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Artificial climate warming positively affects arbuscular mycorrhizae but decreases soil aggregate water stability in an annual grassland

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Despite the importance of arbuscular mycorrhizae to the functioning of terrestrial ecosystems (e.g. nutrient uptake, soil aggregation), and the increasing evidence of global warming, responses of arbuscular mycorrhizal fungi (AMF) to climate warming are poorly understood. In a field experiment using infrared heaters, we found effects of warming on AMF after one growing season in an annual grassland, in the absence of any effects on measured root parameters (weight, length, average diameter). AMF soil hyphal length was increased by over 40% in the warmed plots, accompanied by a strong trend for AMF root colonization increase. In the following year, root weight was again not significantly changed, and AMF root colonization increased significantly in the warmed plots. Concentration of the soil protein glomalin, a glycoprotein produced by AMF hyphae with importance in soil aggregation, was decreased in the warmed plots. Soil aggregate water stability, measured for five diameter size classes, was also decreased significantly. In the following year, soil aggregate weight in two size classes was decreased significantly, but the effect size was very small. These results indicate that ecosystem warming may have stimulated carbon allocation to AMF. Other factors either influenced glomalin decomposition or production, hence influencing the role of these symbionts in soil aggregation. The observed small changes in soil aggregation, if widespread among terrestrial ecosystems, could have important consequences for soil carbon storage and erosion in a warmed climate, especially if there are cumulative effects of warming.

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The occurrence of global warming, the increase in near surface temperature of the earth due to the enhanced greenhouse effect, is increasingly supported (Santer et al. 1996), and may already be detectable as a biological signal (Hughes 2000). Without climate-mitigation policies, the 90% probability interval for 1990–2100 global-mean warming is 1.7–4.9°C (Wigley and Raper 2001). While a strong relationship exists between soil mean annual temperature and soil organic carbon content (Buol et al. 1990), the effects of warming on belowground biota and processes are not very well understood and are likely quite complex, because both direct and indirect effects of warming (and their interactions) have to be considered (Shaver et al. 2000).

Arbuscular mycorrhizae, by virtue of their ubiquity in terrestrial ecosystems, their key position at the root-soil interface, and their influence on plant physiology, plant communities and ecosystems, warrant a detailed consideration in global change biology (O'Neill et al. 1991, Rillig and Allen 1999). Despite the importance of

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warming to ecosystem functioning, and the recognized key role of AMF in terrestrial ecosystems, very little is known about AMF responses to ecosystem warming. Studies of AMF ecophysiology under in vitro conditions have shown that some stages of the fungal life cycle, for example spore germination (e.g. Daniels and Trappe 1980) and initial hyphal growth from a germinated spore (Bowen 1987), can respond to altered growth medium temperature. For example, hyphal elongation increased by 50% for one AMF species with a 5°C warming of the growth medium (15–20°C) (Tommerup 1983). However, responses of the mycorrhizal symbiosis are less understood. In the few cases examined thus far, no consistent responses have emerged, mostly because of differences in experimental design and temperature ranges used. Baon et al. (1994), in a greenhouse study using a single fungal isolate (*Glomus intraradices*), observed that barley roots did not become colonized at 10°C, but were mycorrhizal at 15°C. Monz et al. (1994) studied warming (4°C) effects on AMF colonization for two grass species using intact soil cores. AMF colonization in *Pascopyrum smithii* was decreased (15%), but no effects were found for *Bouteloua gracilis*. Fitter et al. (2000) reported that roots of *Plantago* became more rapidly colonized by *Glomus mossea* at 20°C than 12°C.

None of these studies have examined the effects of warming on the extraradical mycelium of AMF. An important function of this extraradical phase at the ecosystem level is its contribution to the formation and maintenance of soil structure by stabilizing aggregates (Tisdall and Oades 1982). Any global change effects on the extraradical mycelium of mycorrhizal fungi (or on the allocation of carbon from the plant to the fungus) could secondarily impact soil structure (Young et al. 1998, Rillig et al. 1999a). Soil structure is crucial for facilitating water infiltration, soil-borne aspects of biogeochemical cycling processes, success of sustainable agriculture, and for providing resistance against erosional loss of soil (Oades 1984, Elliott and Coleman 1988, Hartge and Stewart 1995, Jastrow and Miller 1997). Rillig et al. (1999a, 2001) have shown that for another factor of global change, elevated atmospheric CO_2, soil aggregate water stability was increased. AMF hyphal length and soil concentrations of the AMF-produced protein glomalin, which is involved in soil aggregate stabilization (Wright and Upadhyaya 1998), were increased concomitantly.

In contrast to global change factors involving atmospheric concentration changes, e.g. elevated atmospheric CO_2, it is important to realize that warming could have direct *and* indirect effects on arbuscular mycorrhizal fungi. Elevated CO_2, for example, can only indirectly affect AMF, since soil serves mostly as a buffer against changes in atmospheric gas composition. Warming can directly affect AMF and the decomposition of their products (e.g. glomalin), but warming can also indirectly affect the fungi by altering carbon allocation from the host to the mycobiont.

We wanted to specifically examine the existence and strength of both kinds of effects in a field study by testing the following hypotheses:

(1) Based on in vitro fungal ecophysiology, warming will increase AMF root colonization and hyphal length, hence providing evidence for increased carbon allocation to the (obligately biotrophic) fungus.
(2) Warming can increase decomposition of soil organic matter (Buol et al. 1990). Glomalin pools in soil, part of organic C, will therefore be decreased.
(3) As glomalin concentrations (immunoreactive fraction) are tightly correlated with soil aggregate water stability across a variety of soils (Wright and Upadhyaya 1998), this will therefore lead to decreased aggregate water stability.

Materials and methods

Field experiment

The Jasper Ridge Global Change Experiment (which in addition to warming also encompasses watering, nitrogen fertilization, and elevated CO_2 treatments) is situated on the Jasper Ridge Biological Preserve, which is located in the eastern foothills of the Santa Cruz Mountains in northern California, USA. The site has a Mediterranean-type climate with cool, wet winters and warm, dry summers. The experiment is located in a fenced grassland area dominated by annual non-native grasses (e.g. *Avena barbata*, *Bromus hordeaceus*, *Lolium multiflorum*) and forbs (e.g. *Anagallis arvensis*, *Centaurea solstitialis*, *Convolvulus arvensis*, *Trifolium hirtum*, *Vicia sativa*, *Crepis vesicaria*, *Plantago lanceolata*, *Geranium dissectum*, *Erodium botrys*), but also contains native annual forbs (e.g. *Hemizonia congesta* ssp. *luzulifolia*, *Epilobium brachycarpum*, *Lotus purshianus*).

The warming of plots in the field was accomplished using overhead infrared heaters, similar in concept to those employed by Harte et al. (1995) and Bridgham et al. (1999). Each circular plot ($n = 8$; 3.14 m^2) had a 250-W heater, for a power input of about 80 W m^{-2}. This power input achieved a resultant warming of 1.5–2°C at the canopy and 1°C at the soil surface. The warming was greatest at night when the winds were low, paralleling the prediction for future warming. Control plots ($n = 8$) had heater installations identical to the treatment plots, except that no warming treatment was applied.

In this Mediterranean-type climate, the growing season begins after the first significant rain, typically November, and ends with the onset of the summer

drought in May or June. Samples were taken 13 and 14 April 1999 and 30 and 31 March 2000, approximately 2 weeks before peak aboveground biomass. Two soil cores each were extracted from heated and control plots to a depth of 15 cm with a 2.2-cm diameter corer. The samples were pooled per plot by gently mixing, cooled on ice in the field, rapidly air-dried (50°C) in the laboratory, and stored in plastic bags until analysis.

Root measurements

Roots were picked out of 5-g soil subsamples with fine forceps (approximate picking time per sample was 20 min), cleaned, weighed and stored in paper bags. Root weight (after drying to constant weight) was expressed per gram of soil weight. Using the WinRhizo V 3.10B root image analysis system (Régent Instruments Inc, Québec, Canada), root lengths and diameters were measured (only for the 1999 samples).

Arbuscular mycorrhizae: percent root colonization

After processing the roots with the image analysis system, they were prepared for mycorrhizal percent root colonization measurements using a standard protocol of clearing in 10% KOH (90°C, 60 min), acidifying with 1% HCl (20°C, 5 min), staining with Trypan Blue in lactoglycerol (90°C, 30 min), and destaining in lactoglycerol. All stained roots were mounted on slides in lactoglycerol, and examined for mycorrhizal structures at 100 ×. For the 1999 season, roots of *Avena barbata* plants were extracted with a knife from all plots (3–5 plants per plot were used), and percent colonization was measured for these root samples as well.

Arbuscular mycorrhizae: soil hyphae (1999 samples only)

Hyphae were extracted from a 4-g soil subsample by an aqueous extraction and membrane filter technique modified after Jakobsen et al. (1992), as described in Rillig et al. (1999b). Soil samples were mixed and suspended in 100 mL of deionized water, to which 12 mL of sodium hexametaphosphate solution (35 g L^{-1}) was added. The soil suspensions were shaken for 30 s (end-over-end), left on the bench for 30 min, and then decanted quantitatively through a 38-μm sieve to retain hyphae, roots and organic matter. The material on the sieve was sprayed gently with deionized water to remove clay particles, and then transferred into a 250-mL Erlenmeyer flask with 200 mL of deionized water. The flask was shaken vigorously by hand for 5 s, left on the bench for 1 min, and a 2-mL aliquot was taken and pipetted onto 25-mm Millipore filters. The filter was transferred to a microscope slide and mounted in 0.05% Trypan Blue solution. Hyphal extractions from aggregates (see below) were carried out in the same way, but we also accounted for coarse material in the aggregates, since this differed among the aggregate size classes. For aggregate hyphal extractions, we randomly selected 4 of the 8 replicates per treatment. Hyphal length was measured with a grid-line intersect method at 200 × magnification, distinguishing hyphae into mycorrhizal and non-mycorrhizal hyphae.

It is not universally accepted that AM fungal hyphae can be reliably distinguished from hyphae of other fungi in field-collected soil samples. We used defined criteria to distinguish AM fungal hyphae from others, and our gentle extraction method was designed to optimize detection of these criteria. Being in the fungal phylum Zygomycota, AM fungal hyphae are characterized by branching dichotomously (rather than at right angles), and by having non-regular septa; however, hyphae vary greatly in diameter depending on their position within the mycelium architecture and their function (Friese and Allen 1991). AMF hyphae are (with the exception of a few species that have never been observed in roots from this field site) not darkly melanized. Hyphal growth is seldom straight, but rather irregular. Hyphae have irregular wall thickness, frequently with "elbow"-like protrusions. Occasionally, hyphae are connected to chlamydospores typical of AM fungi.

Glomalin and soil aggregation

Glomalin extractions from soil (1.00 g; only 1999 samples) were carried out as described by Wright and Upadhyaya (1998). Easily-extractable glomalin (EEG) was extracted with 20 mM citrate, pH 7.0 at 121°C for 30 min. EEG is considered the more recently deposited fraction of the protein in soil (Wright and Upadhyaya 1998). Total glomalin (TG) was extracted with 50 mM citrate, pH 8.0 at 121°C in rounds of 60 min each. For the sequential extractions, the supernatant was removed by centrifugation at 5000 × g for 20 min. Extraction of a sample continued until the supernatant showed none of the red-brown color typical of glomalin. Extracts from each replicate were pooled and then analyzed. After extraction cycles were completed, samples were centrifuged at 10 000 × g to remove soil particles, and immuno-reactive (IR) protein in the supernatant was determined using the monoclonal antibody MAb3211b (Wright and Upadhyaya 1998). Total (glomalin) protein was determined with a Bradford assay, using BSA as standard. Thus, four fractions of glomalin were measured: EEG, TG, IREEG, and IRTG. Concentration of glomalin was extrapolated to mg/g by correcting for the dry weight of coarse fragments (> 0.25 mm) included in the extraction of soil.

Prior to aggregate stability measurements, all soils had been stored as air-dried samples >4 months. Soil (30 g) was dry-sieved to obtain aggregates in five diameter size classes: 2.00–3.00 mm, 1.00–2.00 mm, 0.50–1.00 mm, 0.212–0.50 mm, and 0.053–0.212 mm. Water-stability of aggregates in each size class (using 1.00 g) was measured with a wet-sieving method using the apparatus and procedure described in Kemper and Rosenau (1986) after capillary rewetting for 10 min. The initial and final weights of aggregates were corrected for the weight of coarse particles. Sieve sizes for wet-sieving and coarse matter determination for the different size classes were 0.5 mm, 0.25 mm, 0.1 mm, 0.06 mm, and 0.012 mm, respectively. Aggregate stability is the mass of aggregated soil remaining after wet sieving as a percent of the total mass of soil.

For the 2000 samples, soil aggregate weight (as percentage of soil weight) was measured using wet-sieving (0.5–1.0 mm and 1.0–2.0 mm) following the method of Elliott (1986). Briefly, air-dried soils were sieved under water by moving the sieve 3 cm vertically 50 times over a period of 3 min. Coarse matter was determined on subsamples. This method measures weight of stable aggregates (not % aggregate stability). Due to the limited amount of soils available from this long-term study, it was impossible to measure aggregate stability in both years.

Results

Root responses to warming in 1999 are given in Table 1. Neither root weight nor length or average diameters were changed significantly in the warmed plots. Also there was no significant difference in total root volume or surface area (Table 1). There was no significant difference in the distribution of root length among nine diameter size classes (divided into 0.05-mm intervals from 0.00–0.05 mm to 0.40–0.45 mm; data not shown).

By contrast, several mycorrhizal parameters did respond to the warming treatment (Table 2). There was a trend for percent AMF root colonization of roots obtained from soil cores to be increased in warming, and AMF root colonization of *Avena* roots was significantly increased in the warmed plots. AMF soil hyphal length increased by over 40%. Both immunoreactive glomalin fractions (IREEG and IRTG) decreased in response to warming, whereas EEG and TG were not changed significantly. AMF hyphal length increases did not occur uniformly across all aggregate diameter size classes (Fig. 1), but warming was significant in a MANOVA (Wilks' Lambda $= 0.428$; $F_{1,6} = 7.99$; $P = 0.03$) that simultaneously considered all aggregate sizes. In univariate ANOVAs, hyphal length in the 0.053–0.212, 0.5–1.0 (log-transformed), and 2.0–3.0 mm size classes was changed significantly ($P < 0.05$). The percentage of water-stable soil aggregates (Fig. 2) was decreased in the warmed plots (MANOVA; Wilks' Lambda $= 0.74$; $F_{1,14} = 4.85$; $P = 0.04$). In univariate tests, aggregate stability in the 0.5–1.0 and 0.212–0.5 mm size classes was significantly ($P < 0.05$) decreased.

In 2000 (Table 3), there again was no significant change in root dry weight. AMF root infection (measured on roots extracted from the cores) was significantly increased in the warmed plots, following a similar pattern to the one observed in the previous year. The fraction of stable aggregates per weight of total soil in the two size classes measured was significantly decreased (Table 3) in response to warming; however, the effect size was very small.

Discussion

Mycorrhizal responses to ecosystem warming

In the same grassland ecosystem we have previously shown that arbuscular mycorrhizae responded to another factor of global change, elevated atmospheric CO_2 concentration (Rillig et al. 1999a, b, c). We found increased presence of mycorrhizal fungi in roots and soil, higher soil glomalin concentrations, and increased soil aggregate water stability. These responses confirmed the resource balance model, i.e. as aboveground resource availability increases, increased allocation of plant resources below ground should occur (Chapin 1980). There is no such powerful conceptual framework for carbon allocation to mycorrhizal fungi under increased temperature. However, we showed here, in one of the first field studies of AMF responses to experimental warming, that resource allocation to (obligately biotrophic) AMF can increase, leading to the observed increases in percent AMF root colonization, and extraradical hyphal length. The observed increase in soil

Table 1. Effects of warming on root parameters (1999). Means, standard errors of the mean ($n = 8$) in brackets.

Response variable	Ambient	Warming	F (P) value
Root length (m g^{-1})	0.58 (0.04)	0.49 (0.08)	0.95 (0.34) n.s.
Root dry weight (10^{-3} g g^{-1})	1.04 (0.13)	1.19 (0.16)	0.53 (0.48) n.s.
Average root diameter (10^{-4} m)	1.55 (0.039)	1.60 (0.052)	0.74 (0.41) n.s.
Root volume (cm^{-3})	0.0113 (0.0012)	0.0095 (0.0013)	0.93 (0.35) n.s.
Root surface area (cm^{-2})	2.87 (0.27)	2.45 (0.36)	0.89 (0.36) n.s.

n.s. not significant ($P > 0.15$).

Table 2. Effects of warming on arbuscular mycorrhizal parameters (1999). Means, standard errors of the mean ($n = 8$) in brackets.

Response variable	Ambient	Warming	F (P) value
AMF root colonization (%)[#]			
Soil core roots	63.8 (3.2)	75.6 (4.8)	2.47 (0.13)[+]
Avena roots	60.3 (4.7)	80.5 (3.0)	13.02 (0.003)**
AMF soil hyphal length (m g^{-1})	12.1 (0.85)	17.2 (0.75)	20.51 (0.0005)***
Glomalin (EEG) (mg g^{-1})	0.80 (0.02)	0.80 (0.03)	0.001 (0.98) n.s.
(TG) (mg g^{-1})	1.60 (0.05)	1.51 (0.05)	1.21 (0.29) n.s.
(IREEG) (mg g^{-1})	0.33 (0.02)	0.25 (0.01)	9.11 (0.009)**
(IRTG) (mg g^{-1})[$]	0.38 (0.04)	0.28 (0.03)	3.42 (0.09)[+]

[+] $0.05 < P < 0.15$ (trend), ** $P < 0.01$, *** $P < 0.001$, n.s. not significant ($P > 0.15$).
[#] arcsine-square root transformed.
[$] log transformed.

hyphae could be either due to altered growth physiology of hyphae (Tommerup 1983), due to changes in AMF species composition such that more prolific hypha-producers were favored, changes in carbon allocation from the plants to the mycobionts, or changes in other soil characteristics favorable to hyphal growth, or a combination of these factors.

While soil temperature is known to influence root growth (e.g. Pregitzer et al. 2000), the observed changes in mycorrhizal parameters in this study occurred in the apparent absence of gross changes in root characteristics, such as root length or weight (Tables 1, 3).

It is important to realize that this experiment was an ecosystem warming study. Hence, other soil conditions, which may have changed concurrently with the warming (Shaver et al. 2000), could have also impacted on hyphal growth. For example, soil warming could cause altered nutrient availability through stimulation of microbial mineralization (e.g. Zak et al. 1999, Shaw and Harte 2001), which could in turn influence hyphal growth or host regulation of AMF root colonization (Smith and Read 1997).

Soil nutrient and moisture data will be reported in a separate contribution. However, nitrogen (ammonium and nitrate) and phosphate availability (measured using resin bags) in our study plots showed a similar pattern in 1999 and 2000, which was an overall numerical decrease in the warmed plots, albeit not or only marginally statistically significant (unpubl.). Soil moisture throughout May 1999 was numerically higher in the warmed plots compared to the control, although this difference was not statistically significant (unpubl.). This difference could have arisen due to significantly increased aboveground plant biomass (over 40% increase; $P = 0.03$) in 1999 (unpubl.). By comparison, in 2000, plant aboveground biomass was not increased in the warmed plots. These data illustrate the complexity of warming responses in the field. Testing of hypotheses regarding mechanisms of the observed stimulation in mycorrhizal parameters therefore has to occur in subsequent controlled environment studies (at the expense of decreased realism).

Glomalin and soil aggregation

Immunoreactive glomalin decreased in the warmed plots (Table 2). This is consistent with our hypothesis that glomalin, as other portions of soil organic matter, may be subject to more rapid decomposition at higher temperatures. This is consistent with our data on increased soil moisture availability in soils (0–15 cm) during 1999. This response was not seen at the level of the larger Bradford (total) protein pools, possibly indicating that microbial attack has led to decreased antibody recognition of glomalin protein.

An alternative hypothesis for decreased glomalin pools cannot be excluded with our present understanding of the factors controlling hyphal production of this recently discovered (Wright et al. 1996) protein. Average glomalin production per hyphal length could have decreased in the warmed plots. Further experimentation is needed to distinguish between the two mechanisms.

Supporting our initial hypothesis, a decrease in (immunoreactive) glomalin was associated with a decrease in aggregate water stability, as measured for several size classes. Our data on hyphal lengths from aggregates (Fig. 2) illustrate that AMF hyphal lengths may not be

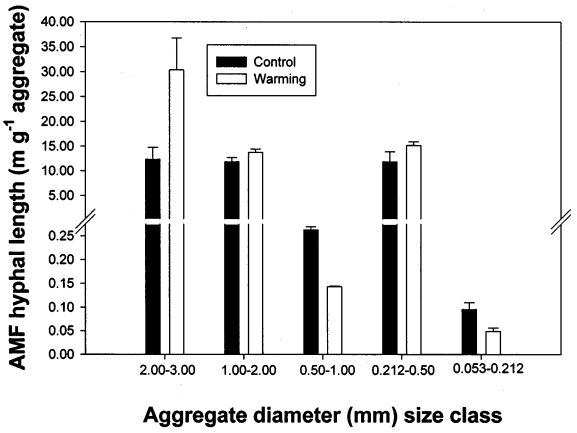

Fig. 1. Effects of warming on hyphal length in soil aggregates of five aggregate diameter size classes. Error bars are standard errors of the mean ($n = 4$).

Table 3. Effects of warming on selected soil, root and arbuscular mycorrhizal parameters (2000). Means, standard errors of the mean ($n = 8$) in brackets.

Response variable	Ambient	Warming	F (P) value
Root dry weight (10^{-3} g g^{-1})[$]	1.28 (0.12)	1.83 (0.30)	1.91 (0.19) n.s.
AMF colonization (soil core) (%)[#]	32.15 (3.77)	53.51 (2.76)	27.75 (0.0012)**
Soil aggregates (% soil weight)			
0.5–1.0 mm	10.92 (0.03)	9.56 (0.61)	4.08 (0.078)[+]
1.0–2.0 mm	15.36 (0.68)	14.12 (0.27)	2.85 (0.12)[+]
(MANOVA)			7.16 (0.02)*

[+] $0.05 < P < 0.15$ (trend), * $P < 0.05$, ** $P < 0.01$, n.s. not significant ($P > 0.15$).
[#] arcsine-square root transformed.
[$] log transformed.

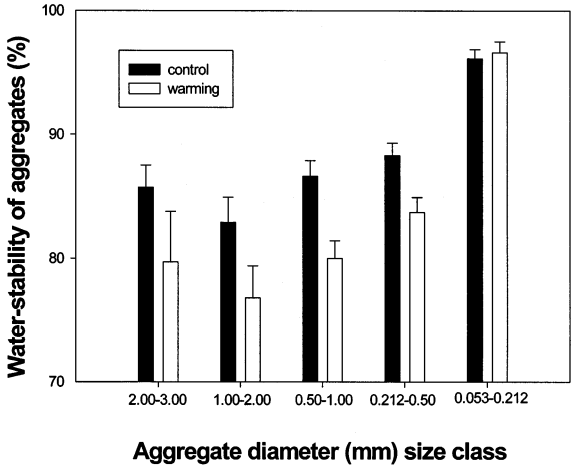

Fig. 2. Effect of warming on water stability (%) of soil aggregates in five aggregate diameter size classes. Error bars are standard errors of the mean ($n = 8$).

as good a predictor of aggregate stability as generally assumed (e.g. Jastrow and Miller 1997). Instead, glomalin could be a useful indicator of soil structure changes in response to global change factors, or other ecosystem treatments in general.

It is remarkable that we observed aggregate stability changed after only one growing season of warming treatment. Importantly, these changes were maintained the following year. However, it will be important to monitor these changes in the long-term: the magnitude of soil aggregation changes was relatively small, but cumulative effects over several years may contribute to greater effect sizes.

Soil carbon storage

Soils are a very large repository for carbon: roughly two thirds of all terrestrial carbon is contained in soil organic matter (e.g. Schlesinger 1977, Post et al. 1982, Schlesinger 1990). About a third of soil carbon has relatively short to intermediate turnover times (time required to convert organic carbon to inorganic carbon, e.g. CO_2) of decades or less (e.g. Schimel et al. 1994, Trumbore et al. 1996, Trumbore 1997). Therefore, small changes in the factors and mechanisms controlling organic matter production or decomposition may cause soils to become sinks or sources of atmospheric CO_2 (Trumbore et al. 1996). Stable aggregates can protect otherwise labile organic material from decomposition (Jastrow 1996, Jastrow and Miller 1997). A decrease in aggregate stability, as observed in this study, could lead to a negative impact on soil carbon storage under conditions of global warming, i.e. accelerate carbon loss from soils. Additionally, soil could be lost to erosion processes.

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