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Degradation of seed mucilage by soil microflora promotes early seedling growth of a desert sand dune plant

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ABSTRACT

In contrast to the extensive understanding of seed mucilage biosynthesis, much less is known about how mucilage is biodegraded and what role it plays in the soil where seeds germinate. We studied seed mucilage biodegradation by a natural microbial community. High-performance anion-exchange chromatography (HPAEC) was used to determine monosaccharide composition in achene mucilage of *Artemisia sphaerocephala*. Mucilage degradation by the soil microbial community from natural habitats was examined by monosaccharide utilization tests using Biolog plates, chemical assays and phospholipid fatty acid (PLFA) analysis. Glucose (29.4%), mannose (20.3%) and arabinose (19.5%) were found to be the main components of achene mucilage. The mucilage was biodegraded to CO_2 and soluble sugars, and an increase in soil microbial biomass was observed during biodegradation. Fluorescence microscopy showed the presence of mucilage (or its derivatives) in seedling tissues after growth with fluorescein isothiocyanate (FITC)-labelled mucilage. The biodegradation also promoted early seedling growth in barren sand dunes, which was associated with a large soil microbial community that supplies substances promoting seedling establishment. We conclude that biodegradation of seed mucilage can play an ecologically important role in the life cycles of plants especially in harsh desert environments to which *A. sphaerocephala* is well-adapted.

Key-words: *Artemisia sphaerocephala*; adaptation; moving sand dunes; mucilage biodegradation; plant–microbe interaction; polysaccharide; soil microbial community.

INTRODUCTION

Plants are adapted to numerous environmental stresses and thereby can survive in a diverse range of habitats. In angiosperms, the extremely diverse external surface of seed coats reflects a multiple adaptation to seed dispersal and germination in different environments (Fahn 1990). Upon imbibition of water, seeds commonly produce a pectinaceous mucilage (myxospermy), which has been shown to have multiple functions in seed maturation, dispersal and germination. In seed maturation, the mucilage of species such as *Cavanillesia platanifolia* permits seeds within fruits to be fully mature until the beginning of the rainy season (Garwood 1985; Thapliyal *et al.* 2008). In seed dispersal, mucilage creates strong adherence to the soil surface once it is dehydrated. This prevents further dispersal of the seed by rain and wind and by ants or other seed predators (Gutterman & Shem-Tov 1997; Huang, Gutterman & Hu 2000; Huang, Gutterman & Osborne 2004). At germination and early seedling growth, mucilage initiates or enhances seed germination by retaining moisture needed for imbibition (Hedge 1970; Swarbrick 1971; Garwood 1985). It also minimizes water loss by enlarging the area of contact of between seed and soil (Harper & Benton 1966; Witztum, Gutterman & Evenari 1969; Gutterman, Witztum & Heydecker 1973; Grubert 1974; Evans, Young & Hawkes 1979). Additionally, our recent reports have shown that mucilage aids seed germination in osmotically stressful and saline habitats of the desert environment (Yang, Dong & Huang 2010) and that mucilage assists seed cells in maintaining DNA integrity when hydrated in desert dew (Yang *et al.* 2011).

In the past decade, steps in the synthesis of seed mucilage during seed development have been investigated using *Arabidopsis thaliana* as a model plant. As a result, the structure, biosynthesis, tissue differentiation and genes involved in the synthesis of seed mucilage have been identified (for a review, see Western 2006). *Arabidopsis* seed mucilage is composed primarily of complex and structurally diverse polysaccharides (pectin), with homogalacturonan (HG) and rhamnogalacturonan I (RG-I) as two key components (Western, Skinner & Haughn 2000; Penfield *et al.* 2001; Usadel *et al.* 2004; Western *et al.* 2004; Macquet *et al.* 2007). Pectinaceous mucilage consists of an outer water-soluble layer and an inner adherent layer; the latter being separated into two domains with the internal domain containing cellulose (Macquet *et al.* 2007). The production of mucilage by epidermal cells is a rather complex developmental process that occurs during seed coat cell growth (Western *et al.* 2000, 2001, 2004; Windsor *et al.* 2000; Dean *et al.* 2007; Young *et al.* 2008).

Despite a large number of studies describing the protective roles of mucilage in seed dispersal and germination, relatively little is known about the role of mucilage within

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the actual soil where seeds germinate and seedlings grow, especially its effect on the soil microbial community and seedling growth *per se*. Moreover, in contrast with our detailed understanding of mucilage biosynthesis, little is known of how the mucilage is biodegraded and how this process affects early seedling growth in the natural environment. Knowledge of the process of mucilage biodegradation would help interpret its ecological and adaptive significance.

Seeds of *Artemisia sphaerocephala* Kraschen. (Asteraceae) produce large quantities of mucilage. Up to 35% of seed dry mass is comprised of mucilage (Yang *et al*. 2010). This species has strong resistance to wind erosion, drought, cold and saline-alkaline soil conditions and is one of the most important pioneer plants of the moving and semi-stable sand dunes in the deserts and steppes of northwest and north-central China (Editorial Board of the Flora of China of Chinese Academy of Sciences 1991). Our previous reports have shown that the achene mucilage of *A. sphaerocephala* is ecologically multifunctional. It can help to maintain DNA integrity in desert dew and aid seed germination in osmotically stressful and saline habitats (Huang *et al*. 2008; Yang *et al*. 2010). However, the mucilage around the seeds appears to be stable, and no enzymatic cleavage or hydrolysis of the mucilage or any products that might be utilized during germination heretofore have been detected (Huang *et al*. 2004). Thus, the question arises as whether the large amount of mucilage (up to 35% of seed mass) has a function that is limited simply to aiding seed hydration. We hypothesized that mucilage has an additional function of providing an indirect source of nutrition during seedling development in the barren desert soil and that the effect is mediated by soil microbial biodegradation. Testing this hypothesis required establishing dynamic changes in the soil microbial community during mucilage biodegradation. The work provides a quantitative demonstration of seed mucilage biodegradation by the microbial community of natural soil and identifies its promoting effect on early seedling growth.

MATERIALS AND METHODS

Achene and soil collection

Freshly matured achenes of *A. sphaerocephala* were collected from dry, unopened infructescences in December 2009 from natural populations in moving sand dunes in Yulin, Shaanxi Province, north-central China. In the laboratory, infructescences were manually shaken to detach the achenes, which were stored dry in a closed cotton bag at 4 °C until used. At the same time, soil was collected under shrubs at three randomly chosen natural habitats of *A. sphaerocephala* in moving sand dunes (38°35′06″N, 109°37′33″E; 38°30′19″N, 109°35′14″E; 38°23′11″N, 109°34′21″E; 1197–1205 m a.s.l.). In each habitat, three soil cores 20 cm deep were collected at random using a sharp-edged metal cylinder with an inner diameter of 8 cm. The cores were thoroughly homogenized, sieved (2 mm mesh size) and placed into plastic bags and transported to the laboratory in a portable cold-box (Bethesda Research Laboratories, Gaithersburg, MD, USA) within 24 h before being stored at 4 °C.

Monosaccharide composition of mucilage

The preparation of mucilage samples (1 mg) for high-performance anion-exchange chromatography (HPAEC) followed Dean *et al*. (2007). Sugar standards included neutral sugars (rhamnose, arabinose, galactose, glucose, xylose and mannose) and acid sugars (glucuronic acid and galacturonic acid). The identity and concentration of monosaccharide sugars were determined using HPAEC analysis on a Dionex ICS 3000 HPAEC-PAD system with a CarboPac PA20 carbohydrate column (Dionex Corp., Sunnyvale, CA, USA). A 15 μL sample was injected and gradient elution was performed according to Anumula & Taylor (1991). The separation consisted of 3% NaOAc buffer isocratic (pH 5.5) for 5 min followed by a linear increase to 10% at 30 min in constant 15% of 0.5 M NaOH. At the end of each run, the column was regenerated by washing with 90% NaOH and 10% NaOAc buffer for 10 min followed by equilibration with initial conditions for 10 min. The HPAEC analysis was repeated six times. Chromeleon Client software (version 6.11) from Dionex was used for data acquisition and processing.

Substrate utilization by soil microbial community

The capability of the soil microbial community to utilize monosaccharides in the mucilage was assessed using Biolog GN2 plates (Biolog Inc., Hayward, CA, USA) by a procedure adapted from Garland & Mills (1991). In the Biolog plate, respiration of sole carbon sources is indicated by colour produced from the reduction of tetrazolium violet (Garland & Mills 1991). Each plate contained 95 different sole carbon sources and a control (blank well) in a 96 well microplate. The carbon sources included seven of the eight monosaccharides identified in HPAEC (glucose, mannose, arabinose, galactose, rhamnose, glucuronic acid and galacturonic acid). The rate of utilization of the carbon sources was indicated by the chemical reduction of tetrazolium, a redox indicator dye, which changes from colourless to purple. Three GN2 plates were prepared for soil samples from each habitat (10 g soil serially diluted) with 150 μL aliquots of a final dilution of 1:1000 added to each of the 96 wells per plate. The plates were incubated at 25 °C in darkness. Optical density (OD) was read on a plate reader (Labsystems Dragon Wellscan MK3, Thermo Labsystems, MA, USA) at 590 nm every 24 h for 11 d, and data were processed in a Genesis Labsystems (version 3.03). OD readings were corrected for the control (blank) well on each plate before data analyses. Average well colour development (AWCD) was calculated as the average OD across all wells

per plate and used to indicate the general microbial activity of each soil sample (Larkin 2003).

CO_2 evolution during mucilage degradation in soil

Soil samples (moisture contents 3.14 ± 0.14% unless otherwise stated) of 20 g each were placed in 250 mL glass flasks. Dry mucilage (2 mg) peeled from intact achenes was added to each bottle and mixed thoroughly into the soil. A soil without mucilage addition (S), a sterilized soil (previously heated to 121 °C for 240 min) and a sterilized soil with mucilage addition (St + M) were also included for each habitat. Thus, there were 96 flasks in this experiment (i.e. 2 achene types × 2 soil types × 3 replicates). After conditioning for 1.5 h, the bottles were sealed with rubber stoppers and incubated at 25 °C in darkness. Every 24 h for 8 d, gas samples (10 mL) were taken from flasks with gas-tight syringes after piercing through the stoppers. After sampling, the flasks were aerated for 10 min with air and re-sealed. The gas was immediately analysed for CO_2 on an HP 5890 Series II gas chromatograph equipped with a Chromosorb 102 column (Hewlett-Packard, Palo Alto, CA, USA). The carrier gas was ultra-high purity helium, and a thermal conductivity detector (TCD) was set at 220 °C. Data acquisition and processing were performed with HW-2000 Chromatography software (Qianpu software Co., Ltd, Shanghai, China), and CO_2 concentrations were calculated using peak area of the samples compared with CO_2 standard peak areas.

Soluble sugar dynamics during mucilage degradation in soil

Soil samples of 10 g each were placed in 50 mL glass beakers and 2 mg dry mucilage peeled from achenes was then added to each beaker and mixed thoroughly into the soil. An S, a sterilized soil and a St + M were also included in the treatments for each habitat. Thus, there were 96 beakers in this experiment (2 achene types × 2 soil types × 8 d × 3 replicates). The beakers were sealed with a breathable plastic membrane that provided a sterile barrier, to reduce the rate of evaporation, and incubated at 25 °C in darkness. Twelve beakers were sampled every 24 h for 8 d, at which time 4 mL of Millipore water was added to each beaker. After vortexing for 3 min, mixtures in the beakers were transferred to test tubes and centrifuged at 6869 *g* for 3 min (Sigma Centrifuge, Germany). After centrifugation, the undegraded mucilage remained in the pellet and the degraded mucilage in the supernatant. Soluble sugar concentrations in the supernatants were determined colourimetrically. Phenol–sulfuric acid assays were used to determine soluble sugar content (DuBois *et al*. 1956). The absorbance was determined by an ultraviolet (UV)-visible spectrophotometer with data processing software (UV-2550, Shimadzu Corp., Kyoto, Japan).

Phospholipid fatty acid (PLFA) analysis of microbial biomass during mucilage degradation

We quantified viable microbial biomass during mucilage degradation using the PLFA technique (*sensu* Zak *et al*. 2003). The quantity of total PLFAs (<20:0) is directly proportional to microbial biomass (Zelles *et al*. 1995). PLFAs are the lipids of the microbial membranes and have been used as biomarkers for specific groups of microorganisms to monitor rapid changes in soil microbial community structure (Zelles 1999). In addition, total concentration of PLFAs can be used as a measure of viable microbial biomass (Zelles 1997). There were 2 achene types × 2 soil types × 8 d × 3 replicates = 96 soil samples in the analysis. Soil samples (8 g dry weight) from each treatment were extracted with a single-phase, phosphate-buffered $CHCl_3$–CH_3OH solvent. The extracted lipids were then separated into functional classes using Cleanert™ solid phase extraction (Agela Techologies Inc., Newark, DE, USA). The polar lipid fraction was transesterified into fatty acid methyl esters (FAMEs) using a mild-alkaline system containing methanol. The resulting FAMEs were separated and identified using an Agilent 6890 GC interfaced to an Agilent 5973 mass selective detector (Agilent, Schaumburg, IL, USA). FAME 19:0 was used as an internal standard.

Fluorescence microscopy of mucilage polysaccharide absorption by seedlings

Dry mucilage (10 mg) peeled from achenes was labelled with fluorescein isothiocyanate (FITC) according to De Belder & Granath (1973) and Schmidgall & Hensel (2002). FITC has a high quantum yield. Approximately half of the absorbed photons is emitted as fluorescent light (520 nm), and it has been widely used for conjugation to antibodies, proteins and polysaccharides. After labelling, mucilage was precipitated with ethanol (final concentration 80%, v/v), and the precipitate was dialysed for 10 d by excessive dialysis against Aqua Millipore® using Spectra/Por® Membrane Kit (MWCO 3500, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 8 °C for purification from unreacted marker and reagents. The labelled mucilage was added to the germinated achenes in plastic containers (10 × 10 × 20 cm) previously filled with 200 g soil from the natural habitat of *A. sphaerocephala*. The seedlings were cultivated at 25 °C in continuous fluorescent light (100 μmol m^{-2} s^{-1}). After 1, 3 and 5 d of growth, they were retrieved from the containers, washed with Aqua Millipore® three times and their roots, shoots and leaves sectioned and mounted onto microscope slides. Slides were viewed with an inverted fluorescence microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Tokyo, Japan) equipped with a Nikon INTENSILIGHT C-HGFI lamp. Fluorescence intensity of images was measured using the measurement tool of ImageJ 1.44p software (National Institutes of Health, Bethesda, MD, USA). The data were collected from randomly chosen seedlings. This experiment was performed

in triplicate to verify the results, and mucilage without FITC labelling was included as controls.

Effect of mucilage degradation on seedling growth and biomass accumulation

To detect the effects of the mucilage biodegradation process on seedling growth, the mucilage of *A. sphaerocephala* achenes was manually peeled from the achene coat using a surgical scalpel under a Nikon type 104 projection microscope (Nikon Instruments Inc.) as described previously (Huang *et al*. 2008; Yang *et al*. 2010). Batches of intact achenes and those from which the mucilage had been removed (demucilaged) were incubated at 25 °C in continuous fluorescent light (100 μmol m^{-2} s^{-1}). When radicle emergence occurred, two types of newly germinated achenes (i.e. intact and demucilaged) were assigned to the following treatments: (1) intact achenes growing in natural soil; (2) demucilaged achenes growing in natural soil; (3) intact achenes growing in sterilized soil; and (4) demucilaged achenes growing in sterilized soil. Germinated achenes were transplanted into plastic containers ($10 \times 10 \times 20$ cm) previously filled with 200 g soil from their natural habitats. The containers were then sealed with breathable plastic membrane. The seedlings were grown at 25 °C in continuous fluorescent light (100 μmol m^{-2} s^{-1}) in a growth chamber. There were 12 germinated achenes (replicates) for each treatment, six of which were harvested at 10 d of growth and six at 30 d. At harvest, root and shoot lengths of seedlings retrieved from each treatments were measured to the nearest millimeter with a ruler. Root and shoot dry weights of seedlings also were determined using an analytical balance (BS221S, Sartorius Group, Göttingen, Germany) after oven-drying at 75 °C for 48 h.

Data analysis

Data in the tables and figures are presented as arithmetic means ± SE. Data were analysed by analysis of variance (ANOVA) followed by multiple comparisons, and differences between means were considered significant at the $P < 0.05$ level. When variances were not homogeneous, data were log-transformed to improve normality and homogeneity of variances before subjecting them to statistical analysis. All statistical procedures were performed using SPSS Version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

A discriminant analysis (DA) was independently performed for PLFAs using SPSS Version 15.0 for Windows. The main purpose of the DA was to confirm that changes in culture treatments had significantly changed the soil microbial community structure, so that soil samples could be correctly classified by treatments. The discriminant functions produced probability of membership of a given sample in each respective treatment. A given sample was assigned to individual treatment with the highest probability.

RESULTS

Monosaccharide composition of mucilage

HPAEC was used to quantify the monosaccharide composition of *A. sphaerocephala* achene mucilage after extraction with Na_2CO_3 and hydrolysis with sulfuric acid. The contents of six neutral sugars and two acid sugars present in achene mucilage were determined by this method (Fig. 1a). Among neutral sugars, the amount of glucose was (on a concentration basis) highest (29.4% of total identified sugars), followed by those of mannose (20.3%) and arabinose (19.5%). These three sugars accounted for approximately 70% of the total identified sugars. By contrast, the amount of rhamnose was very low (<1%). The amounts of the two acid sugars were also low (5.7%), and the amount of glucuronic acid was much greater (more than ten-fold) than that of galacturonic acid (Fig. 1b).

Substrate utilization by soil microbial community

As the Biolog GN2 plate contained seven of the eight identified sugars in the HPAEC experiment, it was a suitable tool to use to make a preliminary assessment of the capacity of microbial communities from natural habitats to utilize the sugars in achene mucilage. Colour development (OD) of the seven wells in the plates all increased with incubation time and generally followed a sigmoidal curve except for L-arabinose, which had a lower OD throughout the incubation (Fig. 2). OD values for the microbial communities from the three habitats were not different from each other for any of the seven sugars. This indicated that the soil microbial communities from each of the three habitats have similar capacities to utilize these sugars.

To determine the capacity of soil microbial communities from natural habitats to utilize a variety of single carbon sources, AWCD was calculated for each plate during 11 d incubation (Table 1). AWCD values increased with incubation time in all plates ($F = 606.273$, $P < 0.001$). AWCD values of microbial communities from the three habitats were not significantly different from each other ($F = 0.683$, $P = 0.509$). As AWCD represents the overall substrate utilization of a microbial community, this result indicates a similarity in general microbial activity across different habitats. Therefore, for simplicity of explanation soil samples from the three habitats were considered as replicates in the subsequent experiments.

CO_2 evolution during mucilage degradation in soil

The results of the test with Biolog plates prompted us to speculate that mucilage polysaccharide can also be utilized by the soil microbial community in the natural habitat. To test this possibility, mucilage was added to natural soil or to sterilized soil, and CO_2 evolution was determined by gas chromatography during incubation (Fig. 3). CO_2

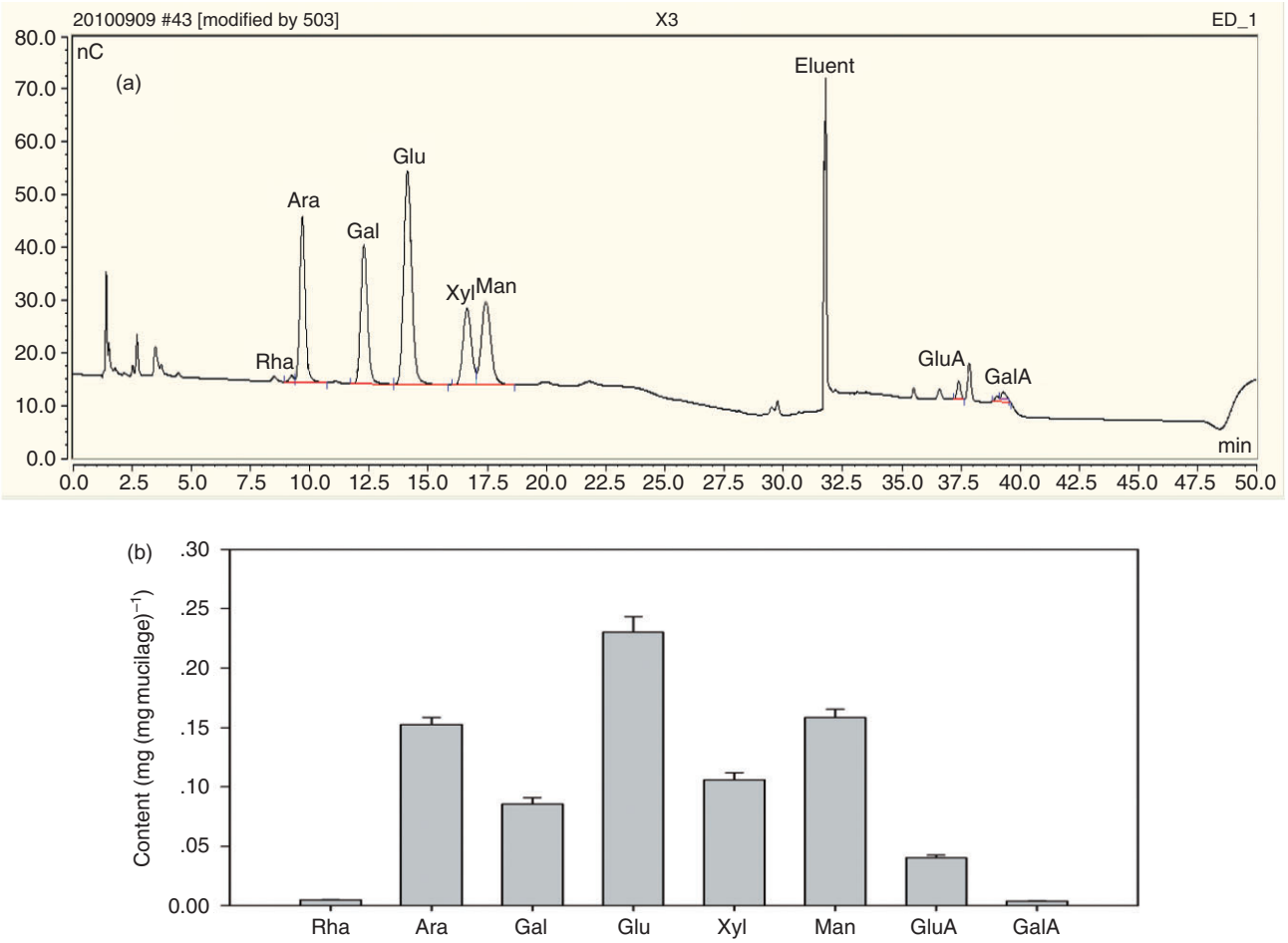

Figure 1. Monosaccharide composition of mucilage determined by high-performance anion-exchange chromatography (HPAEC). (a) Representative HPAEC-PAD chromatographic profiles of achene mucilage of *A. sphaerocephala*. (b) Average content of monosaccharide determined by HPAEC. Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; GluA, glucuronic acid; GalA, galacturonic acid. Values in (b) are the means + 1 SE of six samples (mucilage).

concentration of soil with mucilage addition (S + M) rapidly reached a peak after 1 d of incubation and slightly decreased afterwards. Additionally, CO_2 evolution of S + M was significantly higher than that of sterilized soil without mucilage addition (St), St + M and S throughout the experiment. Three-way ANOVA showed that CO_2 evolution during incubation was significantly affected by mucilage, soil type, incubation time and their interactions (Table 2).

Soluble sugar dynamics during mucilage degradation in soil

To collect more direct information on mucilage degradation, mucilage was added to soil samples, and soluble sugar in the samples was extracted with water and determined colourimetrically (Fig. 4). Soluble sugar concentrations in S + M increased with incubation time, and there was a significant difference in soluble sugar concentration between S + M and other treatments ($P < 0.05$; Fig. 4). By contrast, sugar concentrations in S remained constant and low during incubation and differed significantly from the other treatments ($P < 0.05$; Fig. 4). Soluble sugar concentrations during biodegradation were significantly affected by mucilage, soil type and all the interactions of the three factors, but not by incubation time (Table 2).

PLFA analysis during mucilage degradation

Soil microbial biomass dynamics, assessed as PLFA content, was determined during mucilage degradation. Microbial biomass in S + M increased with incubation time, and significantly exceeded that in the other treatments ($P < 0.05$; Fig. 5a). Microbial biomass in S was lower than that in S + M and remained constant during incubation ($P < 0.05$; Fig. 5a). However, microbial biomass in St + M and St decreased during biodegradation, and there was no significant difference between the two treatments ($P > 0.05$; Fig. 5a). Three-way ANOVA showed that microbial biomass was significantly affected by mucilage, soil type, incubation time, interactions of mucilage and soil type and interactions of soil type and incubation time (Table 2). The DA

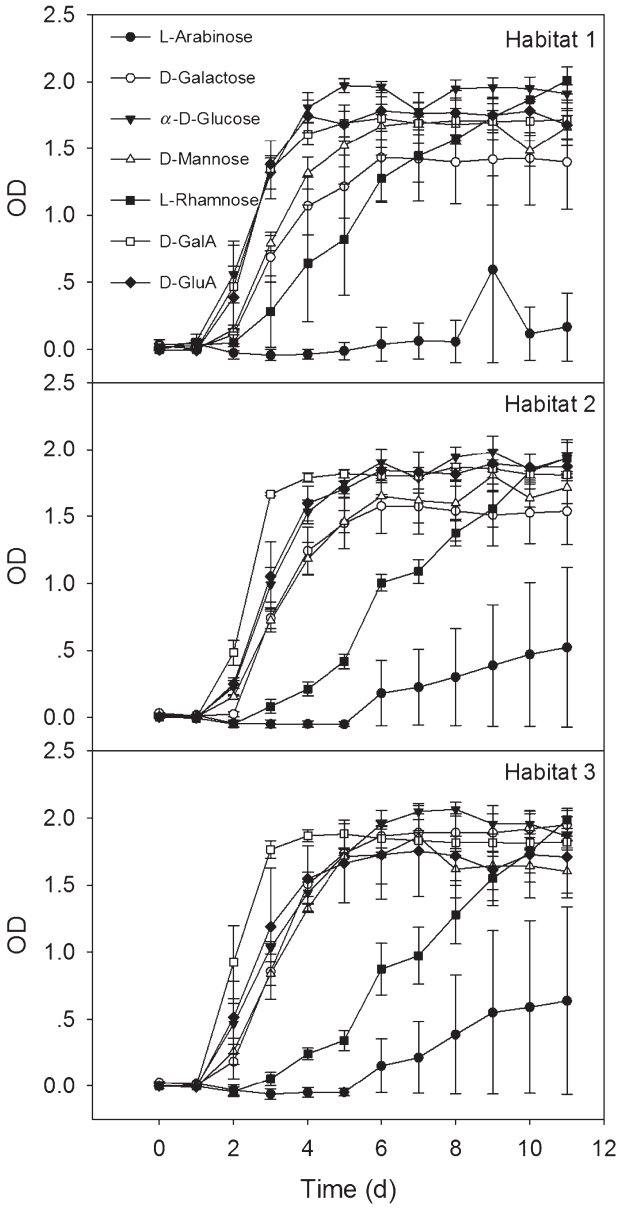

Figure 2. Utilization of seven sugars present in *A. sphaerocephala* achene mucilage by soil microbial communities from three natural habitats on Biolog GN2 plates. Optical density (OD) is mean ± SE of three samples.

performed on the data set of PLFAs resulted in a clear classification of samples in terms of culture treatments, with more than 66% of the samples being correctly classified, with S + M the most clearly classified treatment (Fig. 5b).

Fluorescence microscopy of mucilage polysaccharide absorption by seedlings

We wished to confirm that the mucilage polysaccharide (or its derivatives) was absorbed by the seedlings during development. Seedlings that had been grown for various times were incubated with FITC-labelled mucilage polysaccharide and examined by fluorescence microscopy (Fig. 6). Fluorescence microscopy showed the presence of FITC-labelled substances in the seedling tissues at various growth times. Roots of FITC-labelled seedlings exhibited much stronger fluorescence than that of control seedlings ($F = 155.238$, $P < 0.001$), and fluorescence intensity became stronger as the FITC-labelled seedlings grew older ($F = 29.746$, $P < 0.001$; Fig. 6a,b). Moreover, stems of FITC-labelled seedlings exhibited stronger fluorescence than that of control seedlings ($F = 7.089$, $P < 0.05$), and fluorescence intensity became stronger as the FITC-labelled seedlings grew older ($F = 4.023$, $P < 0.001$; Fig. 6a,c). By contrast, there was no difference in fluorescence intensity in the leaves between FITC-labelled seedlings and control seedlings ($F = 0.050$, $P = 0.827$; Fig. 6a,d). These results indicate that the degraded products of mucilage polysaccharide (or

Table 1. Average well colour development (AWCD) of soil microbial community from three natural habitats of *A. sphaerocephala* determined by Biolog GN2 plates

Time (d)	Habitat 1	Habitat 2	Habitat 3
0	0.008 ± 0.008	0.015 ± 0.008	0.012 ± 0.001
1	0.012 ± 0.003	0.008 ± 0.003	0.005 ± 0.002
2	0.168 ± 0.044	0.125 ± 0.030	0.213 ± 0.064
3	0.568 ± 0.018	0.542 ± 0.044	0.566 ± 0.049
4	0.855 ± 0.035	0.857 ± 0.044	0.821 ± 0.019
5	0.997 ± 0.051	1.020 ± 0.044	0.992 ± 0.016
6	1.249 ± 0.074	1.285 ± 0.062	1.244 ± 0.014
7	1.238 ± 0.067	1.285 ± 0.093	1.303 ± 0.017
8	1.278 ± 0.077	1.346 ± 0.050	1.331 ± 0.015
9	1.322 ± 0.064	1.422 ± 0.076	1.339 ± 0.009
10	1.365 ± 0.086	1.420 ± 0.055	1.412 ± 0.017
11	1.376 ± 0.073	1.446 ± 0.074	1.441 ± 0.007

AWCD values are the means ± SE of three samples.

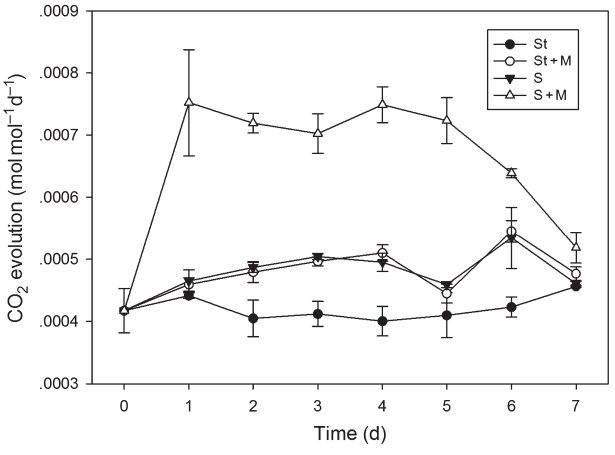

Figure 3. CO_2 evolution during degradation of *A. sphaerocephala* achene mucilage in soil. Concentrations are means ± SE of three samples. St, sterilized soil without mucilage addition; S, soil without mucilage addition; St + M, sterilized soil with mucilage addition; S + M, soil with mucilage addition.

Table 2. Three-way analysis of variance of effects of mucilage, soil type, incubation time and their interactions on CO_2 evolution, sugar concentration, seedling length, seedling weight and root/shoot (R/S) ratio of *A. sphaerocephala* achenes

Source	CO_2 evolution		Sugar concentration		Microbial biomass		Seedling length		Seedling weight		R/S ratio	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Mucilage (M)	134.332	**<0.001**	224.448	**<0.001**	11.300	**0.001**	5.969	**0.019**	9.868	**0.003**	0.396	0.532
Soil type (S)	132.085	**<0.001**	8.018	**0.006**	85.624	**<0.001**	14.246	**<0.001**	28.231	**<0.001**	2.079	0.156
Incubation time (T)	9.576	**<0.001**	0.567	0.780	8.385	**<0.001**	91.897	**<0.001**	73.334	**<0.001**	0.340	0.563
M × S	20.613	**<0.001**	170.914	**<0.001**	6.883	**0.011**	5.155	**0.028**	8.703	**0.005**	3.154	0.083
M × T	4.723	**<0.001**	2.532	**0.023**	1.134	0.353	0.223	0.639	1.043	0.313	0.391	0.535
S × T	5.506	**<0.001**	3.667	**0.002**	17.903	**<0.001**	7.030	**0.011**	0.716	0.402	0.859	0.359
M × S × T	2.721	**0.016**	2.489	**0.025**	0.254	0.969	0.350	0.557	1.480	0.230	1.803	0.186

Values in bold indicate significant effects ($P < 0.05$).

Figure 4. Soluble sugar concentrations during degradation of *A. sphaerocephala* achene mucilage in soil. Concentrations are means ± SE of three samples. St, sterilized soil without mucilage addition; S, soil without mucilage addition; St + M, sterilized soil with mucilage addition; S + M, soil with mucilage addition.

its derivatives) are absorbed by roots and subsequently transported to stems of seedlings.

Effect of mucilage degradation on seedling growth and biomass accumulation

To investigate further the effect of mucilage degradation on seedling growth, length and biomass of seedlings under various combinations of achene type and soil treatment were determined (Fig. 7). Seedling lengths in soil with intact achenes (S + I) were significantly longer than that in soil with demucilaged achenes (S + D), sterilized soil with intact achenes (St + I) or sterilized soil with demucilaged achenes (St + D) at 10 d of growth ($P < 0.05$), but this difference became insignificant by 30 d ($P > 0.05$; Fig. 7a). Seedling weight in S + I was significantly higher than that in S + D, St + I or St + D at both 10 and 30 d ($P < 0.05$; Fig. 7b). Root/shoot (R/S) ratio in S + I was significantly higher than that in S + D or St + I ($P < 0.05$), but this difference became insignificant by 30 d ($P > 0.05$; Fig. 7c).

Three-way ANOVA showed that seedling length and weight were significantly affected by mucilage, soil type, incubation time and interactions of mucilage and soil type. In addition, seedling length was significantly affected by interactions of soil type and incubation time. None of these factors had a significant effect on R/S ratio of seedlings (Table 2).

DISCUSSION

Fruits and seeds of many desert plants are mucilaginous, and their development appears to be one of the evolutionary adaptations of plants to desert environments (Evenari & Gutterman 1976; Gutterman 1993, 2002). The mucilaginous layer covering fruits and seeds is an important functional trait that provides many ecological benefits (Zohary

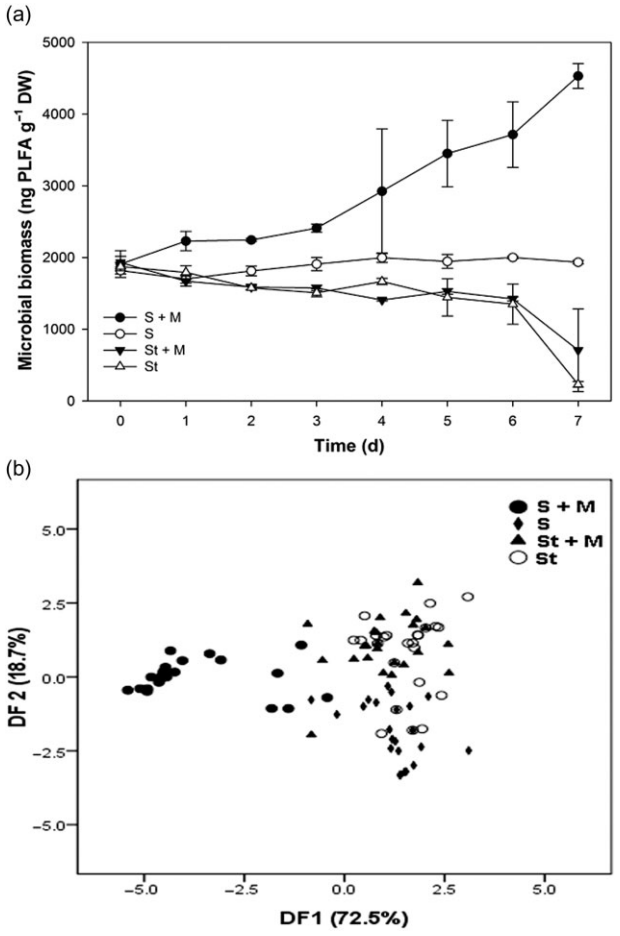

Figure 5. Microbial biomass (a) and discriminant analysis for each treatment (b) during degradation of *A. sphaerocephala* achene mucilage in soil. St, sterilized soil without mucilage addition; S, soil without mucilage addition; St + M, sterilized soil with mucilage addition; S + M, soil with mucilage addition. Microbial biomass is mean ± SE of three samples.

1962; Harper & Benton 1966; Witztum *et al*. 1969; Hedge 1970; Swarbrick 1971; Gutterman *et al*. 1973; Grubert 1974; Evans *et al*. 1979; Garwood 1985; Gutterman 1993; Gutterman & Shem-Tov 1997; Huang *et al*. 2000). Previous studies on the function of mucilage have mainly focused on aspects of seed dispersal strategy such as its role in flotation (Huang & Gutterman 1999), seed adherence to the sand crust to prevent the further dispersal by wind or to protect against predation by seed eaters (Huang *et al*. 2000). In the present study, we extended the knowledge on the ecological function of mucilage by providing evidence that it could promote seedling growth in barren sand soils through its interaction with soil microbes.

Our previous studies have shown that the achene mucilage of *A. sphaerocephala* can maintain seed DNA integrity following repeated partial seed hydration in desert dew and aid seed germination in osmotically stressful and saline habitats, thus playing ecologically important roles in the survival of this species in its harsh desert habitats (Huang *et al*. 2008; Yang *et al*. 2010). In the present study we have shown that the monosaccharide composition of *A. sphaerocephala* achene mucilage is very complex, with eight sugars being identified in the HPAEC experiment. We have also shown that achene mucilage can be utilized by the soil microbial community in the natural habitat, resulting in an increase in microbial respiration of CO_2 and an increase in PLFA contents, which indicate an enriched soil microbial community. Additionally, we have provided evidence that the products of biodegraded mucilage could be absorbed by seedlings. Finally, our results indicate that this biodegradation process could promote early seedling growth. Our data give further insight into the ecological importance as an adaptation of plants to the infertile sand desert environment.

In Biolog plates, rapid colour development was observed during incubation in the seven wells containing the same sugars present in achene mucilage. The rapid colour development in these wells gives preliminary evidence of the capacity of the soil microbial community to use these sugars as respiratory substrates. These results provide evidence that soil microbes able to degrade and utilize the sugars in the achene mucilage of *A. sphaerocephala* are present in its natural habitat.

CO_2 evolution rates significantly increased when mucilage was added into the natural soil, indicating that the mucilage can be used as a substrate by the soil microbial community and ultimately degraded to CO_2. In addition, CO_2 evolution in S + M rapidly peaked after 1 d of incubation, suggesting that mucilage enters into soil mainly as a dissolved organic carbon (DOC) pool that has been reported to be turned over several times per day (Schimel & Weintraub 2003; Bengtson & Bengtsson 2007). These findings suggest that achene mucilage can be rapidly degraded by the soil microbial community in the natural habitat, resulting in a rapid release of CO_2 from the soil.

Sugar concentrations in S + M increased with incubation time and were higher than they were in other treatments (Fig. 4). The higher concentrations of soluble sugar in this treatment may result from the degradation of mucilage by the microbial community. These results further confirm that the soil microbial community is capable of utilizing the monosaccharides of mucilage as shown by the Biolog plates (Fig. 2). It is notable that soluble sugars determined in this experiment may be only part of the products of mucilage biodegradation. Although we cannot know the precise pathways of biodegradation of mucilage polysaccharides and biosynthesis of new components from this study, our findings demonstrate that complex processes are involved in the biodegradation of mucilage. Further studies to elucidate the mechanisms underlying the biodegradation of mucilage are warranted.

Our PLFA analysis showed that microbial biomass increased during mucilage biodegradation and that mucilage had a significant effect on PLFA composition (Fig. 5a,b). Mucilage enters into the soil as a soil organic matter (SOM), which may have influence on the soil microbial processes. Soil microbial communities have been shown to play an important role in SOM decomposition processes

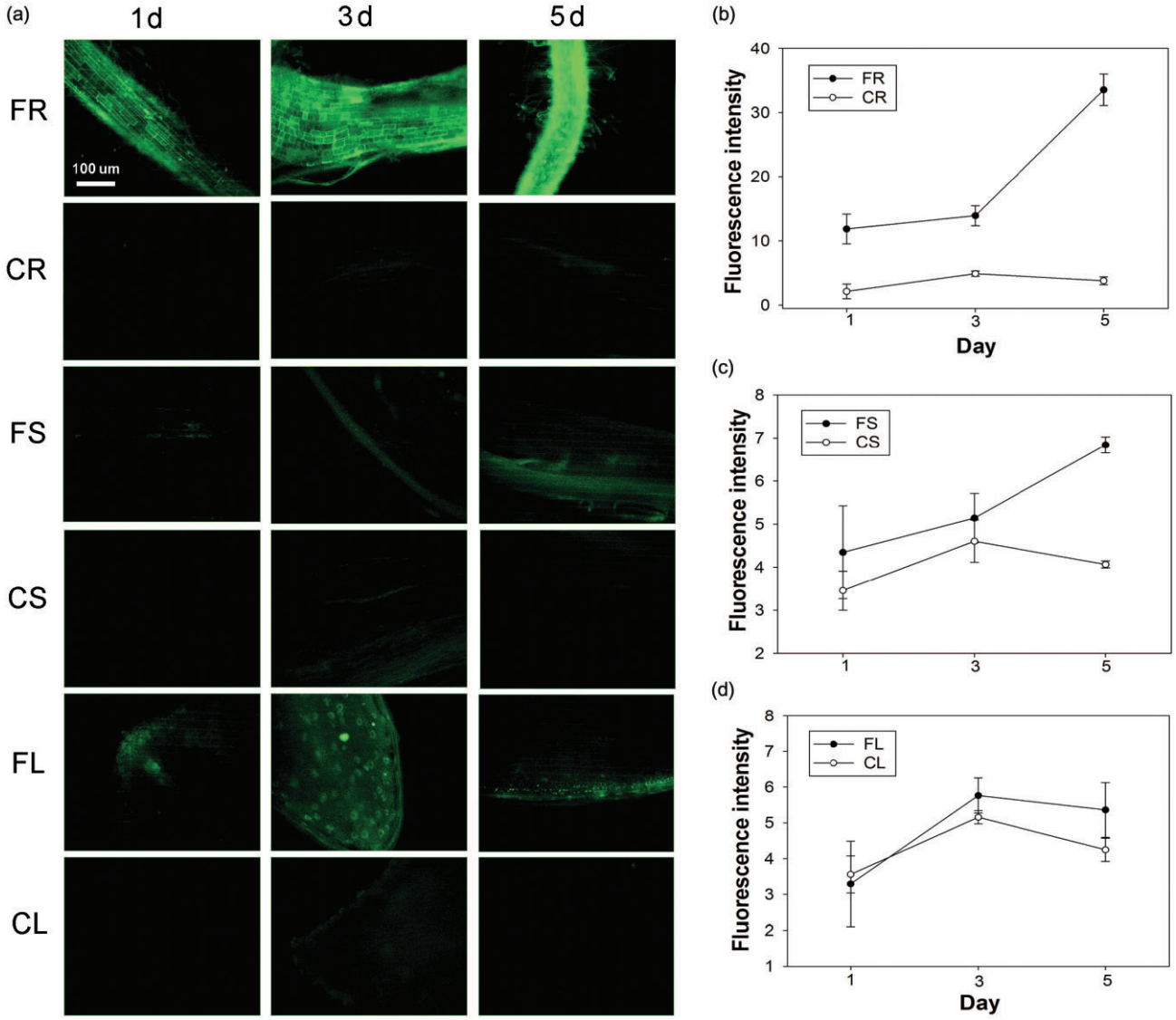

Figure 6. Fluorescence microscopy of absorption of mucilage polysaccharide by seedlings of *A. sphaerocephala*. (a) Representative fluorescence images of seedlings at 1, 3 and 5 d after mucilage addition. (b) Quantification of fluorescence intensity in roots of fluorescence-labelled and control seedlings. (c) Quantification of fluorescence intensity in stems of fluorescence-labelled and control seedlings. (d) Quantification of fluorescence intensity in leaves of fluorescence-labelled and control seedlings. Vertical bars represent ±SE. IR, root of seedlings after fluorescence-labelled mucilage was added; CR, root of control seedlings (mucilage had not been fluorescence-labelled); IS, stem of seedlings after fluorescence-labelled mucilage was added; CS, stem of control seedlings; IL, leaf of seedlings after fluorescence-labelled mucilage was added; CL, leaf of control seedlings.

and to respond quickly to changes in labile soil C additions because they are generally limited by the availability of labile C in soil (Tate 1995; Darrah 1996; de Graaff *et al.* 2010). For example, it has been reported that microbial community structure and C-use efficiency are sensitive to root C inputs (De Deyn, Cornelissen & Bardgett 2008). Addition of exudates to the rhizosphere of *Panicum virgatum* enhanced both overall CO_2 respiration and bacterial and fungal gene copy numbers (de Graaff *et al.* 2010). Thus, the increase in PLFA concentrations during mucilage biodegradation is likely to have resulted from the increase in SOM inputs that may subsequently enrich the soil microbial community. However, our present experiments do not provide complete information on changes in the soil microbial community during mucilage biodegradation. In future work, it would be valuable and interesting to isolate and characterize the microbes capable of degrading achene mucilage and to determine the dynamics of degradation.

Our previous studies have demonstrated that early seedling growth of intact achenes of *A. sphaerocephala* did not differ significantly from that of demucilaged achenes under either osmotic or saline stress (Yang *et al.* 2010), and that there is no enzymatic cleavage or hydrolysis of

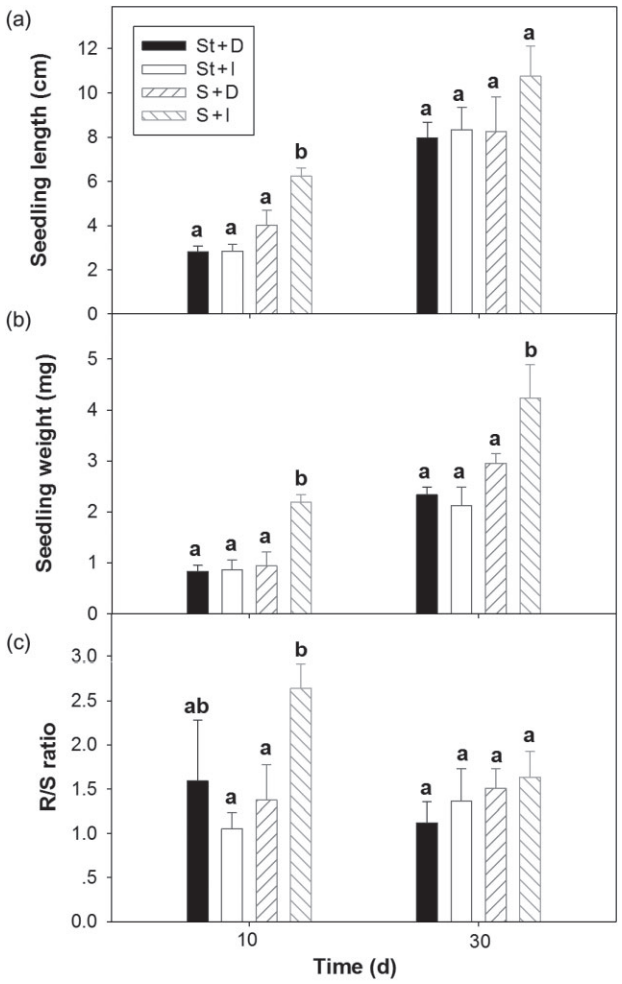

Figure 7. Seedling length (a), seedling weight (b) and root/shoot (R/S) ratio (c) of *A. sphaerocephala* seedlings during degradation of achene mucilage in soil. St + I, sterilized soil with intact achenes; S + D, soil with demucilaged achenes; S + I, soil with intact achenes; St + D, sterilized soil with demucilaged achenes. Different lowercase letters indicate significant difference between treatments at an incubation time. Vertical bars represent ±1 SE.

the mucilage that produces any products utilized during germination as the mucilage is very stable (Huang *et al*. 2004). However, results of the present study clearly indicate that, after being added to natural soil, mucilage improved seedling growth of this species as evidenced by a higher biomass accumulation during incubation (Fig. 7). This discrepancy may be accounted for by the use of different substrates in these studies. The present study used natural soil containing a soil microbial community in its natural habitat as substrates for seedling growth, and previous studies have used solutions or distilled water that were unlikely to contain a natural soil microbial community. Seedling growth in sterilized soil with demucilaged or intact achenes and natural soil with demucilaged achenes did not differ from each other, indicating that the soil microbial community or mucilage alone can not account for the promotion of seedling growth. However, seedling growth in natural soil with intact achenes was significantly higher than that in the other treatments, suggesting a synergistic effect of the microbial community and mucilage in promoting early seedling growth.

Fluorescence microscopy showed that the mucilage polysaccharides (or its derivatives) are absorbed by roots and subsequently transported to shoots of seedlings (Fig. 6), which provides direct evidence for the absorption of mucilage-derived molecules in early seedling growth. As discussed earlier, mucilage biodegradation would lead to an increase in soil-labile C inputs, thus enriching the soil microbial community. Increased soil-labile C inputs could regulate decomposition of more-recalcitrant soil C by controlling the activity and relative abundance of fungi and bacteria (de Graaff *et al*. 2010; Phillips, Finzi & Bernhardt 2011), leading to a further stimulation of rhizosphere N cycling, a mechanism some plants employ for increasing N availability in low-fertility soils (Phillips *et al*. 2011). The increase of seedling growth in the present study may be explained by such mechanisms. However, we cannot rule out the possibility that fluoroscein isothiocyanate released by microbial action was subsequently taken up by the plants. To confirm this possibility, further works is required. For example, hexoses labelled by stable isotopes could be used to verify that these compounds can be taken up. It also would be useful to determine whether sugar supplementation causes the same growth effects as mucilage. The alternative explanation is that the microbial community provides some micronutrient or hormonal signal for plant growth. Moreover, it would be interesting to provide a mix of hexoses to the soil to determine if there are compounds in the mucilage other than the sugars that promote seedling growth. Taken together, then, these results further substantiate that the soil microbial community in the natural habitat can degrade the mucilage and thereby possibly promote seedling growth of *A. sphaerocephala*.

In light of the findings in the present study, a model summarizing the biodegradation process of achene mucilage of *A. sphaerocephala* is presented in Fig. 8. Mucilage is developed in the epidermal cells of the fruit wall during achene maturation on the mother plant. After dispersal, the achenes enter into the soil, where the soil microbial community is present. Mucilage polysaccharides are then degraded to small molecules by the microbial community. These small molecules may be used directly by seedlings for growth. Alternatively, they could be used as substrates for metabolism by the soil microbial community and ultimately degraded to CO_2. As a result, the microbial community is enriched, leading to promotion of early seedling growth. Thus, our results provide a framework for further understanding the ecological function of mucilage in the life history of plants inhabiting desert environments, and such an ecological role is functioning through plant–microbe interactions.

The present study demonstrated the biodegradation process of seed mucilage in the presence of soil microbial communities from the natural habitats. Such

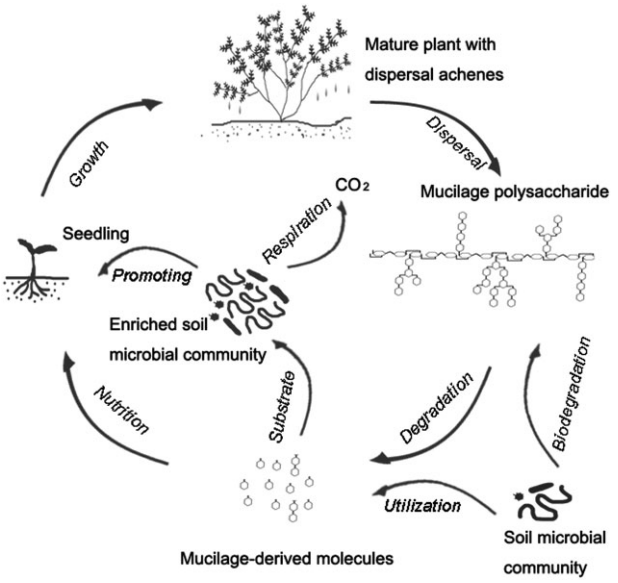

Figure 8. A conceptual model for biodegradation of achene mucilage of *A. sphaerocephala* in the natural habitat.

biodegradation may subsequently exert a beneficial effect on enrichment of the soil microbial community and promote early seedling growth, which would thus enable successful establishment of plants that produce seed mucilage. Therefore, in addition to the many beneficial functions of seed mucilage before it is degraded, biodegradation also presumably plays an ecologically important role in promoting growth of seedlings of *A. sphaerocephala* in the harsh desert soils and possibly in other species adapted to different environments.

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REFERENCES

Anumula K.R. & Taylor P.B. (1991) Rapid Characterization of asparagine-linked oligosaccharides isolated from glycoproteins using a carbohydrate analyzer. *European Journal of Biochemistry* **195,** 269–280.

Bengtson P. & Bengtsson G. (2007) Rapid turnover of DOC in temperate forests accounts for increased CO_2 production at elevated temperatures. *Ecology Letters* **10,** 783–790.

Darrah P.R. (1996) Rhizodeposition under ambient and elevated CO_2 levels. *Plant and Soil* **187,** 265–275.

Dean G.H., Zheng H., Tewari J., *et al*. (2007) The *Arabidopsis MUM2* gene encodes a β-galactosidase required for the production of seed coat mucilage with correct hydration properties. *The Plant Cell* **19,** 4007–4021.

De Belder A. & Granath K. (1973) Preparation and properties of fluorescein-labelled dextrans. *Carbohydrate Research* **30,** 375–379.

De Deyn G.B., Cornelissen J.H.C. & Bardgett R.D. (2008) Plant functional traits and soil carbon sequestration in contrasting biomes. *Ecology Letters* **11,** 516–531.

DuBois M., Gilles K.A., Hamilton J.K., Rebers P.A. & Smith F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28,** 350–356.

Editorial Board of the Flora of China of Chinese Academy of Sciences (1991) *Flora of China*, vol. 76. Science Press, Beijing. (in Chinese).

Evans R.A., Young J.A. & Hawkes R. (1979) Germination characteristics of Italian thistle (*Carduus pycnocephalus*) and slenderflower thistle (*Carduus tenuiflorus*). *Weed Science* **27,** 327–332.

Evenari M. & Gutterman Y. (1976) Observations on the secondary succession of three plant communities in the Negev desert, Israel. I. *Artemisietum herbae albae*. In *Etudes de Biologie Végétale* (eds R. Jacques & H.P.P. Chouard), pp. 57–86. CNRS, Gif sur Yvette.

Fahn A. (1990) *Plant Anatomy* 4th edn, Pergamon Press, Oxford.

Garland J.L. & Mills A.L. (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* **57,** 2351–2359.

Garwood N. (1985) The role of mucilage in the germination of Cuipo, *Cavanillesia platanifolia* (H. & B.) H.B.K. (Bombacaceae): a tropical tree. *American Journal of Botany* **72,** 1095–1105.

de Graaff M.A., Classen A.T., Castro H.F. & Schadt C.W. (2010) Labile soil carbon inputs mediate the soil microbial community composition and plant residue decomposition rates. *The New Phytologist* **188,** 1055–1064.

Grubert M. (1974) Studies on the distribution of myxospermy among seeds and fruits of Angiospermae and its ecological importance. *Acta Biologica Venezuelica* **8,** 315–551.

Gutterman Y. (1993) *Seed Germination in Desert Plants (Adaptations of Desert Organisms)*. Springer-Verlag, Berlin.

Gutterman Y. (2002) *Survival Strategies of Annual Desert Plants*. Springer-Verlag, Berlin.

Gutterman Y. & Shem-Tov S. (1997) The efficiency of the strategy of mucilaginous seeds of some common annuals of the Negev adhering to the soil crust to delay collection by ants. *Israel Journal of Plant Sciences* **45,** 317–327.

Gutterman Y., Witztum A. & Heydecker W. (1973) Studies on the surfaces of desert plant seeds. II. Ecological adaptations of the seeds of *Blepharis persica*. *Annals of Botany* **37,** 1051–1055.

Harper J.L. & Benton R.A. (1966) The behaviour of seeds in soil II. The germination of seeds on the surface of a water supplying substrate. *Journal of Ecology* **54,** 151–166.

Hedge I.C. (1970) Observation on the mucilage of *Salvia* fruits. *Notes from the Royal Botanic Garden, Edinburgh* **30,** 79–95.

Huang Z. & Gutterman Y. (1999) Water absorption by mucilaginous achenes of *Artemisia monosperma*: floating and germination as affected by salt concentrations. *Israel Journal of Plant Sciences* **47,** 27–34.

Huang Z., Gutterman Y. & Hu Z. (2000) Structure and function of mucilaginous achenes of *Artemisia monosperma* inhabiting the Negev Desert of Israel. *Israel Journal of Plant Sciences* **48,** 255–266.

Huang Z., Gutterman Y. & Osborne D.J. (2004) Value of the mucilaginous pellicle to seeds of the sand-stabilizing desert

woody shrub *Artemisia sphaerocephala* (Asteraceae). *Trees* **18,** 669–676.

Huang Z., Boubriak I., Osborne D.J., Dong M. & Gutterman Y. (2008) Possible role of pectin-containing mucilage and dew in repairing embryo DNA of seeds adapted to desert conditions. *Annals of Botany* **101,** 277–283.

Larkin R.P. (2003) Characterization of soil microbial communities under different potato cropping systems by microbial population dynamics, substrate utilization, and fatty acid profiles. *Soil Biology & Biochemistry* **35,** 1451–1466.

Macquet A., Ralet M.C., Kronenberger J., Marion-Poll A. & North H.M. (2007) In situ, chemical and macromolecular study of the composition of *Arabidopsis thaliana* seed coat mucilage. *Plant & Cell Physiology* **48,** 984–999.

Penfield S., Meissner R.C., Shoue D.A., Carpita N.C. & Bevan M.W. (2001) *MYB61* is required for mucilage deposition and extrusion in the *Arabidopsis* seed coat. *The Plant Cell* **13,** 2777–2791.

Phillips R.P., Finzi A.C. & Bernhardt E.S. (2011) Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO_2 fumigation. *Ecology Letters* **14,** 187–194.

Schimel J.P. & Weintraub M.N. (2003) The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. *Soil Biology & Biochemistry* **35,** 549–563.

Schmidgall J. & Hensel A. (2002) Bioadhesive properties of polygalacturonides against colonic epithelial membranes. *International Journal of Biological Macromolecules* **30,** 217–225.

Swarbrick J.T. (1971) External mucilage production by seeds of British plants. *Botanical Journal of the Linnean Society* **64,** 157–162.

Tate R.L. (1995) *Soil Microbiology*. John Wiley & Sons, Inc., New York.

Thapliyal R.C., Phartyal S.S., Baskin J.M. & Baskin C.C. (2008) Role of mucilage in germination of *Dillenia indica* (Dilleniaceae) seeds. *Australian Journal of Botany* **56,** 583–589.

Usadel B., Kuchinsky A.M., Rosso M.G., Eckermann N. & Pauly M. (2004) RHM2 is involved in mucilage pectin synthesis and is required for the development of the seed coat in *Arabidopsis*. *Plant Physiology* **134,** 286–295.

Western T.L. (2006) Changing Spaces: the *Arabidopsis* mucilage secretory cells as a novel system to dissect cell wall production in differentiating cells. *Canadian Journal of Botany* **84,** 622–630.

Western T.L., Skinner D.J. & Haughn G.W. (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiology* **122,** 345–356.

Western T.L., Burn J., Tan W.L., Skinner D.J., Martin-McCaffrey L., Moffatt B.A. & Haughn G.W. (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiology* **127,** 998–1011.

Western T.L., Young D.S., Dean G.H., Tan W.L., Samuels A.L. & Haughn G.W. (2004) *MUCILAGE-MODIFIED4* encodes a putative pectin biosynthetic enzyme developmentally regulated by *APETALA2*, T*RANSPARENT TESTA GLABRA1*, and *GLABRA2* in the *Arabidopsis* seed coat. *Plant Physiology* **134,** 296–306.

Windsor J.B., Symonds V.V., Mendenhall J. & Lloyd A.L. (2000) *Arabidopsis* seed coat development: morphological differentiation of the outer integument. *The Plant Journal* **22,** 483–493.

Witztum A., Gutterman Y. & Evenari M. (1969) Integumentary mucilage as an oxygen barrier during germination of *Blepharis persica* (Burm.) Kuntze. *Botanical Gazette* **130,** 238–241.

Yang X., Dong M. & Huang Z. (2010) Role of mucilage in the germination of *Artemisia sphaerocephala* (Asteraceae) achenes exposed to osmotic stress and salinity. *Plant Physiology and Biochemistry* **48,** 131–135.

Yang X., Zhang W., Dong M., Boubriak I. & Huang Z. (2011) The achene mucilage hydrated in desert dew assists seed cells in maintaining DNA integrity: adaptive strategy of desert plant *Artemisia sphaerocephala*. *PLoS ONE* **6,** e24346. doi: 10.1371/journal.pone.0024346.

Young R.E., McFarlane H.E., Hahn M.G., Western T.L., Haughn G.W. & Samuels A.L. (2008) Analysis of the Golgi apparatus in *Arabidopsis* seed coat cells during polarized secretion of pectin-rich mucilage. *The Plant Cell* **20,** 1623–1638.

Zak D.R., Holmes W.E., White D.C., Peacock A.D. & Tilman D. (2003) Plant diversity, soil microbial communities and ecosystem function: are there any links? *Ecology* **84,** 2042–2050.

Zelles L. (1997) Phospholipid fatty acid profiles in selected members of soil microbial communities. *Chemosphere* **35,** 275–294.

Zelles L. (1999) Fatty acids patterns of phospholipids and lipopolysacharides in the characterisation of microbial communities in soil: a review. *Biology and Fertility of Soils* **29,** 111–129.

Zelles L., Bai Q.Y., Rackwitz R., Chadwick D. & Beese F. (1995) Determination of phospholipid- and lipopolysaccharidederived fatty acids as an estimate of microbial biomass and community structures in soils. *Biology and Fertility of Soils* **19,** 115–123.

Zohary M. (1962) *Plant Life of Palestine*. Ronald, New York.

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