profils polypeptidiques de spores de *G mosseae*, germées dans l'eau ou dans les exudats de deux génotypes hôtes (myc+) (cv Frisson et le mutant non nodulant P56) et d'un génotype non-hôte (P2) de *P sativum* a aussi été effectuée. Bien que de faibles stimulations dans la croissance des hyphes aient été obtenues dans les exudats racinaires par rapport à l'eau, aucune différence dans le profil polypeptidique n'a été observée.

génotype de pois / champignon mycorhizogène arbusculaire / profils polypeptidiques / germination de spores

* Correspondence and reprints

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi belonging to the order Glomales are ubiquitous and form obligate symbiotic associations with the majority of land plants (Morton and Benny, 1990). The establishment of an AM association involves a sequence of events which lead to complex interactions between the two symbionts (Gianinazzi-Pearson et al, 1988; Bonfante and Perotto, 1995). Hyphal growth of AM fungi is considerably stimulated by the presence of living host roots before there is appressorium formation and subsequent root colonization (Hepper, 1984; Giovannetti et al, 1993b), and there are several reports that root exudates and volatile compounds from host plants promote spore germination and hyphal growth of *Gigaspora margarita* (Gemma and Koske, 1988; Bécard and Piché, 1989a, b; Gianinazzi-Pearson et al, 1989). Furthermore, root exudates not only stimulate hyphal growth, but also induce hyphal ramification (Gianinazzi and Gianinazzi-Pearson, 1990; Giovannetti et al, 1993b). Interestingly, root exudates from *Pisum sativum* L cv Frisson (myc$^+$) and its isogenic mycorrhiza-resistant mutant P2 (myc$^-$) (Duc et al, 1989) both have stimulatory effects on hyphal growth of *Glomus mosseae* (Giovannetti et al, 1993a). The presence of CO_2 or flavonoid compounds similarly have a positive effect on hyphal growth of *Gigaspora* and *Glomus* species (Gianinazzi-Pearson et al, 1989; Bécard et al, 1992; Morandi et al, 1992; Poulin et al, 1993). All these phenomena, indicating the ability of AM fungi to grow before the process of root colonization begins, imply that they have the ability to synthesize new molecules necessary for enhanced metabolism and hyphal growth. Beilby and Kidby (1982) reported that protein synthesis was essential for spore germination in *G caledonium*, but at present, information dealing with qualitative protein/polypeptide modifications during spore germination and/or hyphal growth prior to plant infection is not available. The aim of the present work was to study by two-dimensional gel electrophoresis (2D-PAGE): i) polypeptide contents of spores of four AM fungal species from different genera (*Gigaspora, Acaulospora, Scutellospora* and *Glomus*) and ii) the influence of *P sativum* root exudates on polypeptide modifications during spore germination and hyphal growth of the AM fungus *G mosseae*. Three kinds of exudates were tested: two from AM pea genotypes (myc$^+$) (cv Frisson and its mycorrhiza-forming, non-nodulating [nod$^-$] mutant P56), and one from the mycorrhiza-resistant, non-nodulating (myc$^-$ nod$^-$) pea genotype (P2) (Duc et al, 1989).

MATERIALS AND METHODS

Fungi

Spores of *Acaulospora laevis* Gerd and Trappe (BEG13), *Gigaspora rosea* Nicolson and Schenck (BEG9), *Scutellospora castanea* Walker (BEG1) and *Glomus mosseae* (Nicol and Gerd) Gerdemann and Trappe (BEG12) were sampled from 1-year-old stock cultures of *Allium porrum* L cv Verino of the BEG (Banque Européenne des Glomales [Dodd et al, 1994]). Spores were separated from soil by wet sieving (Gerdemann and Nicolson, 1963) and centrifugation on a Percoll gradient (Hosny et al, 1996), then selected individually under a stereomicroscope. To test the effects of root exudates, spores from *G mosseae* (50 mg fresh weight spores [about 4 000]/treatment) were surface sterilized with a solution containing chloramine T (2%), streptomycine (0.02%), gentamycine (0.01%) and Tween 80 (0.01%) for 8 min and washed with 1 L of sterilized deionized water. Surface-sterilized spores were incubated in the exudates (see later) or in sterilized deionized water in the dark at 4 °C for 15 days. To analyze polypeptide contents, 500 spores from each fungal species were individually collected under a stereomicroscope and then washed several times in 500 mL deionized water. Spores were gently dried and stored at −70 °C until subsequent protein extraction.

Collection of root exudates

Seeds of *P sativum* cv Frisson (myc$^+$ nod$^+$), its mutants P56 (myc$^+$ nod$^-$) and P2 (myc$^-$ nod$^-$) were surface sterilized in 3.5% calcium hypochlorite for 10 min followed by 10 min in ethanol, and pre-germinated at 20 °C in the dark for 8 days in sterilized vermiculite. Five seedlings of each genotype were aseptically transferred to 25 mL sterilized deionized water so that their root systems were submerged, and placed for 5 days in a growth chamber (20 °C, 70% relative humidity, 16 h photoperiod, 220 µmol m^{-2} s^{-1}). Root exudates were collected aseptically and filtered on 0.2 µm Sartorius filters.

Spore germination and hyphal growth

Five mL of root exudates from each of the three pea genotypes were used to germinate 50 mg fresh weight of spores of *G mosseae*. Controls consisted of replacing root exudates with autoclaved deionized water. All spore samples were germinated in 25 mL sterilized bottles at 4 °C in the dark for 15 days (Pons and Gianinazzi-Pearson, 1984). Germinated spores were

collected on nitrocellulose membranes by vacuum filtration using a Millipore (Millipore evase) column, washed several times with autoclaved deionized water, surface dried and stored at –70 °C until extraction.

Protein extraction for 2D-PAGE

Proteins were extracted according to Dumas-Gaudot et al (1994) with the following modifications. The frozen spores were carefully ground in a small mortar with liquid nitrogen; the resulting powder was suspended in 300 µl of extraction buffer (500 mM Tris-HCl pH 7.5, 50 mM EDTA, 0.1 mM KCl, 10 mM Thiourea, 700 mM sucrose, 2 mM phenylmethylsulfonylfluoride [PMSF], 2% (v/v) 2-mercaptoethanol [2-MCE]). An equal volume of redistilled phenol saturated with Tris-HCl 100 mM pH 7.5 was immediately added to the spore extracts. The mixture was shaken at 4 °C for 15 min, and then centrifuged at 13 000 *g* for 30 min. The phenolic phase was collected, mixed with an equal volume of extraction buffer, and centrifuged 20 min at 10 000 *g*. Five volumes of methanol containing 100 mM ammonium acetate were added to the phenolic phase and proteins were precipitated overnight at –20 °C. The protein pellet was collected by swing centrifugation and washed once with methanol, and twice with acetone at –20 °C. The pellet was dried under N_2 for 10 min at room temperature, resuspended in 300 µl O'Farrell lysis buffer (950 mM urea, 1% Nonidet P40, 5% ampholytes [50% LKB 3.5-10, 25% LKB 5-8 and 25% Servalytes 3-10], 2% 2-MCE) 2 h at room temperature and clarified in an Eppendorf air-drive ultracentrifuge for 15 min at 170 000 *g*. Supernatant samples were stored at –70 °C. Protein content was determined according to Bradford (1975) with the modifications suggested by Ramagli and Rodriguez (1985). Twenty-five µg protein were loaded on gels for comparisons of protein profiles from spores of the four AM fungal species, while 70 µg were loaded for comparisons between ungerminated *G mosseae* spores and spores germinated either in water or in root exudates.

2D-PAGE was carried out according to O'Farrell (1975) with the following modifications. The first dimension was in isoelectrofocussing gels (IEF) (18 x 1.5 mm) containing 950 mM urea, 0.5% (v/v) Nonidet P40, 27 mM CHAPS, 4.73% (v/v) polyacrylamide, 0.27% (v/v) N-N' bis-acrylamide, 5.5% (v/v) ampholytes (20% LKB 5-8, 40% LKB 3.5-10, 40% Servalytes [Serva] 3-10), 0.1% Temed and 0.2% ammonium persulphate. The lower reservoir was filled with 10 mM H_3PO_4, while the upper reservoir contained 100 mM NaOH. After 1 h pre-electrofocussing at 1 000 V, the protein samples were applied at the basic end of the IEF gels and submitted to electrophoresis at 1 000 V for 15 h. After extrusion, each gel was equilibrated in 5 mL 70 mM Tris-HCl pH 6.8, 5% sodium dodecylsulphate (SDS), 5% (v/v) 2-MCE and 0.03% bromophenol blue for 20 min and then in the same buffer supplemented with 270 mM iodoacetamide (Görg et al, 1987) for 20 min. The second dimension was carried out in 12% polyacrylamide SDS-PAGE (20 x 16 cm) according to Dumas-Gaudot et al (1994). After SDS-PAGE, proteins were fixed overnight in a mixture of methanol/acetic acid/water (50/12/13). Visualization of polypeptides was by silver staining (Blum et al, 1987). Analyses of the four AM species were repeated three times, and experiments on *G mosseae* spore germination twice. The molecular weight (MW) and isoelectric point (pI) values of polypeptides were estimated from their migration in the gel in relation to that of standard proteins with known MW and pI (Bio-Rad 2D-standards). Furthermore, in each IEF electrophoresis, the range of pH was also determined according to O'Farrell (1975). Changes in the relative accumulation of polypeptides were visually evaluated.

RESULTS

Spore protein concentrations and polypeptide patterns of AM fungi belonging to four different species

Spores of similar size within each fungal species were taken for protein analyses. Spore diameters varied among the species examined ranging from an average of about 100 µm for the smallest (*G mosseae*) to about 300 µm for the largest (*S castanea*) (Morton et al, 1993; Hosny et al, 1996). The protein concentration in spores of *S castanea* (0.63 µg protein/spore) was very close to that of *Gig rosea* while those of the two other genera, *A laevis* and *G mosseae,* were significantly lower with 0.465 µg/spore and 0.189 µg/spore, respectively (table I). Interestingly, the protein concentration of spores belonging to the four species was proportional to their respective spore size (table I).

A total of about 200 polypeptides from *S castanea* and *Gig rosea*, 80 polypeptides from *A laevis* and at least 100 polypeptides from *G mosseae* spore extracts were resolved with good reproducibility by 2D-PAGE. The four species had polypeptides ranging from 76 to 14 kDa with a pI between pH 4.5 to 8.0. For more detailed comparisons between spore extracts of the different fungi, identical areas were selected in the gels in the range of pH 5 to 7.5 and molecular weights 66 to 15 kDa. These areas were those in which major polypeptide differences were observed (fig 1). Two-dimensional polypeptide patterns of *Gig rosea* spore extracts were chosen as the reference (fig 1A). The altered polypeptides were characterized by both their MW and pI and were labelled by the letters r, c or d according to whether they were present in *Gig rosea*, *S castanea* or *A laevis* respectively (table II). Some

Table I. Spore diameter, protein concentration and number of polypeptides resolved by 2D-PAGE in different species from four arbuscular mycorrhizal fungi.

Spore species	Scutellospora castanea	Gigaspora rosea	Acaulospora laevis	Glomus mosseae
Mean spore diameter* (μm)	300	250	190	100
Protein content (μg) per spore	0.630 (± 0.17)	0.603 (± 0.298)	0.465 (± 0.057)	0.189 (± 0.085)
Number of polypeptides resolved by 2D-PAGE	200 (± 9)	200 (± 8)	80 (± 5)	100 (± 7)

Values of the protein content represent the mean of three replications. Standard errors are indicated in parentheses. * According to Hosny et al (1996).

polypeptides were common to all four species, while in *S castanea*, *G mosseae* and *A laevis* others were either additional to or missing from the reference profile of *Gig rosea*. At first sight, polypeptide profiles of *Gig rosea* (fig 1A) and *S castanea* (fig 1B) looked rather similar, and strongly differed from those of *A laevis* (fig 1C) and *G mosseae* (fig 1D). However, closer comparison between the 2D-PAGE profiles of *Gig rosea* and *S castanea* revealed differences which concerned at least 34 polypeptides. Twenty-two of these (labelled r1 to r22 in fig 1A and table il) were only present in *Gig rosea*, while 12 other polypeptides (labelled c1 to c12 in fig 1B and table II) were only detected in *S castanea*. When the 2D-PAGE profiles of *Gig rosea* and *A laevis* were compared, as many as 120 polypeptides were different (table I). Attention was focussed on 18 polypeptides showing a greater staining intensity in the selected area. Seven of these polypeptides (labelled d1 to d7 in fig 1C and table II) were only detected in *A laevis*, whereas the others were only present in *Gig rosea* (labelled r8, r18 to r20 and r23 to r29 in fig 1A and table II). Differences in polypeptide patterns between *Gig rosea* and *G mosseae* were also very important. Among the 100 polypeptides that were different between the two species (table I), 36 more intensely staining polypeptides only present in *Gig rosea* were analyzed in more detail for their MW and pI (r3, r4, r8, r12, r15 to r17, r21 to r27 and r30 to r51 in fig 1A as compared to fig 1D, and table II). All of the polypeptides found in *G mosseae* extracts were also detected in *Gig rosea* (fig 1A, D).

Besides these obvious differences in polypeptide patterns, some resolved polypeptides appeared to be common to the different fungal species. These are given in table III. For example, polypeptides labelled r15, r17 were common to *Gig rosea* and *A laevis*, while polypeptides r18 and r19 were common to *Gig rosea* and *G mosseae*, and those labelled r23 to r27 were common to *Gig rosea* and *S castanea*. Finally, some polypeptides were only present in one of the species such as the polypeptides c5 to c8 in *S castanea*, or d1 to d3 in *A laevis*.

Fungal mass and polypeptide patterns in germinated spores of G mosseae

In G mosseae spores germinated in water or root exudates for 15 days, increases in fungal mass were observed which varied from 16 mg for spores germinating in water to a mean of 20 mg for those germinating in pea root exudates (table IV). However, these values were not statistically different. A small fraction of germinating spores of *G mosseae* from each treatment were sampled and stained with 0.1% trypan blue. Observations under the stereomicroscope confirmed the presence of germ-tube hyphae growing from spores of *G mosseae*.

Soluble protein concentrations in extracts from spores of *G mosseae* were very similar (data not shown), whether they germinated in water or in pea root exudates from myc$^+$ nod$^+$ cv Frisson, myc$^+$ nod$^-$ P56 or myc$^-$ nod$^-$ P2 genotypes. 2D-PAGE of the fungal extracts, representing a mix-

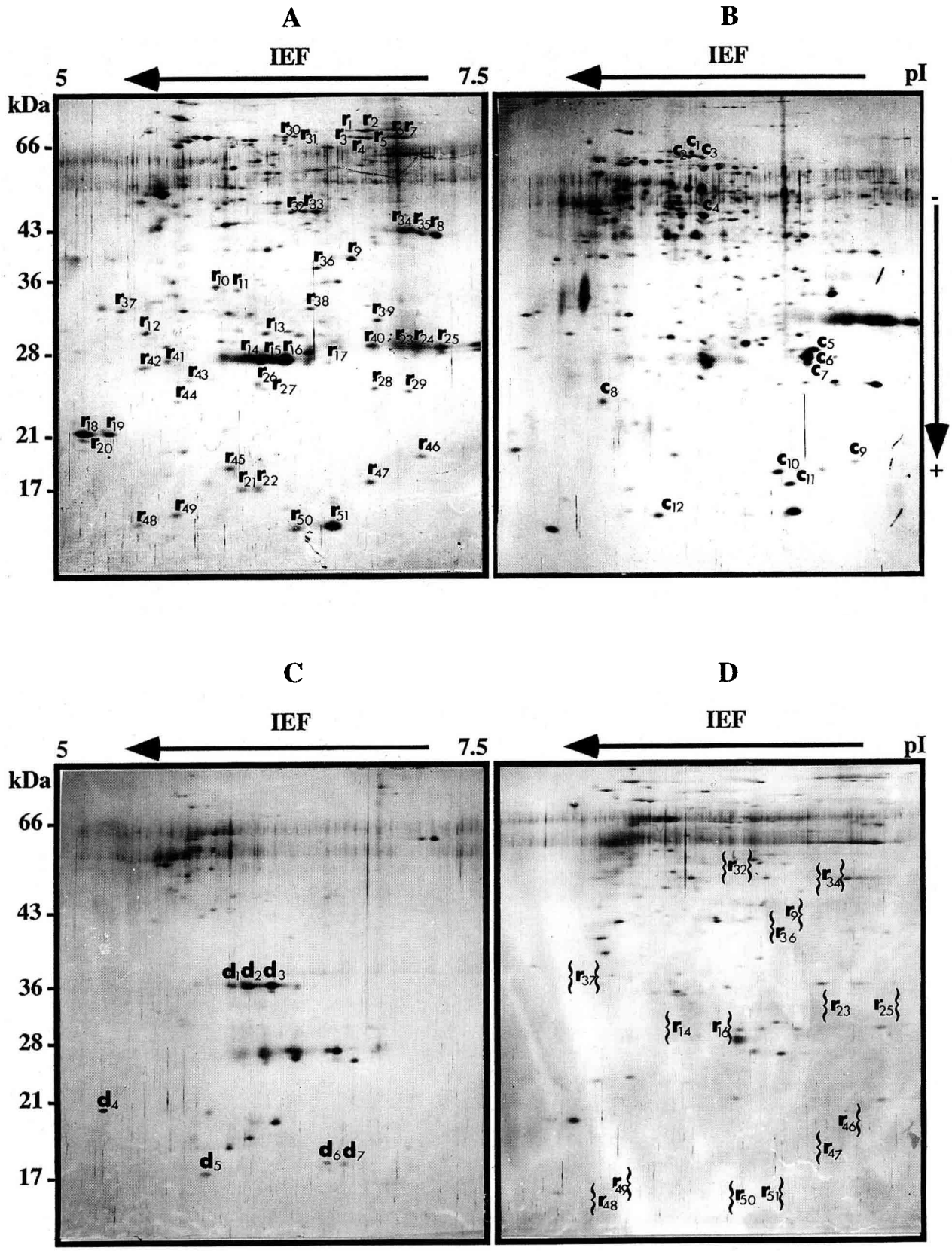

Fig 1. Silver-stained 2D-PAGE gels of soluble proteins from spores of *Gigaspora rosea* (**A**), *Scutellospora castanea* (**B**), *Acaulospora laevis* (**C**) and *Glomus mosseae* (**D**). Similar amounts of protein (25 μg) were loaded onto the gels. The expression of polypeptides labelled r, c and d was altered between spore extracts of *Gig rosea*, *S castanea*, *A laevis* and *G mosseae*, respectively. The first dimension isoelectrofocussing (IEF) was performed at 1 000 V/h for 16 h using wide range ampholytes (pH 3–10) supplemented with pH 5–8 ampholytes. The second dimension SDS gel was in 12% polyacrylamide. Molecular weight markers are in kilodaltons (kDa).

Table II. Polypeptide differences detected in fungal spores of *Gigaspora rosea* (r), *Scutellospora castanea* (c), *Acaulospora laevis* (d) and *Glomus mosseae*.

Gig rosea *compared to* S castanea			S castanea *compared to* Gig rosea			Gig rosea *compared to* A laevis			A laevis *compared to* Gig rosea			Gig rosea *compared to* G mosseae		
Pp	*MW*	*pI*	*Pp*	*MW*	*pI*	*Pp*	*MW*	*pI*	*Pp*	*MW*	*pI*	*Pp*	*MW*	*pI*
r1	67.6	6.70	c1	67.9	6.14	r8	43.3	7.26	d1	37.5	6.00	r3	63.9	6.72
r2	67.6	6.80	c2	67.6	6.14	r18	21.3	5.10	d2	37.5	6.10	r4	63.9	6.77
r3	63.9	6.72	c3	67.6	6.20	r19	21.3	5.30	d3	37.5	6.25	r8	43.3	7.26
r4	63.9	6.77	c4	43.3	6.23	r20	20.4	5.13	d4	20.4	5.23	r12	30.1	5.51
r5	63.9	6.80	c5	27.2	6.87	r23	29.5	7.00	d5	18.0	5.84	r15	28.4	6.20
r6	63.9	6.97	c6	26.7	6.84	r24	29.5	7.13	d6	19.3	6.60	r16	28.4	6.35
r7	63.9	7.04	c7	26.2	6.84	r25	29.5	7.26	d7	19.3	6.65	r17	27.8	6.60
r8	43.3	7.26	c8	24.3	5.62	r26	25.1	6.20				r21	17.4	6.08
r9	37.5	6.72	c9	20.9	7.12	r27	25.1	6.21				r22	17.4	6.18
r10	35.3	5.91	c10	19.0	6.67	r28	25.6	6.87				r23	29.5	7.00
r11	35.3	6.05	c11	18.0	6.72	r29	25.1	7.10				r24	29.5	7.13
r12	30.1	5.51	c12	15.0	5.94							r25	29.5	7.26
r13	30.1	6.21										r26	25.1	6.20
r14	28.4	6.10										r27	25.1	6.21
r15	28.4	6.20										r30	67.6	6.33
r16	28.4	6.35										r31	67.6	6.40
r17	27.8	6.60										r32	44.2	6.37
r18	21.3	5.10										r33	44.2	6.45
r19	21.3	5.30										r34	43.3	7.04
r20	20.4	5.13										r35	43.3	7.08
r21	17.4	6.08										r36	37.5	6.52
r22	17.4	6.18										r37	33.3	5.37
												r38	33.3	6.49
												r39	30.6	6.89
												r40	28.0	6.86
												r41	27.1	5.64
												r42	26.7	5.50
												r43	25.1	5.76
												r44	24.1	5.69
												r45	19.1	6.01
												r46	19.1	7.16
												r47	18.8	6.84
												r48	14.3	5.47
												r49	15.0	5.69
												r50	14.3	6.40
												r51	14.7	6.62

Pp: polypeptide; MW: molecular weight; pI: isoelectric point.

ture of spores and growing hyphae, resolved at least 300 polypeptides with molecular sizes ranging from 76 to 14 kDa and a pI ranging from pH 4.5 to 8.0. When polypeptide profiles of ungerminated spores (fig 2A) were compared with those of spores germinated in water (fig 2B), they showed an increase in polypeptide number following the germination process. Whereas about 130 polypeptides were found in extracts from ungerminated spores of *G mosseae*, more than 300 polypeptides could be detected after spore germination. All the polypeptides detected in ungerminated spores were still present in germinated spores.

Polypeptide profiles obtained from spores germinated in the non-host P2 (myc^-) root exudates (fig 2C), or in exudates from either cv Frisson or P56 genotypes (data not shown), were very similar to those of spores germinated in water (fig 2B).

DISCUSSION

In this study, we observed a relation between spore size and total soluble protein content in four species of AM fungi, and showed for the first time that 2D-PAGE can be used to resolve the polypeptides extracted from glomalean spores. Important qualitative differences existed between polypeptide profiles of spore extracts from the four fungal species belonging to different genera of Glomales. Although some polypeptides were common to the four fungi, additional ones were found in *Gig rosea* compared to *S castanea*, *A laevis* and *G mosseae*, while others were missing in comparison. Only a few reports, using 1D-PAGE electrophoresis, have dealt with protein analyses in AM fungi (Rosendahl, 1989; Schellenbaum et al, 1992; Giovannetti and

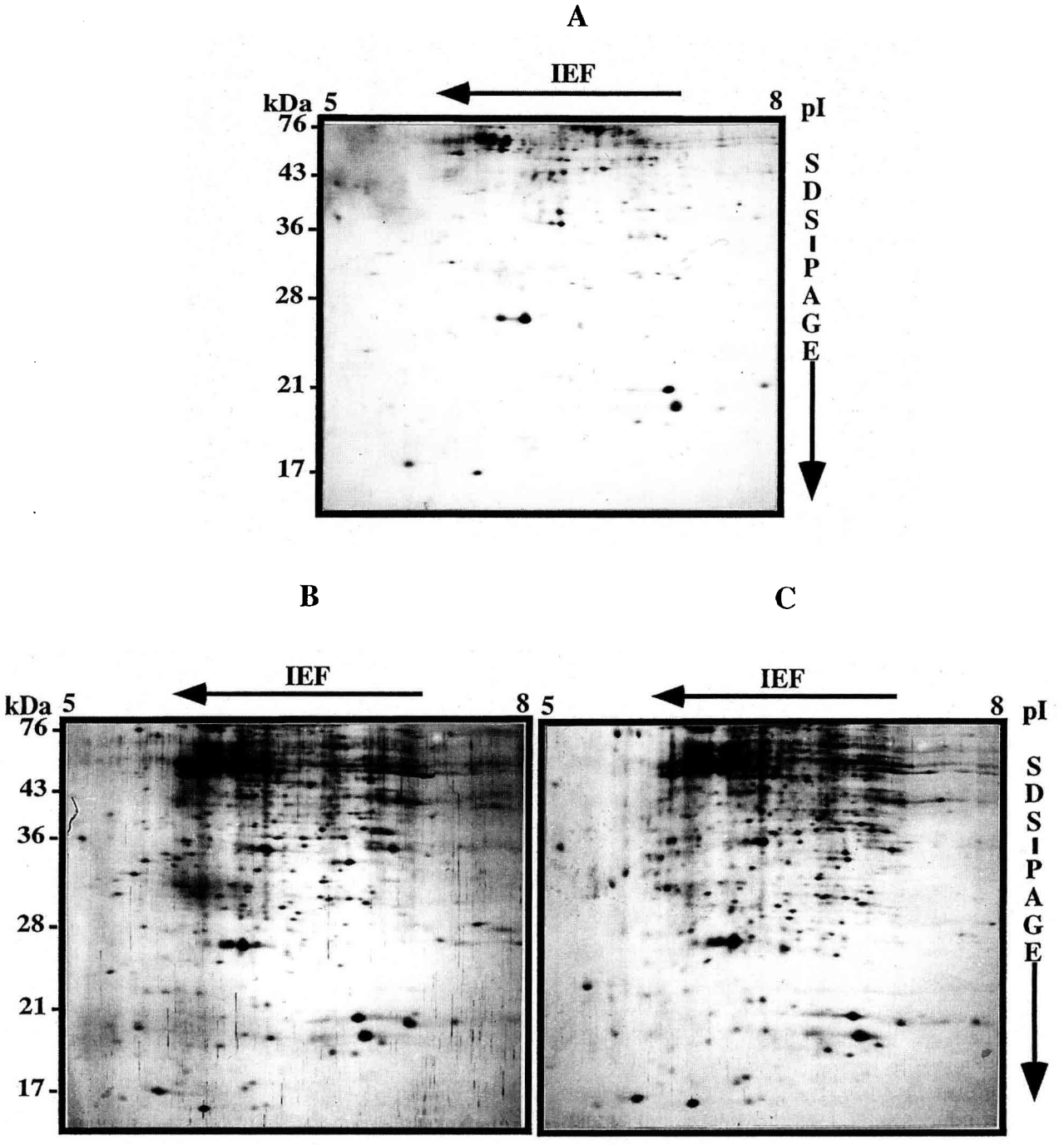

Fig 2. Silver-stained 2D-PAGE gels of soluble protein extracts from ungerminated spores of *Glomus mosseae* (**A**) and spores germinated in sterilized water (**B**) or in root exudates from the mycorrhiza-resistant pea mutant P2 (**C**). Similar amounts of proteins (70 μg) were loaded onto the gels. Electrophoretic conditions and legends are as in figure 1.

Gianinazzi-Pearson, 1994). Schellenbaum et al (1992) and Giovannetti and Gianinazzi-Pearson (1994) reported some differences in soluble protein patterns from AM fungi with different species or genus affinity, but these were limited in number as compared to the qualitative differences in polypeptide profiles we report here.

Identification of AM fungal species within root tissues is hampered by the lack of sufficiently distinguishable morphological characters. Approaches using isozymes, antibodies and DNA probes have been, or are being, explored but they have met with limited success, largely due to their low specificity (Hepper et al, 1988; Rosendahl, 1989; Rosendahl and Sen, 1992; Hahn et al, 1993; Cordier et al, 1994, 1996; Giovannetti and Gianinazzi-Pearson, 1994; Sanders et al, 1996). An alternative approach could be through the identification of taxon-specific proteins that, after purification, can be used for antibody production or sequencing to produce specific oligonucleotide probes. Indeed, the pre-

Table III. Main polypeptide differences and similarities between the four arbusucular mycorrhizal fungal species.

Polypeptide	G rosea	S castanea	A laevis	*Polypeptides in* G mosseae
r3	+	–	–	–
r4	+	–	–	–
r8	+	–	–	–
r14	+	–	–	–
r15	+	–	+	–
r17	+	–	+	–
r18	+	–	–	+
r19	+	–	–	+
r20	+	–	–	–
r21	+	–	+	–
r22	+	–	+	–
c5	–	+	–	–
c6	–	+	–	–
c7	–	+	–	–
c8	–	+	–	–
d1	–	–	+	–
d2	–	–	+	–
d3	–	–	+	–
r23	+	+	–	–
r24	+	+	–	–
r25	+	+	–	–
r26	+	+	–	–
r27	+	+	–	–

+: presence of polypeptide; –: absence of polypeptide.

Table IV. Germination of 50 mg of spores of *Glomus mosseae* 15 days in the presence of either sterile water or root exudates from host and non-host plants of *Pisum sativum* L.

Root exudates	*Sterilized deionized water*	*cv Frisson* (*myc*$^+$ *nod*$^+$)	*P56* (*myc*$^+$ *nod*$^-$)	*P2* (*myc*$^-$ *nod*$^-$)
Fresh weight (mg) before germination	50	50	50	50
Fresh weight (mg) after germination	66.15 (± 1.62)	71.85 (± 0.91)	72.45 (± 2.47)	70.85 (± 1.34)

Values represent the mean of two experiments. Standard errors are indicated in parentheses. Statistical analysis was done by the Student's *t*-test; increases in fungal mass were not different at $P = 0.01$.

sent study of 2D-PAGE polypeptide profiles of spore extracts has revealed that considerable qualitative differences can exist between different AM fungi. In the four species analyzed, the polypeptides named r3, r4, r8, r14, r15, r16, and d1, d2, d3, and c5, c6, c7, which form a group with similar MWs and different pI, are interesting for further studies since they may be taxon-specific and they could therefore be good candidates to discriminate between AM fungi. If this hypothesis is confirmed by further analyses of a wider range of AM fungal species, they could represent, after isolation and purification, appropriate antigens for production of highly specific antibodies or for synthesis of oligonucleotide probes based on their amino acid sequences.

Spores of *G mosseae* were able to germinate not only in water and in pea root exudates from the host genotypes cv Frisson (myc^+ nod^+) or the non-nodulating mutant P56 (myc^+ nod^-), but also from the non-host P2 mutant (myc^- nod^-). These results agree with those previously reported for other AM fungi or root exudates (Bécard and Piché, 1989a, b; Gianinazzi-Pearson et al, 1989). Furthermore, increases in fungal mass as spores germinated in water or in the various pea root exudates were similar, suggesting that the latter did not stimulate hyphal growth. Interestingly, root exudates of the non-host mycorrhiza-resistant pea mutant P2 (myc^- nod^-), in which mycorrhizal colonization is inhibited but appressoria are formed (Duc et al, 1989), did not have any inhibitory effects on *G mosseae* hyphal growth as compared to those from the compatible pea genotypes. This lack of fungitoxic effects in root exudates of non-host roots is in good agreement with results obtained by Bécard and Piché (1990), Giovannetti et al (1993a) and Balaji et al (1994) using the non-host beet and mycorrhiza-resistant mutant pea genotypes.

Although several studies have been undertaken to determine the stimulatory root components implicated in enhanced AM spore germination and hyphal growth (Bécard and Piché, 1989a; Gianinazzi-Pearson et al, 1989; Vierheilig and Ocampo, 1990; Bécard et al, 1992; El Ghachtouli et al, 1996), there has been no research up to now on the influence of the stimuli on the protein metabolism of AM fungi. In order to detect fungal protein modifications induced by root exudates, we compared the polypeptide patterns of ungerminated spores to those of spores germinated in water, or in pea root exudates. The strong qualitative and quantitative modifications in polypeptide patterns which occurred in germinated spores demonstrate the important activation in protein synthesis related to spore germination and hyphal growth of *G mosseae*, and corroborate Beilby and Kidby's (1982) observations that protein synthesis is essential to these processes. Surprisingly, the 2D-PAGE profiles of extracts from spores germinated in the root exudates of the different pea genotypes, cv Frisson, the non-nodulating mutant P56 and the mycorrhiza-resistant mutant P2, did not differ from those of spores germinated in water. Thus, any differences which may exist in the nature of host (myc^+) and non-host (myc^-) root exudates in pea did not act upon protein expression associated with the germination of spores of *G mosseae.* These observations also strengthen Giovannetti et al's (1993a) conclusion that it is not the nature of root exudates in non-host (myc^-) pea which contribute to the mycorrhiza-resistant phenotype of the mutant but rather events occurring after fungal contact with the root surface.

REFERENCES

Balaji B, Ba A, La Rue T, Tepfer D, Piché Y (1994) *Pisum sativum* mutants insensitive to nodulation are also insensitive to invasion *in vitro* by the mycorrhizal fungus, *Gigaspora margarita. Plant Sci* 102, 195-203

Bécard G, Piché Y (1989a) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. *Appl Environ Microbiol* 55, 2320-2325

Bécard G, Piché Y (1989b) New aspects on the acquisition of biotrophic status by a VAM fungus *Gigaspora margarita. New Phytol* 112, 77-83

Bécard G, Piché Y (1990) Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and non-host Ri T-DNA transformed roots. *Can J Bot* 68, 1260-1264

Bécard G, Douds DD, Pfeffer PE (1992) Extensive *in vitro* hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. *Appl Environ Microbiol* 58, 821-825

Beilby JP, Kidby DK (1982) The early synthesis of RNA protein and some associated metabolic events in germinating vesicular-arbuscular fungal spores of *Glomus caledonius. Can J Bot* 28, 623-628

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8, 93-99

Bonfante P, Perotto S (1995) Strategies of arbuscular mycorrhizal fungi when infecting host plants. *New Phytol* 130, 3-21

Bradford MM (1975) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann Biochem* 72, 248-254

Cordier C, Gianinazzi-Pearson V, Gianinazzi S (1994) Immunodétection de champignons endomycorhiziens à arbuscules *in planta*. *Acta Bot Gallica* 141, 465-468

Cordier C, Gianinazzi-Pearson V, Gianinazzi S (1996) An immunological approach for the study of spatial relationships between arbuscular mycorrhizal fungi *in planta*. In: *Mycorrhizas in Integrated Systems: From Genes to Plant Development* (C Azcón-Aguilar, JM Barea, eds), European Commission, EUR 16728, Luxembourg, 25-30

Dodd JC, Gianinazzi-Pearson V, Rosendahl S, Walker C (1994) European Bank of Glomales. An essential tool for efficient international and interdisciplinary collaboration. In: *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems* (S Gianinazzi, H Schüepp, eds), Birkhäuser-Verlag, Basel, Switzerland, 41-46

Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S (1989) First report of non-mycorrhizal plant mutants (myc$^-$) obtained in pea (*Pisum sativum* L) and fababean (*Vicia faba* L). *Plant Sci* 60, 215-222

Dumas-Gaudot E, Guillaume P, Tahiri-Alaoui A, Gianinazzi-Pearson V, Gianinazzi S (1994) Changes in polypeptide patterns in tobacco roots colonized by two *Glomus* species. *Mycorrhiza* 4, 215-221

El Ghachtouli N, Paynot M, Martin-Tanguy J, Morandi D, Gianinazzi S (1996) Effect of polyamines and polyamine biosynthesis inhibitors on spore germination and hyphal growth of *Glomus mosseae*. *Mycol Res* 100, 597-600

Gemma JN, Koske RE (1988) Pre-infection interactions between roots and the mycorrhizal fungus *Gigaspora gigantea*: chemotropism of germ-tubes and root growth response. *Trans Br Mycol Soc* 91, 123-132

Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Trans Br Mycol Soc* 46, 235-244

Gianinazzi S, Gianinazzi-Pearson V (1990) Cellular interactions in vesicular arbuscular (VA) endomycorrhizae: the host's point of view. In: *Endocytobiology IV* (P Nardon, V Gianinazzi-Pearson, AM Grenier, L Margulis, DC Smith, eds), INRA Press, Paris, France, 83-90

Gianinazzi-Pearson V, Gianinazzi S, Dexheimer J, Morandi D, Trouvelot A, Dumas-Gaudot E (1988) Recherche sur les mécanismes intervenant dans les interactions symbiotiques plantes-champignons endomycorhizogènes VA. *Cryptogam Mycol* 9, 201-209

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) *In vitro* enhancement of spore germination and early hyphal growth of vesicular-arbuscular mycorrhizal fungus by root exudates and plant flavonoids. *Symbiosis* 7, 243-255

Giovannetti M, Gianinazzi-Pearson V (1994) Biodiversity in arbuscular mycorrhizal fungi. *Mycol Res* 98, 705-715

Giovannetti M, Avio L, Sbrana C, Citernesi S (1993a) Factors affecting appressorium development in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol & Gerd) Gerd & Trappe. *New Phytol* 123, 115-122

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993b) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. *New Phytol* 125, 587-593

Görg A, Postel W, Weser J, Günther S, Strahler JR, Hanash SM, Somerlot L (1987) Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis* 8, 122-124

Hahn A, Bonfante P, Horn K, Pausch F, Hock B (1993) Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure. *Mycorrhiza* 4, 69-78

Hepper CM (1984) Isolation and culture of VA mycorrhizal (VAM) fungi. In: *VA Mycorrhizas* (CL Powell, DJ Bagyaraj, eds), CRC Press, Boca Raton, FL, USA, 95-130

Hepper CM, Sen R, Azcón-Aguilar C, Grace C (1988) Variation in certain isozymes amongst different geographical isolates of the vesicular-arbuscular mycorrhizal fungi *Glomus clarum*, *Glomus monosporum* and *Glomus mosseae*. *Soil Biol Biochem* 20, 51-59

Hosny M, Dulieu H, Gianinazzi-Pearson V (1996) A simple and rapid method for collecting Glomales spores from soil. In: *Mycorrhizas in Integrated Systems: From Genes to Plant Development* (C Azcón-Aguilar, JM Barea, eds), European Commission, EUR 16728, Luxembourg, 541-543

Morandi D, Branzanti B, Gianinazzi-Pearson V (1992) Effect of some plant flavonoïds on *in vitro* behaviour of an arbuscular mycorrhizal fungus. *agronomie* 12, 811-816

Morton JB, Bentivenga SP, Wheeler WW (1993) Germ plasm in the international collection of arbuscular and vesicular-arbuscular mycorrhizal fungi (INVAM) and procedures for culture development, documentation and storage. *Mycotaxon* 48, 491-528

Morton JN, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (*Zygomycetes*): a new order, *Glomales*, two new suborders, *Glomineae* and *Gigasporineae*, and two new families *Acaulosporaceae*, and *Gigasporaceae*, with an emendation of *Glomaceae*. *Mycotaxon* 37, 471-491

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250, 4007-4021

Pons F, Gianinazzi-Pearson V (1984) Influence du phosphore, du potassium, de l'azote et du pH sur le comportement *in vitro* de champignons endomycorhizogènes à vésicules et arbuscules. *Cryptogam Mycol* 5, 87-100

Poulin MJ, Bel-Rhlid R, Piché Y, Chênevert R (1993) Flavonoids released by carrot (*Daucus carota*) seedlings stimulate hyphal development of vesicular

arbuscular mycorrhizal fungi in the presence of optimal CO_2 enrichment. *J Chem Ecol* 19, 2317-2327

Ramagli LS, Rodriguez LV (1985) Quantification of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6, 559-563

Rosendahl S (1989) Comparisons of spore-cluster forming *Glomus* species (*Endogonaceae*) based on morphological characters and isozyme banding patterns. *Acta Bot* 100, 215-223

Rosendahl S, Sen R (1992) Isozyme analysis of mycorrhizal fungi and their mycorrhiza. In: *Methods in Microbiology* (JR Norris, DJ Read, AK Varma, eds), Academic Press, New York, NY, USA, 169-194

Sanders I, Clapp J, Wiemken A (1996) The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems – a key to understanding the ecology and functioning of the mycorrhizal symbiosis. *New Phytol* 133, 123-134

Schellenbaum L, Gianinazzi S, Gianinazzi-Pearson V (1992) Comparison of acid soluble protein synthesis in roots of endomycorrhizal wild type *Pisum sativum* and corresponding isogenic mutants. *J Plant Physiol* 141, 2-6

Vierheilig H, Ocampo JA (1990) Role of root extract and volatile substances of non-host plants on vesicular-arbuscular mycorrhizal spore germination. *Symbiosis* 9, 199-202