Mycorrhiza (2011) 21:523–535
DOI 10.1007/s00572-010-0354-y

ORIGINAL PAPER

Arbuscular mycorrhizal fungi associated with *Artemisia umbelliformis* Lam, an endangered aromatic species in Southern French Alps, influence plant P and essential oil contents

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Received: 11 August 2010 / Accepted: 16 December 2010 / Published online: 18 January 2011

Abstract Root colonization by arbuscular mycorrhizal (AM) fungi of *Artemisia umbelliformis*, investigated in natural and cultivated sites in the Southern Alps of France, showed typical structures (arbuscules, vesicles, hyphae) as well as spores and mycelia in its rhizosphere. Several native AM fungi belonging to different Glomeromycota genera were identified as colonizers of *A. umbelliformis* roots, including *Glomus tenue*, *Glomus intraradices*, *G. claroideum/etunicatum* and a new *Acaulospora* species. The use of the highly mycorrhizal species *Trifolium pratense* as a companion plant impacted positively on mycorrhizal colonization of *A. umbelliformis* under greenhouse conditions. The symbiotic performance of an alpine microbial community including native AM fungi used as inoculum on *A. umbelliformis* was evaluated in greenhouse conditions by comparison with mycorrhizal responses of two other alpine *Artemisia* species, *Artemisia glacialis* and *Artemisia genipi* Weber. Contrary to *A. genipi Weber*, both *A. umbelliformis* and *A. glacialis* showed a significant increase of P concentration in shoots. Volatile components were analyzed by GC–MS in shoots of *A. umbelliformis* 6 months after inoculation. The alpine microbial inoculum increased significantly the percentage of E-β-ocimene and reduced those of E-2-decenal and (E,E)-2-4-decadienal indicating an influence of alpine microbial inoculum on essential oil production. This work provides practical indications for the use of native AM fungi for *A. umbelliformis* field culture.

Keywords *Artemisia umbelliformis* L. · Arbuscular mycorrhiza fungi colonization · Alpine microbial communities · Shoot P · Shoot N · Essential oils

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Introduction

The impact of arbuscular mycorrhizal (AM) on plant growth and physiology is influenced by environmental conditions and dependent on the specific combination of plant and fungus involved (Johnson et al. 1997). Mycorrhizal colonization has been studied in highly stressed environments like areas in the Arctic, Antarctic, and alpine habitats (Gardes and Dahlberg 1996; Olsson et al. 2004; Haselwandter 1987) where plants are under short growing periods, low temperatures, low nutrient status, and low decomposition rates (Grime 1979). Alpine plant populations are characterized by a lower proportion of mycorrhiza-dependent plant species than in other plant environments (Read and Haselwandter 1981; Trappe 1988). One striking feature of plants growing at high latitudes and altitudes is the extensive occurrence in their roots of dark septate endophyte (DSE) (Haselwandter and

Read 1980; Read and Haselwandter 1981; Christie and Nicolson 1983; Currah and Van Dyk 1986; Kohn and Stasovski 1990; Väre et al. 1992; Treu et al. 1995; Jumppoonen and Trappe 1998; Ruotsalainen et al. 2002; Upson et al. 2009). When AM fungi are observed, they are often formed by the fine endophyte *G. tenue*, and colonization increases with altitude (Haselwandter and Read 1980; Olsson et al. 2004).

Although information on the mycorrhizal status of alpine plants is available for many species, only one study has addressed the mycorrhizal status of alpine wormwood (Artemisia) species (Read and Haselwandter 1981). Alpine *Artemisia* species (*Asteraceae*) are herbaceous aromatic plants growing wild at an altitude of between 2,000 and 3,700 m above sea level (a.s.l.) on rocks, screes, or moraines (Bonnier and de Layens 1986; Chas 1994). The highly scented, dried flowering aerial parts were originally used to prepare herbal infusions in traditional medicine against coughs. Its use to prepare "genepi", a highly prized liquor, appeared over a century ago in different gastronomic and commodity treatises in France and Italy (Bicchi et al. 1984; Pieroni and Giusti 2009; Rubiolo et al. 2009). The indiscriminate picking of these plants has diminished their already rare presence almost everywhere and their harvesting is forbidden in Switzerland and Italy. In France, these plants are collected in a regulated manner according to regions. Furthermore, regions like the Hautes-Alpes aim to reduce, or even to stop, derogations for collecting wild flowers of alpine *Artemisia* species destined to liquor production, creating an urgent need to develop for crop production systems.

Artemisia umbelliformis is the only alpine *Artemisia* species that has so far been cultivated with successful agronomic results in some Alpine areas of France and on a larger scale in the Western Alps of Italy (Rey and Sclacanin 1997; Rey et al. 2002). This type of production offers (1) protection of endangered habitats and preservation of natural plant resources in the Alps area, (2) possibilities for development of sustainable small-scale agricultural/gathering activities and eco-tourism (Pieroni and Giusti 2009). *A. umbelliformis* culture needs specific conditions such as altitude (above 1,600 m a.s.l.), exposition (south-east or south-west), drained soils and alpine climatic conditions and propagation is essentially by seed production. *A. umbelliformis* plants in culture have a short life cycle of 3–5 years when compared to the 10 years observed in nature. A leaf rosette is formed the first year, flowers are produced the second year, and the floral yield diminishes over the third and fourth years. The culture is then initiated on another land plot; a crop rotation system is used to reduce *A. umbelliformis* diseases caused by fungi pathogens such as *Puccinia absinthii*, *Verticillium* sp., *Sclerotinia* sp., *Fusarium* sp., or *Rhizoctania* sp. (Rey et al. 2002; Tamietti et al. 2007).

Information on the impact of mycorrhiza on the physiology of *A. umbelliformis* are however lacking. Some previous investigations have focused on AM effects on mineral nutrient uptake and productivity, as well as yield and composition of essential oils, in other aromatic plants (Gupta et al. 2002; Kapoor et al. 2002a, b; Khaosaad et al. 2006; Freitas et al. 2004; Copetta et al. 2006; Kappoor et al. 2007; Chaudhary et al. 2008). Reported results include the increased production of essential oils in coriander and dill colonized by *Glomus fasciculatum* or *Glomus macrocarpum* (Kapoor et al. 2002a, b), in mint colonized by *G. fasciculatum* or a suite of AM fungi (Gupta et al. 2002; Freitas et al. 2004), in sweet basil colonized by *Gigaspora rosea* (Copetta et al. 2006), in oregano colonized by *Glomus mosseae* (Khaosaad et al. 2006) and in annual wormwood colonized by *G. fasciculatum* (Kappoor et al. 2007; Chaudhary et al. 2008).

The aim of our study was to examine the ability of *A. umbelliformis* root system to establish a reciprocally beneficial symbiotic relationship with AM fungi in alpine habitats, as a prerequisite for applications of mycorrhiza biotechnology to *A. umbelliformis* cultivation in alpine areas. The mycorrhizal status of *A. umbelliformis* was investigated in natural and cultivated sites located in the Southern French Alps, extending from 2,100–2,900 m a.s.l. Our interest has focused on AM fungi present in the complex soil microbial communities in associations with *A. umbelliformis* roots from an alpine culture site. Morphological and molecular approaches were used to determine the identity of AM fungal species occurring in the *A. umbelliformis* roots. The entire communities present in the rhizosphere of mycorrhizal *A. umbelliformis* plants from an alpine culture site were used as inoculum for experiments. The influence of *Trifolium pratense* as a companion plant on mycorrhizal colonization of *A. umbelliformis* was investigated under greenhouse conditions and the effects of alpine microbial inoculum on plant growth and nutrition were compared with those of two other alpine *Artemisia* species, *Artemisia glacialis* and *Artemisia genipi* Weber, in order to evaluate its impact on different species of alpine wormwood. Mineral and essential oil composition was analyzed in *A. umbelliformis* plants inoculated with alpine microbial inoculum compared to non-inoculated plants.

Materials and methods

The study area

The study was conducted in the Southern French Alps in three sites of wild *A. umbelliformis* (1, 2, and 3) and two sites of cultivated *A. umbelliformis* (4 and 5). The main features of selected sites are detailed in Table 1. Soil

Table 1 Features of field sites selected for this study

	Site 1	Site 2	Site 3	Site 4	Site 5
Altitude (m)	2,897	2,888	2,238	2,300	2,100
Geographic position	44°39′32.81″ N	44°41′12.28″ N	44°47′51.55″ N	44°42′57.28″ N	44°35′08″ N
	06°57′15.72″ E	06°57′01.40″ E	06°41′25.05″ E	06°20′26.35″ E	06°43′05″ E
Type of habitat	Schist, scattered vegetation	Schist, scattered vegetation	Schist, scattered vegetation	Grassland	Grassland
Cultivation intensity	Natural	Natural	Natural	Cultivated since 2005	Cultivated since 2000
Fertilization	None	None	None	Horse manure	Horse manure
Soil pH	8.29	8.73	7.98	5.34	7.08
P (Olsen method, g/kg)	0.011	0.005	0.034	0.014	0.082

samples were collected in summer 2005 at a depth of 5–15 cm, depending of the amount of soil available, in an area of approximately 20 m in diameter. Soil pH and available phosphorus was measured for each site (INRA, Arras, France) according to standard methods.

Evaluation of mycorrhizal status of *A. umbelliformis* plants in natural and cultivated environments

Root samples of five *A. umbelliformis* plants from each site were collected in summer 2005. The age of wild plants collected in natural sites 1, 2, and 3 was undetermined. Plants collected in site 4 were seedlings obtained in pot culture in 2004 and transplanted in site 4 in spring 2005. Collected plants in site 5 were seedlings obtained into pot culture in 2003 and transplanted in site 5 in autumn 2004. AM fungal colonization was estimated after partial digestion in KOH and staining with trypan blue (Phillips and Hayman 1970) in glycerol as the level of mycorrhizal frequency and root colonization as described by Trouvelot et al. (1986) using the MYCOCALC program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). Mycorrhizal frequency (*F*%) gives the percentage of root fragments in contact with AM fungi. Mycorrhizal root colonization (*M*%) gives an estimation of the amount of colonized root cortex in the whole root system.

Trap culture experiments

Soil samples from each site collected in summer 2005 were used as a source of inoculum and pot cultured with a genotype (*B2*) of micropropagated *A. umbelliformis* (Gautheret et al. 1984) and *Medicago truncatula* cv. Jemalong line J5 seedlings as trap plant species. *M. truncatula* was included for comparison of mycorrhization levels with those obtained for *A. umbelliformis*. Plants were transplanted into pots containing a 400 ml mix of an equal volume of soil and sterilized sand, and grown in greenhouse for 3 or 8 months. They received weekly, 20 ml of Long Ashton nutrient solution (Hewitt 1966) containing 13.4 μM of phosphate (provided as NaH_2PO_4, $2H_2O$) and 8 mM of nitrate (provided as KNO_3 and $Ca(NO_3)_2$, $2H_2O$). To monitor mycorrhizal root colonization, roots of trap plants were prepared and examined at different culture times in the same way as those of field plants.

DNA extraction from spores and roots

Spores from rhizospheric soil and root systems of *A. umbelliformis* were collected in site 4 in summer 2008. Spores were extracted by wet sieving through nested sieves (500, 250, 160, and 63 μm). Spores, apparently healthy when examined through a dissecting microscope, were

selected and rinsed in water. Sporal DNA was extracted in TE buffer as described by Farmer et al. (2007). Root DNA was extracted from randomly selected 1-cm root fragments using the NucleoSpin Plant II extraction kit (Macherey-Nagel). All DNA samples were stored at −20°C until use.

Identification of AM fungi by polymerase chain reaction of ribosomal DNA from spores

Polymerase chain reaction (PCR) reactions were performed on spore DNA in a final volume of 20 µl containing 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl pH 8.8, 2.0 mM $MgCl_2$ 10 mM 2-mercaptoethanol, 200 mg/ml bovine serum albumin, 0.05% (*w*/*v*) polyoxyethylene ether W-l (Sigma Chemical Co., St. Louis, MO), 200 µM of each dNTP, 200 nM of each of the eukaryotic primer pair ITS3 (White et al. 1990) and NDL22 (van Tuinen et al. 1998), 0.2 U of *Taq* DNA polymerase (QBiogene) and 5 µl of spore extract were used as template DNA. Amplification was performed in a Biometra T3 thermal programmed as follows: initial denaturation cycle at 93°C (5 min), followed by 40 cycles of denaturation at 92°C (45 s), annealing at 58°C for (1 min), and extension at 72°C (1 min). The last cycle was followed by a final elongation of 5 min at 72°C. Amplification products obtained using the primer pair ITS3-NDL22 were diluted 1/50, 1/100, or 1/500 and served as template for a second reaction using either the primer pair LR1-FRL2 (FRL2 used as a fungal specific primer; van Tuinen et al. 1998; Trouvelot et al. 1999) or ITS3-FRL4 (FRL4 used as a Glomeromycota specific primer; Gollotte et al. 2004). PCR conditions were as above except for using 35 amplification cycles. PCR amplification products were cloned into the vector TOPO (TOPO TA Cloning Kit for Sequencing, Invitrogen) according to the manufacturer's instructions. Plasmid preparation was done using the Nucleoplasmid kit (Macherey-Nagel) and sequencing performed by Eurofin MWG Operon (D).

Nested PCR detection of AM fungi in roots

The arbuscular mycorrhizal population colonizing *A. umbelliformis* roots was partially characterized by a nested PCR approach using taxon-specific primers detecting, *G. mosseae*, *Glomus intraradices*, *Glomus geosporum*, *Glomus etunicatum/claroideum*, and a new *Acaulospora sp.* PCR reactions were performed in a final volume of 20 µl DNA root extract as described above, except that the primer pair LR1 and NDL22 (van Tuinen et al. 1998) was used. Amplification products were diluted 1/100 and served as template for a second reaction using a taxonspecific primers in combination with LR1. Taxon-specific primers used in this study were 8.24 (*G. intraradices*; Farmer et al. 2007), 5.25 (*G. mosseae*; van Tuinen et al. 1998), 53.12 (*G. geosporum*; Jacqot-Plumey et al. 2001), Getunsp2 (*G. etunicatum/claroideum*; Farmer et al. 2007) and AS1-2 (*Acaulospora* sp.; 5′ACCAGGGTCTATAAACACTTCA3′). PCR conditions were as above except for 28 amplification cycles and annealing at 60°C. Only the presence or absence of detection of a specific fungus in root systems was estimated.

Phylogenetic analysis

Multiple alignments were performed using the MAFFT, over 710 bp, and the alignment was manually optimized using Se-Al version 2 software (University of Oxford). Sequences from the GenBank database were also included in the analysis. A phylogenetic analysis was performed using the neighbor-joining (NJ) method (Saitou and Nei 1987). The reliability of the internal branches was assessed using the boostrap method with 1000 replicates, and the best tree drawn using NJplot (http://biom3.univ-lyon1.fr).

Inoculum preparation

The microbial inoculum in further experiments consisted of the rhizospheric soil and the roots collected from four mycorrhizal *A. umbelliformis* plants (age: 2 years old) in site 4 in summer 2008. The level of mycorrhizal frequency (*F*%) and root colonization (*M*%) of the four *A. umbelliformis* plants reached 76.7±20.2% and 20±8.9%, respectively, as average. Microbial inoculum included spores, mycelium and native AM fungi whom a part was characterized as *G. tenue*, *G. intraradices*, *G. claroideum/etunicatum*, and a new *Acaulospora* species as described in the results.

Companion plant experiment

In order to study the effect of a companion plant on the mycorrhizal status of *A. umbelliformis*, *T. pratense* was chosen as a companion plant as it is a highly mycorrhizal species found among species growing in the grassland site 4. *A. umbelliformis* (*B2*) vitroplants were transplanted into 400 ml pots containing 50% of γ-irradiated (10 kGray) soil of the site 4 and 50% of sterilized sand and inoculated with 20 g of the microbial inoculum in monoculture or coculture with one companion plantlet of *T. pratense* and grown in a greenhouse. Granulometry analysis performed by LCA, Bordeaux (France) of the site 4 soil was as follow: clay, (<2 µm) 37.5%; silt, (2–20 µm) 31.6% and (20–50 µm) 11.3%; sands, (50–200 µm) 5% and (200–2,000 µm) 6.7%. Plants were watered weekly with 20 ml of Long Ashton nutrient solution (Hewitt 1966) containing 13.4 µM of phosphate (provided as NaH_2PO_4, $2H_2O$) and 8 mM of nitrate (provided as KNO_3 and $Ca(NO_3)_2$, $2H_2O$). *T.*

pratense was cultivated alone to control mycorrhizal colonization without *A. umbelliformis*. The roots of the trap plants were prepared and examined after 3 months of culture in the same way as those of the field plants to monitor the mycorrhizal root colonization.

Inoculation experiments

To study the impact of microbial inoculum including AM fungi on three alpine *Artemisia* species, inoculation experiments were performed with *T. pratense* as a companion plant given the results obtained with companion plant experiment. Three genotypes of micropropagated alpine *Artemisia* species (*A. umbelliformis* (*B2*), *A. glacialis* (*G1*), and *A. genipi* Weber (*W4*) (Gautheret et al. 1984) were cocultured with one companion plant, *T. pratense*, per pot and inoculated in the same way as for the companion plant experiment described above. The control was a sterilized mixture of the microbial inoculum. The companion plant was cut back after 3 months of culture. The microbial inoculum was also tested on plantlets of *Allium porum* L. in monoculture in order to study its efficiency on a different plant species. Plants were watered weekly with 20 ml of Long Ashton nutrient solution (Hewitt 1966) containing 13.4 μM of phosphate (provided as NaH_2PO_4, $2H_2O$) and 8 mM of nitrate (provided as KNO_3 and $Ca(NO_3)_2$, $2H_2O$). Each treatment comprised 10 replicates. All experiments were duplicated and the results shown are from one representative experiment. *Artemisia* plants were analyzed for mycorrhizal intensity, plant biomass, shoot nitrogen (N) and phosphorus (P) concentrations in inoculated plants compared to non-inoculated controls after 3 months of culture under greenhouse conditions. N and P concentrations were measured by the INRA laboratory of Arras (France), according to standard methods. The inoculation experiments were extended to 6 months for *A. umbelliformis* (*B2*) in order to evaluate the effect of AM fungi on essential oil production.

Chemical analyses of essential oils

After 6 months growth, essential oil constituents were analyzed in *A. umbelliformis* (*B2*) plants by a semi quantitative analysis. Oils were isolated from dried aerial parts of *A. umbelliformis* (0.3 g) by a Likens–Nickerson hydrodistillation system with distilled dichloromethane for 1 h, and octan-2-one was added as an internal standard to normalize the extraction and quantification processes (Likens and Nickerson 1964). The extracts were dried over anhydrous sodium sulfate and concentrated to 1 ml with a Kuderna-Danish apparatus. Samples were frozen at −20°C before being analyzed. Gas chromatography/mass spectrometry analyses were carried out on two distinctive systems: (1) Shimadzu GCMS QP2010 system provided with an AOC-20i AutoInjector and a J&W DB 5MS UI capillary (30 m, 0.25 mm internal diameter, 0.25 μm film thickness) and (2) Agilent 6890GC provided with an Agilent MSD5973N detector, Agilent 7683 autosampler, and a J&W DB Wax capillary (30 m, 0.32 mm internal diameter, 0.5 μm film thickness).

For the Shimadzu system, the analytical conditions were: injection mode, splitless; injector temperature, 240°C; transfer line temperature, 320°C; ion source temperature, 200°C; carrier gas, helium; flow rate, 1.16 ml/min in constant flow mode; temperature program, 40°C to 320°C with 6°C/min. The MS detector was in electron impact ionization mode (EI) at 70 eV, the scan rate was 1,500 atomic mass unit/s and the mass range was *m*/*z* 25–400. For the Agilent system, the analytical conditions were: injection mode, splitless; injector temperature, 240°C; transfer line temperature, 250°C; ion source temperature, 230°C; carrier gas, helium; flow rate, 1.5 ml/min in constant flow mode; temperature program, 40–240°C with 4°C/min. The MS detector was in electron impact EI at 70 eV, the scan rate was 1,605 atomic mass unit/s, and the mass range was *m*/*z* 29–350. Both autosamplers used the same injection method. The injection cycle was: two solvent washes, five sample pumps, 1 μl injection, then two solvent washes. Three injections from each essential oil samples were performed. The results were all processed by Agilent MSD ChemStation software. Essential oils compounds were identified by comparison of both their linear retention indices, calculated versus a C_8–C_{30} hydrocarbon mixture, and their mass spectra with those of mass spectrometry database or with published data (Rubiolo et al. 2009).

Statistical analyses

Data were processed by analysis of variance (one-way ANOVA) for repeated measures using the SuperANOVA program (version 1.11, Abacus Concepts Inc., Berkley, CA, USA). Significant differences between means were determined by the Student−Newman−Keuls test ($P<0.05$).

Results

AM fungal root colonization in *A. umbelliformis*

A. umbelliformis root colonization by AM fungi was investigated in one acid (pH 5.34) and four alkaline (pH from 7.98 to 8.73) soils collected in French alpine areas located between 2,100 and 2,900 m a.s.l. Soil analysis established low available *P* values in each site (Table 1). Both wild and cultivated plants formed AM with a significant higher colonization intensity in natural plants from site 1. The

mycorrhizal frequencies (*F*%) revealed the presence of active fungi in the soil with an average of 23.9±14.3 across sites (Table 2). An important variability in the levels of root colonization was observed within sites and the AM fungal colonization intensity (*M*%) remained quite low among sites with an average of 13±10.2% (Table 2). The soil pH did not seem to have an influence on AM fungal colonization with no significant differences between acid and alkaline soils for sites 2, 3, 4, and 5. Levels of AM fungal colonization of *A. umbelliformis* from trap cultures in greenhouse (3 months) also exhibited low values with no significant differences between natural and cultivated soils (Table 3). However, mycorrhizal intensity increased with time in trap culture roots and after 8 months of culture, it reached between 38±12% (soil sites 4 and 5) and 89±6.1% (soil site 3). The greatest increase was observed in soils where *A. umbelliformis* grows naturally and at highest altitude. Moreover, mycorrhizal intensity was significantly higher in *M. truncatula* roots as compared to *A. umbelliformis* roots in trap cultures after 3 months with soil from sites 1, 2, 4, and 5 (Table 3).

The vegetation of sites 1–3 was scattered in a rocky environment with a limited amount of soil available. It was not possible to establish subcultures of the mycorrhizal fungi from these sites. On the other hand, sites 4 and 5 were grasslands with no real limitation in the top soil, and subculturing of the indigenous fungi from site 4 is in progress. For these reasons, site 4 was retained for a more thorough analysis.

Identification of spores associated with the rhizosphere of field-grown *A. umbelliformis* roots

One spore morphotype isolated from the rhizosphere of *A. umbelliformis* collected in site 4 formed small (40–100 μm diameter) yellow spores with an ornamented outer spore wall having regular and circular pits (0.2–1 μm diameter). Sequences of approximatively 970 bp long were obtained, comprising ITS3 and the 5′ end of the 25 s rDNA subunit covering the D1 and D2 variable regions. Phylogenetic analysis with the LSU sequence firmly placed all obtained sequences into the genus *Acaulospora* (Fig. 1), but being clearly distinct from all the *Acaulospora* sequences already present in public databases. Even the use of the ITS2 region did not facilitate identification of this morphotype at the species level (data not shown). Another spore morphotype found in the rhizosphere of colonized *A. umbelliformis* roots from site 4 corresponded to *G. intraradices* which was confirmed by nested PCR analyses (data not shown).

Identification of autochtonous AM fungal species associated with *A. umbelliformis* roots

Forty *A. umbelliformis* plants collected in site 4 were studied for mycorrhizal colonization using morphological and molecular approaches and 24 out of them were mycorrhized. Two different fungal morphotypes (Fig. 2) were observed: one morphotype has the distinctive morphology of *G. tenue*, characterized by very thin hyphae and small hyphal swellings as they spread intra-and intercellulary (Fig. 2a; Gianinazi-Pearson et al. 1981); the second morphotype developed «H-branched» and irregular hyphae, arbuscules and large oval vesicles (Fig. 2b). Colonization by DSE-like was very rare (data not shown). Using a nested PCR reaction with taxon-specific primers, the presence of various Glomeromycota fungi was detected in the root systems of five mycorrhizal *A. umbelliformis* plants. In four of the five plants, the presence of the *Acaulospora sp.* identified from the rhizospheric spores was detected in roots using the AS1-2 primer. The presence of *G. intraradices* was confirmed in root systems of two of the five plants and *G. claroideum/etunicatum* in three plants. On the other hand, neither *G. mosseae* nor *G. geosporum* were detected in the root system of *A. umbelliformis* from site 4. Due to the lack of molecular information on *G. tenue*, molecular detection of this fungus could not be performed. The rhizospheric soil and roots of the four *A. umbelliformis* plants colonized by native AM fungi from site 4 were mixed and used as microbial inoculum for further experiments.

T. pratense, an effective companion plant for *A. umbelliformis*

The influence of *T. pratense* as a companion plant on the mycorrhizal status and growth of *A. umbelliformis* was analyzed using the microbial inoculum (Table 4). After 3-month culture in the greenhouse, mycorrhizal colonization of

Table 2 AM fungal colonization of *A. umbelliformis* roots collected in field sites

Field sites	Colonization parameters in roots	Site 1	Site 2	Site 3	Site 4	Site 5
Roots of *A. umbelliformis*	F%	44.3±7.2 b	18.3±25.9 a	20.5±18.7 a	25.9±9.9 a	10.6±10.2 a
	M%	30.5±12.2 b	9.3±13.3 a	12.7±11.14 a	7.8±5 a	4.7±5 a

F% mycorrhizal frequency, *M%* mycorrhizal root colonization

Data represent mean root colonization by AM fungi of five plants per site

Different lowercase letters indicate differences between field sites; Student–Newman–Keuls test at $P<0.05$

Table 3 Mycorrhizal root colonization (*M*%) of micropropagated *A. umbelliformis* (*B2*) and of *M. truncatula* seedlings in greenhouse trap cultures

Origin of soil samples	Site 1	Site 2	Site 3	Site 4	Site 5
Roots of *M. truncatula* from trap cultures for 3 months	49.5±8.4 aA	37.8±8.9 aA	30.6±14.5 aB	40.8±2.7 aA	29.8±13.8 aA
Roots of *A. umbelliformis* from trap cultures for 3 months	10.3±11.3 aB	8.9±8.3 aB	14.2±15.1 aB	16.8±7.9 aB	3.6±4.6 aB
Roots of *A. umbelliformis* from trap cultures for 8 months	65.1±14.0 b	72±19.3 b	89±6.1 b	38.0±15.6 a	38.8±10.3 a

Data represent mean root colonization by AM fungi of five replicates/trap culture

Different uppercase letters in columns indicate differences between plant species and different lowercase letters in lines indicate differences between soil samples; Student–Newman–Keuls test at $P<0.05$

A. umbelliformis and *T. pratense* in monoculture were 20.8± 7% and 62±19.1%, respectively. The mycorrhizal status of *A. umbelliformis* was increased 2.4-fold in coculture with *T. pratense* as compared to a monoculture, but *A. umbelliformis* had no effect on colonization levels of *T. pratense.* Moreover, companion plant did not affect *Artemisia* plant growth by comparing total biomass in monoculture and coculture conditions. Given these results, *T. pratense* was used as companion plant for experiments to study responsiveness of *A. umbelliformis* to inoculation with microbial inoculum.

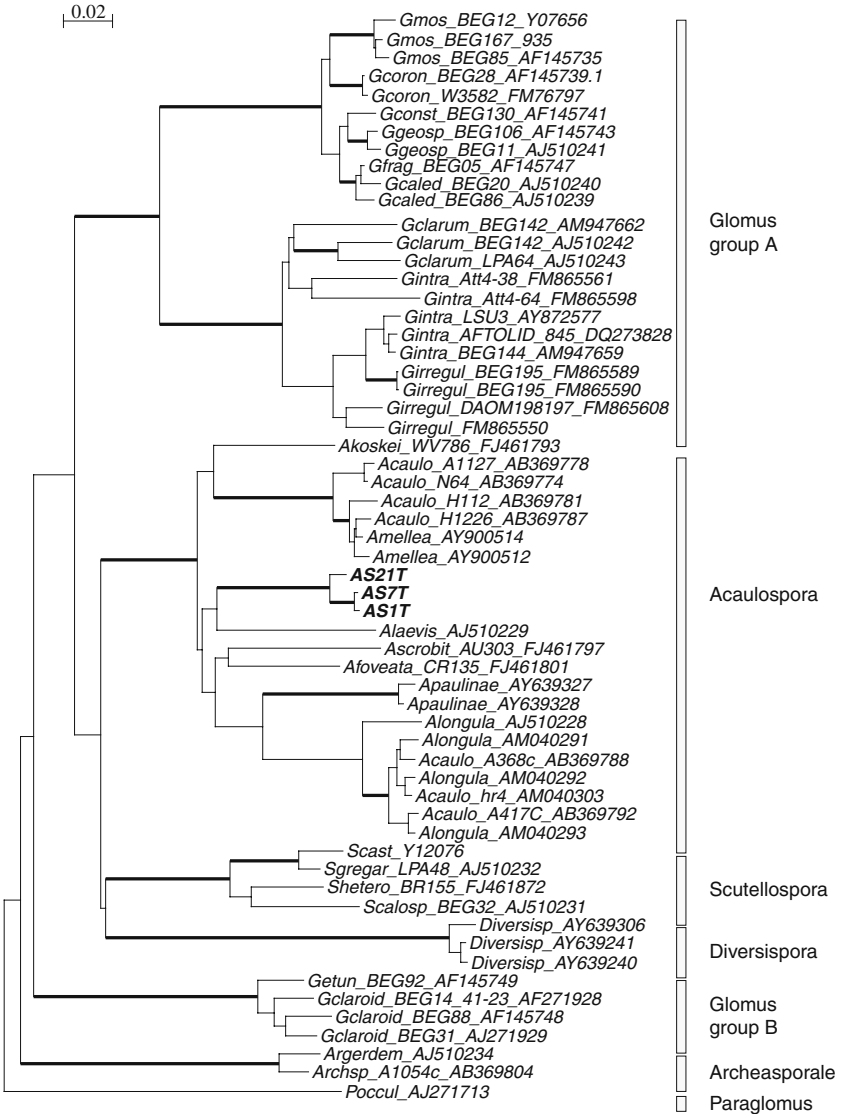

Fig. 1 Neighbor-joining tree of the LSU sequences (*bold*) of the *Acaulospora* isolated from *A. umbelliformis* roots. Branches with bootstrap values >95% are shown in heavy lines. *Paraglomus occultum* was used as outgroup

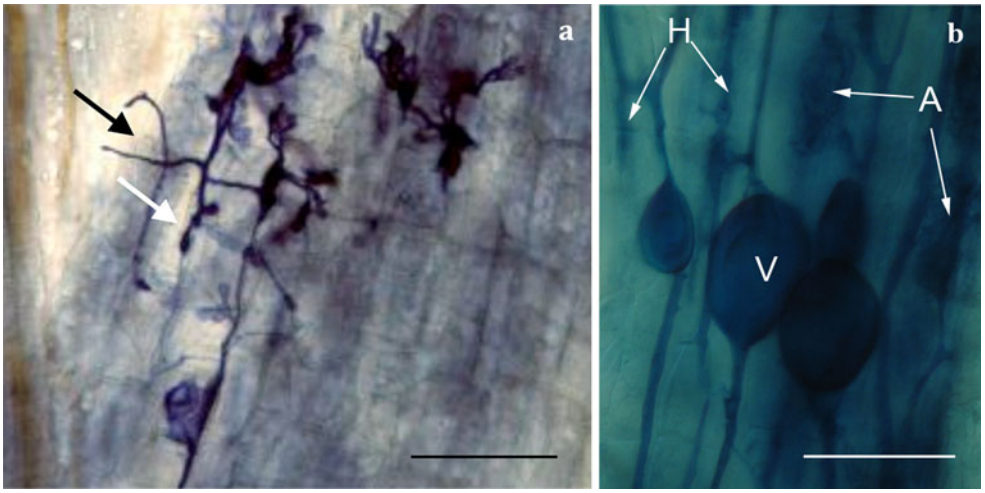

Fig. 2 Mycorrhizal structures in roots of *A. umbelliformis* from site 4 vizualized after staining with Trypan Blue. **a** *G. tenue* typically grows with thin hyphae (*black arrow*), small hyphae swellings as they spread intra- and intercellulary (*white arrows*). *Scale bar* 50 μm. **b** Development of «H-branched» and irregular hyphae (*I*, *white arrows*), arbuscules (*A*, *white arrows*) and vesicles (*V*) in cortical root cells. *Scale bar* 10 μm

Growth and physiological responses of *Artemisia* species to microbial inoculum including native AM fungi from an alpine culture site

After 3 months in the greenhouse in coculture with *T. pratense*, inoculated plants of *A. umbelliformis*, *A. glacialis*, and *A. genipi* Weber were colonized to a level of 34.44±8.4%, 50±2.5%, and 50±5.2%, respectively (Table 5). No colonization was detected in non-inoculated plants. Mycorrhizal colonization by the microbial inoculum had no positive effect on the growth of alpine wormwood plants. There were no significant differences in shoot DW and root DW between controls and mycorrhizal plants for *A. umbelliformis* and *A. glacialis*. However, for the *A. genipi* Weber plants, the shoot DW was significantly reduced (−60%) when compared to controls although root DW biomass was not changed. In contrast, the microbial inoculum induced a significantly positive growth response in leek plants (*Allium porrum* L.). At 3-month harvest, shoot DW (0.2 g) and root DW (0.065 g) in non-inoculated leeks were increased 2.5-fold and twofold, respectively, in inoculated plants. The three alpine wormwood species differed in their N and P responses to the inoculation with microbial inoculum (Table 5). Shoot N concentration was not altered in mycorrhizal plants for either *A. umbelliformis* or *A. glacialis* while a significant decrease in shoot N concentration was observed in mycorrhizal *A. genipi* Weber, plants with a reduction of 30% compared to controls. The P concentration in shoots of *A. umbelliformis* and *A. glacialis* was significantly enhanced in mycorrhizal plants compared to controls, with an increase of 1.4 and 1.7-fold, respectively. Shoot P concentration in *A. genipi* Weber was not altered by inoculation.

Table 4 Mycorrhizal root colonization (*M*%) and growth of micropropagated *A. umbelliformis* (*B2*) and *T. pratense* seedlings in 3 month-old greenhouse grown monocultures or cocultures using microbial inoculum including native AM fungi

	%*M*	Total DW (g)
A. umbelliformis in monoculture	20.8±7.6 a	0.33±0.01 a
T. pratense in monoculture	62±19.1 b	n.d.
A. umbelliformis in coculture	54.8±14.5 b	0.35±0.07 a
T. pratense in coculture	57.3±18.2 b	n.d.

Data represent mean of five plants/culture from one representative experiment

The data presented in each column followed by the same letter are not significantly different (*n.d.* not determined); Student–Newman–Keuls test at $P<0.05$

Essential oil composition

The composition of essential oils was studied in response to the microbial inoculum in shoots of *A. umbelliformis* 6 months in the greenhouse in coculture with *T. pratense*. After this period of culture, mycorrhizal colonization by microbial inoculum was maintained at a level of 39.1% and a mycorrhizal effect was still observed with an increase in P concentrations of 1.5-fold in inoculated *A. umbelliformis* plants as compared to non-inoculated plants (Table 5). The oil composition of *A. umbelliformis* samples was characterized by the presence of a wide range of monoterpenes (hydrocarbons as β-pinene, (E)-β ocimene, ketones as α-thujone and β-thujone, aldehydes as (E,E)-2,4-decadienal, ethers as 1,8-cineole, esters, alcohols, and oxides), and sesquiterpenes (hydrocarbons as β-caryophyllene and germacrene D, oxides and alcohols) with a total of 23 compounds identified (Table 6). Chemical analyses showed that in *A. umbelliformis* shoots, α-thujone was the most abundant oil, followed by β-thujone, hexadecane and all the other oils in decreasing order (Table 6). The three volatile compounds (E)-β-ocimene, (E)-2-decenal and (E, E)-2-4-decadienal which were minor components of the essential oils were significantly affected by the microbial inoculum. The relative percentage of the monoterpene (E)-

Table 5 Growth, nutrient uptake and root colonization of three micropropagated *Artemisia* species in coculture with *T. pratense* in response to microbial inoculum including native AM fungi in greenhouse

		A. umbelliformis (*B2* genotype)		*A. glacialis* (*G1* genotype)		*A. genipi Weber* (*W4* genotype)	
		Control	Inoculated	Control	Inoculated	Control	Inoculated
3 months	Shoot DW (g)	0.29± 0.13 a	0.19±0.03 a	0.14±0.06 a	0.08±0.01 a	0.19±0.03 b	0.08±0.00 a
	Root DW (g)	0.07±0.02 a	0.09±0.01 a	0.05±0.02 a	0.03±0.00 a	0.03±0.00 a	0.03±0.00 a
	Shoot N (mg g^{-1}. DW)	27.16±4.14 a	25.23±1.34 a	26.7±30 a	24.3±1.50 a	33.93±1.83 b	22.5±2.68 a
	Shoot P (mg g^{-1}. DW)	4.1±0.90 a	5.9±0.64 b	3.22±0.10 a	5.66±1.68 b	5.44±0.49 a	6.18±1.01 a
	M%	0±0.0 a	34.4±8.4 b	0±0.0 a	50±2.5 b	0±0.0 a	50±5.2 b
6 months	Shoot DW (g)	0.39±0.12 a	0.29±0.10 a				
	Root DW (g)	0.15±0.05 a	0.10±0.03 a				
	Shoot N (mg g^{-1}. DW)	31.64±1.43 b	29.02±1.45 a				
	Shoot P (mg g^{-1}. DW)	4.50±0.64 a	6.57±0.69 b				
	M%	0±0.0 a	39.1±3.1 b				

Data represent the mean of ten plants per treatment from one representative experiment. For each plant species, the data presented in each line followed by the same letter are not significantly different; Student–Newman–Keuls test at $P<0.05$

β-ocimene was increased by 1.3-fold and those of (E)-2-decenal and (E,E)-2-4-decadienal reduced by half indicating slight changes in the relative quantities of single volatile compounds in mycorrhizal plants compared to control plants.

Discussion

The present work is the first report on responsiveness of *A. umbelliformis*, an alpine *Artemisia* species to inoculation with microbial communities including native AM fungi

Table 6 Percentage composition of oil extracts from *A. umbelliformis* shoots in response to microbial inoculum including native AM fungi in greenhouse conditions

Compound	Control	Inoculated
β-Pinene	0.17±0.01 a	0.18±0.04 a
2-Heptanone	0.20±0.02 a	0.21±0.02 a
Heptanal	0.13±0.03 a	0.16±0.02 a
Dodecane	0.65±0.04 a	0.67±0.05 a
1,8-Cineole (eucalyptol)	0.55±0.03 a	0.54±0.06 a
(E)-β-ocimene	**0.32±0.04** a	**0.42±0.04** b
1-Dodecene	0.06±0.01 a	0.08±0.02 a
Tetradecane	1.07±0.11 a	1.11±0.14 a
α-Thujone	60.79±2.87 a	63.75±3.26 a
β-Thujone	16.39±0.93 a	15.54±1.35 a
Cis sabinene hydrate	1.64±0.26 a	1.14±0.49 a
Trans sabinene hydrate	0.50±0.06 a	0.37±0.17 a
(E,E)-3,5-Octadien-2-one	1.63±0.16 a	1.58±0.28 a
Hexadecane	8.31±3.20 a	7.41±6.85 a
β-Caryophyllene	1.59±0.99 a	1.05±0.18 a
Terpinen-4-ol	0.85±0.85 a	1.40±0.85 a
(E)-2-decenal	**2.75±0.43** b	**1.55±0.64** a
Germacrene-D	0.41±0.12 a	0.63±0.28 a
(E,E)-2,4-Decadienal	**0.96±0.13** b	**0.58±0.29** a
Octadecane	0.31±0.11 a	0.29±0.06 a
Methyl hexadecanoate	0.30±0.15 a	0.29±0.09 a
Hexadecanoic acid, isopropyl ester	0.31±0.12 a	0.39±0.11 a
Ethyl hexadecanoate	0.99±0.33 a	1.26± 0.56 a

Data represent the mean of five replicates/treatment and three repetitions per replicate from two independent experiments. Data (shown in bold type) in the same line followed by different letters are significantly different; Student–Newman–Keuls test at $P<0.05$

from one alpine culture site. In the five Southern French Alps sites studied, *A. umbelliformis* formed AM associations irrespective of the soil pH in the calcareous bedrock or cultivated grassland zones where mineral soils were poor in phosphorus nutrient. However, relatively low levels of mycorrhizal colonization were observed under the different natural or agricultural conditions. The other *Artemisia* species *A. glacialis* and *A. genipi* Weber growing in the study area also showed low AM fungal colonization, comparable to that of *A. umbelliformis* (data not shown). Our results are consistent with those reported by Read and Haselwandter (1981) with 10% of AM infection in *A. umbelliformis* and no AM in *A. genipi* Weber in the Tyrolean Central Alps in Austria. Even under greenhouse conditions, AM fungal colonization was only lightly developed in *A. umbelliformis* after 3 months in the trap cultures. The dramatic increase after 8 months may reflect the presence of slow colonizing AM fungal species requiring time to be detected in the roots (Hart and Reader 2002) or it may also be due to the presence of less infective structures (spores or infective hyphae) in these inocula. Impact of the growth condition present in a greenhouse, on the ability of some AM fungi to colonize plant roots has already been reported (Sýkorová et al. 2007). The higher AM colonization rate of *M. truncatula* compared to that obtained by *A. umbelliformis* in trap cultures suggests that the low AM levels in the latter is inherent to the plant species.

Among the existing vascular plants that have been examined for the presence of AM, plant species range from non-mycorrhizal to obligate, through facultative, dependency on mycorrhizal associations in some environmental settings (Johnson et al. 1997). The present observations indicate that, in the absence of other surrounding plants, *A. umbelliformis* may to be a low mycorrhiza-dependent species, as reported for other alpine plants (Read and Haselwandter 1981; Trappe 1988). In any given interactions between AM fungi and host plants, the colonizing ability of the AM fungal species as well as the AM sensitivity of the host plant must be taken into account as well as the environmental factors influencing both (Johnson et al. 1997; Hart and Reader 2002). Colonization with native AM fungi was enhanced in *A. umbelliformis* roots by coculture with *T. pratense* under greenhouse conditions; coexistence with a highly mycorrhizal species such as *T. pratense* may have a positive impact on the AM mycelium networks so increasing the fungal propagation and density. These results are consistent with previous investigations using nurse plants as a donor plant of AM fungi. Chen et al. (2005) reported that highly mycorrhizal species *Kummerowia striata*, can increase the colonization rate of poorly mycorrhizal species *Digitaria ciliaris*, while poorly mycorrhizal species *D. ciliaris* have either no effect (*K. striata*) or negative effects on highly mycorrhizal species (*Ixeris denticulate*). In field experiment, Püschel et al. (2008) reported that highly mycotrophic annual *Tripleurospermum inodorum* enhanced the proliferation of AM fungi, which resulted in increased colonization of facultative mycotrophic *Arrhenatherum elatius* or even non-mycotrophic annual *Atriplex sagittata*. In pot experiment, Kovárová et al. (2010) showed that the melilot acts as a donor plant of mycorrhizal fungi and stimulate the production of resveratrol and its derivatives in the knotweed *Reynoutria x bohemica*.

Several native AM fungal isolates coexisted in the plant root systems of *A. umbelliformis* cultivated in a grassland site at 2,300 m a.s.l., indicating a potential adaptation of these autochtonous AM fungal populations to alpine environmental conditions. A part of the AM fungi community associated to *A. umbelliformis* roots was identified as *G. tenue*, *G. intraradices*, *G. claroideum/etunicatum*, and a new *Acaulospora* species. Haselwandter and Read (1980) reported a progressive increase in root colonization by *G. tenue* compared to AM fungi in parallel to increases in altitude in the Austrian Alps, which indicates that this fungal taxon is well adapted to plants with a short growing season and that it encompasses symbionts that are more successful under adverse conditions. A large diversity of AM fungal species has been described in Swiss alpine regions extending from 1,000–3,000 m a.s.l. (Oehl et al. 2006). Species belonging to the *Acaulospora* genus were found particularly present, and relatively much more abundant than in the lowlands of Switzerland. A new *Acaulospora* species, under the epithet, *Acaulospora alpina* was found exclusively in the Swiss Alps at altitudes> 1,300 m a.s.l. (Oehl et al. 2006) but no sequence similarity was found with this or other well identified *Acaulosporaceae* from LSU sequences available in databases. The presence of DSE was very rare in *A. umbelliformis* roots growing above 2,000 m in contrast to their high abundance and varied effects reported in several studies on plants growing in alpine situations (Mandyam and Jumpponen 2005).

In the present work, the impact of the entire microbial inoculum containing AMF community, enabled to compare several mycorrhizal and non-mycorrhizal *Artemisia* species. AM colonization did not improve the growth of *A. umbelliformis* and *A. glacialis* (non-responsive species) and growth depression was observed in shoots of AM *A. genipi Weber* plants (negatively responsive species). Nevertheless, the AM plant leeks showed a positive mycorrhizal growth response to the same microbial inoculum, revealing a possible physiological specificity of the relationship of the mycorrhizal isolates with the host genotype. Results are in agreement with other reports of growth depression in AM plants for a wide variety of species

(Tawaraya 2003; Sudovà 2009) although increased growth and development upon AM colonization was reported for many plant species (Smith and Read 1997). Recently, Grace et al. (2009) reported that AM inhibition of growth in barley (*Hordeum vulgare*) was not attributed to the extent of root colonization, fungal phosphorus uptake or effects on the expression of plant phosphate transporter genes.

The three alpine *Artemisia* species responded differently at the level of P nutrition to inoculation with microbial inoculum under greenhouse conditions. Both mycorrhizal *A. umbelliformis* and *A. glacialis* plants that are non-responsive in terms of weight had higher P concentrations than non-mycorrhizal plants. Smith et al. (2009) suggested another level of regulation, or limiting factor, that prevents the improved P nutrition from being immediately converted to additional vegetative biomass. The present experiments did not show benefits for two *Artemisia* species in terms of plant growth and for nutrition in *A. genipi Weber*, which may suggest other benefits of the symbioses such as resistance to disease expression (Smith and Read 2008). This underlines the necessity to consider all aspects and functions of mycorrhizas in addition to implications in growth and nutrition, such as effects on secondary metabolic pathways (Vosatka and Dodd 2002; Copetta et al. 2006).

Volatile organic components in shoots of *A. umbelliformis* contained monoterpenes and sesquiterpenes, which reflect those previously found in flower essential oils of this species (Rubiolo et al. 2009). Mycorrhiza-related effects were observed on levels for (E)-β-ocimene which was significantly higher in mycorrhizal plants and E-2-decenal and (E,E)-2-4-decadienal which were both lower in mycorrhizal plants. The monoterpene (E)-β-ocimene, wich is a defensive compound in *Plantago lanceolata*, did not differ in its relative release rate between herbivore-damaged mycorrhizal and non-mycorrhizal plants (Fontana et al. 2009). In *Artemisia annua*, Rapparini et al. (2008) reported that mycorrhization did not influence the amount of total terpenes, while it did affect single terpene production and emission. The effectiveness of AM fungi in increasing the production of essential oils has been demonstrated in several aromatic plant species (Gupta et al. 2002; Kapoor et al. 2002a, b; Khaosaad et al. 2006; Freitas et al. 2004; Copetta et al. 2006; Kappoor et al. 2007; Chaudhary et al. 2008). The increase in essential oil concentration in AM plants has been attributed either or not to enhanced P-nutrition depending on the plant species or genotype (Chaudhary et al. 2008). However, the significance of the altered composition of essential oils in mycorrhizal aromatic plants requires further investigations.

In conclusion, *T. pratense* as a companion plant impacted positively on mycorrhization of *A. umbelliformis* by microbial inoculum including native AM fungi from alpine grassland site. Mycorrhizal *A. umbelliformis* showed benefits in terms of P nutrition in shoots indicating fungal contribution to nutrient uptake. The relative proportion of volatile components was modified for three compounds in shoots of mycorrhizal *A. umbelliformis*.

Studies of mycorrhizal responses under edaphoclimatic conditions with measurements of P accumulation and flower essential oil production are needed for field application. Inoculation experiments with different genotypes of *A. umbelliformis* and with other sources of fungi will also be useful to extend data on mycorrhizal responses in alpine *Artemisia* species. Sub-cultures of native AM fungal isolates from this original microbial inoculum are in progress to produce pure indigenous inoculum and to test its performance in field.

Acknowledgments We are thankful to the Region Bourgogne for financial support. We gratefully acknowledge Raymonde Gesseaume for her technical work and Bruno Gonnon and Eric François for use of their production sites for soil and plant samples. Vivienne Gianinazzi-Pearson and Daniel Wipf are thanked for critical reading of the manuscript.

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