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Fluorescein Diacetate Hydrolysis as a Measure of Fungal Biomass in Soil

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Abstract. The fatty acid methyl esters of lipids extracted from an agricultural soil in the preharvest period of soybean or middle growth cycle from wheat were characterized and quantified by gas-liquid chromatography. The fatty acids 18:2ω6 and 16:1ω5 were used as markers of saprotrophic and arbuscular mycorrhizal fungi. In parallel, biomass estimation through plate counts in selective media for cellulolytic and saprotrophic fungi was also performed all throughout a soybean crop or middle growth cycle of wheat. As an enzymatic method, the fluorescein diacetate (FDA) hydrolytic activity of the samples was determined. Owing to the high relationship exhibited by FDA hydrolysis with organic carbon and total nitrogen content of soil, the enzymatic activity was correlated with the microbial biomass estimated through marker lipids or plate counts. The results obtained point out that FDA hydrolysis may be used as a rapid, cheap, and reliable estimator of fungal biomass.

Soil microbial biomass comprises 1%–4% of the total organic carbon [3], and 2%–6% of the total organic nitrogen [19] in soil. Microorganisms, despite their relatively low amounts, play the crucial role of keeping the main nutrient cycles in soil (C, N, P, S) through recycling from organic matter. The latter is fundamental not only for primary production, but for the long-term functioning of ecosystems as well [13, 14, 32].

Microbial biomass is very dynamic in soil and responds to weather, crop input, season, soil type, and landscape position [15, 28]. Because of its rapid turnover, microbial biomass is a sensitive indicator of changes in climate, tillage systems, crop rotations, and pollutant toxicity [28]. Thus, the quantitative determination of the amounts of microbial biomass in soil in a rapid, reliable, and cheap way is an important task for the appraisal of fundamental biological and biochemical activities, e.g., microbial respiration, enzymatic activities. Furthermore, microbial biomass must be taken into account in any set of data for the assessment of soil quality [13] and to estimate microbial respiration in soil [9]. It has been stated that the ability to assess the effect of perturbations (e.g., tillage, herbicides, fertilizers) on the status of soil microbial diversity and amount is crucial for the understanding of their impact on soil quality and the sustainability of agricultural practices [33].

Enzymatic activities as a simple approach to the study of microbially mediated processes may correlate with microbial biomass. However, few studies have attempted to make such a correlation between microbial biomass and soil enzymatic activities, and with the presence and activities of specific components of the microbial community. β-*N*-acetylglucosaminidase activity was significantly correlated with estimates of fungal biomass, based on the content of two fungus-specific indicator molecules, 18:2ω6 phospholipid fatty acid (PLFA) and ergosterol [20]. Endo 1,4-β-glucanase/cellobiohydrolase activity was significantly correlated with the PLFA-based estimate of fungal biomass in the soil, but no correlation was found with ergosterol-based estimates of fungal biomass [20].

Within enzymatic activities, the hydrolytic activity of 3′,6′-diacetylfluorescein (FDA) encompasses a wide spectrum of them. In fact, FDA is hydrolyzed by a number of different enzymes such as proteases, lipases,

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Table 1. Soil physico-chemical variables for the same agricultural soil under summer (soybean) and winter (wheat) crops

Crop	OC (%)	TN (%)	C/N	P (ppm)	pH	$EC_{1:1}$ (dS/m)	BD (g/cm^3)
Soybean	3.59 ± 0.41	0.24 ± 0.02	15.2 ± 1.9	37 ± 15	5.42 ± 0.27	0.17 ± 0.02	0.9 ± 0.1
Wheat	3.72 ± 0.08	0.28 ± 0.03	13.2 ± 1.5	20 ± 4	5.67 ± 0.11	0.05 ± 0.01	0.91 ± 0.11

The normal P content of soils from the El Salado river basin is 3–7 ppm. The high P values determined in the top soil profile (0–10 cm) are owing to superphosphate triple addition (SPT 0-46-0: 74 kg/ha on the row).

and esterases [30]. Moreover, it has been used to determine amounts of active fungi and bacteria [7, 18, 30, 31]. When taken up by cells, FDA is cleaved by esterases and retained inside as fluorescein, i.e., the product of this enzymatic conversion, which can be visualized by fluorescence microscopy or quantified by fluorometry or spectrophotometry [11, 26].

Our previous data showed that fungal biomass was higher (10–30:1) with respect to the bacterial one in soils from the El Salado river basin (Buenos Aires, Argentina; Aon et al. unpublished results). Soil fungi often make up at least 75%–95% of the soil microbial biomass and, together with bacteria, are responsible for about 90% of the total energy flux of organic matter decomposition in soil [8, 26]. Saprotrophic fungi and mycorrhiza are among the main groups of microorganisms associated with plant roots. As obligate symbionts, mycorrhizal fungi are associated with nearly all plants. Particularly, arbuscular mycorrhiza (AM) or vesicular-arbuscular mycorrhiza (VAM) (depending upon whether the fungi produce vesicles in the root cortex of the host plant) are the most frequently found in nature and widely distributed geographically and in the plant kingdom [17]. The most important advantage obtained by plants involved in mycorrhizal symbiosis is related to nutrient uptake and translocation contributed by the fungus, especially that of phosphorus [26]. Given the geographical ubiquity of fungi that take part of AM or VAM, and that the great majority of plants are associated with them, we expect them to contribute to soil fertility. This is a timely topic for soils from the El Salado river basin owing to the well-known fact that they are poor in P [9]. Thus, the estimation of mycorrhizal fungi biomass is of importance. For AM fungi, the signature fatty acid 16:1ω5 provides a promising tool for the estimation of VAM fungal biomass in soil and roots [22, 23]. For ectomycorrhizal fungi, 18:2ω6,9 dominates among fatty acids and can be used as an indicator of mycelial biomass of these fungi. The fatty acid 18:2ω6 accounts for 43% of the total fatty acids of 47 species of soil fungi, whereas the fatty acid 16:1ω5 is found especially in the AM fungi genus *Glomus* [26].

Thus, the main objective of the present work was to check whether the FDA hydrolytic activity could be used as a simple, rapid, and reliable method for estimating fungal biomass. In order to achieve this aim, we used different methods (marker lipids and CFU counting) for the estimation of mycorrhizal and saprotrophic fungal biomass.

Materials and Methods

3′,6′-diacetylfluorescein (FDA) and fluorescein sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used were of analytical grade.

Soil characteristics and sampling procedure. The soil examined (Argiudoll aquic) in the El Salado river basin (center east of the province of Buenos Aires, Argentina) presents a high content of OC (ca. 3%) and acid pH (between 5 and 6) (see Table 1), corresponding to a natural grass land (28 ha) that was conventionally managed only two years earlier to the date of the startup of the present experiment (October 10, 1998) [9]. In this work, microbiological, enzymatic, physical, and chemical variables were monitored in two depths, D1 (5–10 cm) and D2 (15–20 cm), before plowing and seeding (T0), at the flowering stage (T1), and preharvest period (T2), respectively, of the soybean crop [*Glycine max* (L.) Merr.], or middle growth cycle of wheat [*Triticum aestivum*] (T3). We did a composite stratified random (i.e., serpentine [10]) sampling method distributed in row and inter-row locations. Soil samples were collected for enzymatic, microbial, and physico-chemical tests, by using a 20-mm (i.d.) hand probe to a depth of 20 cm. The soil columns corresponding to the 5–10 cm or 15–20 cm depth were directly stripped from the hand probe. At the places where each sample was collected, we pooled five to six subsamples in a square of *ca.* 3 m^2.

Analysis of lipid fatty acids and fungal biomass estimation. In order to detect the presence of mycorrhiza and saprotrophic fungi in the top soil profile (D1 = 5–10 cm) with the molecular markers fatty acid 16:1ω5 and 18:2ω6, respectively, a lipid extraction from the soil samples (200 g dry soil) was performed with a 2:1 chloroform:methanol mixture and sonication (3 × 20 min). The methanolic phase was discarded, and the chloroformic phase containing the total of lipids was dried, concentrated, and saponified with 10% KOH in ethanol at 80°C for 45 min in N_2 atmosphere. The fatty acids were extracted with hexane after acidification and were esterified with boron trifluoride 10% in methanol. Fatty acid methyl esters (FAME) were analyzed by gas liquid chromatography on a 30-m DB-23 phase J&W Scientific capillary column in a Hewlett Packard model 6890 gas chromatograph equipped with a flame ionization detector. The column temperature was programmed for a linear increase of 3°C/min from 160° to 220°C. Identification of the fatty acids 16:1ω5 and 18:2ω6 was achieved by using the retention times on a chromatogram with soil fatty acids

previously determined through gas chromatography and mass spectrometry.

The amount of fungal biomass was estimated from molecular markers, phospholipid fatty acid (PLFA) 16:1ω5 (mycorrhiza) and 18:2ω6 (saprotrophic fungi). The total of fatty acid methyl esters of lipids for each sample was quantified, and the percentage of phospholipids (25%) as well. The amount of 16:1ω5 (MW = 268.4) or 18:2ω6 (MW = 294.5) methyl ester present in each sample was determined by comparison with an internal standard from chromatograms and was converted to saprotrophic or mycorrhizal fungal biomass through the following conversion factors: 1.5 nmol PLFA 16:1ω5 mg^{-1} dry biomass and 2 nmol PLFA 18:2ω6 mg^{-1} dry biomass [24].

Microbial counts and fungal biomass estimation. Viable counts for fungi were performed in a rose bengal-streptomycin agar containing (per liter): 10 g glucose, 5 g peptone, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.033 g rose bengal, 15 g agar. Streptomycin was added after autoclaving in the form of a filter-sterilized solution at a final concentration of 30 μg/ml [27].

Cellulolytic fungi were counted in a slightly modified cellulose-yeast extract agar [1] containing (per liter): 2.5 g cellulose powder (swollen in 0.1 *N* HCl for 12 h in the cold chamber, and then washed by 5 times repeated suspending and decanting with distilled water until neutral), 1.2 g KH_2PO_4, 1.0 g K_2HPO_4, 2.0 g NH_4Cl, 0.89 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 10 mg adenine, 0.1 mg thiamine, 15 g agar. Cellulolytic fungi were counted in the presence of streptomycin at a final concentration of 30 μg/ml. After inoculation, plates (duplicates) were incubated either aerobically or anaerobically at room temperature (22 ± 3°C).

Biomass estimates based on microbial counts can be performed according to the following general expression [32]:

$$\text{Biomass} = \text{number of cells} * \text{volume} * \text{density} \quad (1)$$

The number of cells is given by CFU g^{-1} dry soil obtained as described above. Fungal biomass was estimated using eqn. (1) with the following parameters: density 1.2 g cm^{-3} [26], and the hyphal volume was calculated considering an average diameter of 5 μm for filaments and 10 m length of fungal mycelium per gram of surface soil [2], so that in 1×10^4 cells g^{-1} dry soil (CFU), the average volume of a filamentous fungus will be given by: $(2.5 \times 10^{-6}\ m)^2 * 3.1416 * 10\ m\ g^{-1}$ soil/1×10^4 cells g^{-1} soil, that is, equivalent to 1.96×10^{-8} ml $cell^{-1}$. Applying Eqn. (1), we obtain 0.235 g biomass fungi kg^{-1} soil. In order to express the latter result in kg fungal biomass ha^{-1}, a depth of 20 cm was considered and the corresponding bulk density (see Table 1). Considering a bulk density of 1.0 g cm^{-3}, we obtain 470 kg fungal biomass ha^{-1}.

Measurement of fluorescein diacetate (3′,6′-diacetylfluorescein) (FDA) hydrolysis in soil. FDA hydrolysis was measured as described in [11], with slight modifications. Briefly, soil samples were conditioned by passing them through a 2-mm sieve before use. For FDA hydrolytic activity measurement, soil was kept moist. Sieved soil (0.5 g) was mixed with 5 ml of sodium phosphate buffer 60 mM, pH 7.6, and the reaction was started with the addition of 50 μl 4.8 mM FDA. After 2 h incubation with shaking, the reaction was stopped by adding 5 ml of acetone. Before reading at 490 nm, the tubes were centrifuged for 5 min at 5000 rpm. FDA hydrolytic activity was expressed in the following units: mg fluorescein (Fluor) kg^{-1} soil h^{-1}.

Fungal identification. The method described in [25] was utilized to observe fungi species as active mycelium. Briefly, after shaking a soil sample in water and fractionation, soil particles retained in 0.5 mm mesh were washed and transferred to a sterile filter paper in a Petri dish and dried for one day to suppress vigorous bacterial and yeast growth after plating [34]. Eighty to 100 soil particles were plated in malt agar with glucose in the presence of 0.5% sulfate streptomycin and 0.25% chloramphenicol at a rate of four particles per plate. Plates were incubated at 25°C and observed microscopically at 1-week intervals. Original taxonomic papers and reference [12] were used for identifying sporulating fungi. The percentage frequency of occurrence for each fungi species was calculated as: (number of particles bearing a specified fungus/total number of particles) × 100 [16]. Those particles that did not give colonies or that became contaminated were not scored in the total number of particles.

Table 2. Quantitative comparison between culture-dependent (CFU) and culture-independent (fatty acids) methods of estimation of soil microbial biomass

Method and Treatment		Fungi (g biomass kg^{-1} d.s.)	
		Saprotrophic	AM
CFU	Soybean	2.3 ± 0.8	—
	Wheat	1.37 ± 0.38	—
FAME	Soybean	0.43 ± 0.33	0.57 ± 0.18
	Wheat	0.46 ± 0.08	1.26 ± 0.53

For the procedure of calculation and assumptions, see Materials and Methods. *Key for abbreviations*: CFU, colony-forming units; FAME, fatty acid methyl ester; AM, arbuscular mycorrhiza; d.s., dry soil.

Physico-chemical measurements. Total organic carbon (OC) [21] and Kjeldahl N [6] were determined on dry soil samples (12 h at 105°C). Soil pH (pHmeter Orion SA230, Orion Research, Boston, MA) and conductivity (conductivity/TDS meter Hach, Loveland, CO, USA) were measured on a soil:water 1:1 paste. Phosphorus levels were quantitated according to [5].

Soil water content was determined gravimetrically on samples taken with stainless steel cores of 72-mm diameter and 25-mm height. Bulk density was determined as described in [4].

Statistical analysis. Correlation and regression analysis were performed with the software GraphPad Prism version 2.0 (San Diego, CA). A *t*-test (small samples, paired *t*-test with two-tail *P* values, with unknown variances assumed equal) was used to compare data between both crops.

Results and Discussion

The physico-chemical variables of the soil analyzed are shown in Table 1. They did not differ significantly in both crops analyzed except for P and electrical conductivity ($EC_{1:1}$).

Fungal biomass estimation in soil. Fungal biomass estimated through the fatty acids 18:2ω6 (lipid marker of saprotrophic fungi), 16:1ω5 (lipid marker of mycorrhizal fungi), and CFU plate counting is shown in Table 2. Data of Table 2 correspond to the same agricultural soil but two different crops, i.e., soybean preharvest and half-growth cycle of wheat. Values obtained through both methods differed only by a factor of two (soybean) or were similar (wheat) when the values obtained by FAME

for saprotrophic and mycorrhizal fungi were added (Table 2). The rationale for doing the latter is that according to pot culture studies, more than 80% of the AM fungal mycelium can be found outside the root [22]. When both methods of fungal biomass estimation were plotted against each other, a clear correlation, although not significant ($r = 0.62$; $P > 0.05$), appeared (results not shown). As a caveat, the values obtained by lipid markers corresponding to saprotrophic fungi were plotted for biomass, since plate counting does not quantify mycorrhizal fungi.

The amounts of saprotrophic or mycorrhizal fungal biomass estimated in soil were comparable to those already reported in the literature [24]. Indeed, the amount of mycorrhizal fungi was 0.35 g/kg soil in a soil from Denmark with lower pH (5.2 against ~5.5) and organic matter content (4% against ~6.3%) than those exhibited by the soil analyzed in this work (see Table 1).

It is now accepted that culture-dependent methods (e.g., CFU counting) do not allow access to many soil microorganisms and that we can study only about 1% of the cells in a sample, which does not mean that we can culture only 1% of the species in the sample. The latter is owing to the fact that, although 90–99% of the cells cannot be cultured, they would be represented by the culturable kin because they are phylogenetically similar or identical to the culturable minority [29]. Thus, the coincidence in fungal biomass estimation between both culture-dependent and -independent methods is at least surprising.

According to CFU counting, saprotrophic fungal biomass was 10- to 30-fold higher than bacterial biomass [Aon et al., unpublished results]. Table 3 shows the percentage occurrence of each fungi species in both crops (i.e., *Fusarium oxysporum*, *F. solani*, *Trichoderma koningii*, *T. saturnisporum*, *T. harzianum*, *Penicillium thomii*, *P. rubrum*, *Zygorrhynchus moelleri*). Several of the genera presented in Table 3 have been shown to be able to hydrolyze FDA, e.g., *Penicillium*, *Fusarium* [18, 30]. Given the abundance of species belonging to both genera, it is likely that they are mainly responsible for FDA hydrolysis.

FDA hydrolysis. FDA hydrolytic activity exhibited a linear relationship with fungal biomass estimated through plate counting in both depths (D1: $r^2 = 0.608$, $P < 0.001$; D2: $r^2 = 0.596$, $P < 0.001$) (Fig. 1A, B) of two crops (soybean and wheat). A weaker correlation was observed with fungal biomass quantified through lipid markers ($r = 0.49$; NS, $P > 0.05$) (Fig. 1C). Indeed, the range of FDA (i.e., between 7 and 9 mg fluorescein kg^{-1} soil h^{-1}) for which the correlation with saprotrophic fungal biomass (according to lipid markers) is observed was much more restricted than plate counting.

Table 3. Relative percentage of each fungi species determined in an agricultural soil from the El Salado river basin with soybean or wheat crops

Fungi spp.	Soybean	Wheat
Fusarium oxysporum	27	28.6
Trichoderma koningii	18.2	12.5
Penicillium thomii	14.6	4
Zygorrhynchus moelleri	11.7	8.6
Trichoderma saturnisporum	7.2	19.8
Humicola fusco-atra	6.1	2.8
Penicillium rubrum	6.0	3.9
Gliocladium roseum	2.9	3.1
Penicillium restrictum	1.6	0.5
Talaromyces helicus	1.6	0.3
Fusarium solani	1.1	6.0
Gongronella butleri	0.7	0.3
Trichoderma harzianum	0.2	5.4
Thielavia basicola	1.3	—
Rhizopus nigricans	1.1	—
Myrothecium verrucaria	0.5	—
Aspergillus fumigatus	1.2	—
Mucor hiemalis	0.4	—
Penicillium megasporum	0.3	—
Chaetomium globosum	—	0.5
Cylindrocarpon didymum	—	0.7
Cylindrocarpon obtusisporum	—	0.2
Mortierella sp.	—	0.8
Penicillium lilacinum	—	0.2

The percentage value for each fungi species was determined as described under Materials and Methods. The number of samples analyzed in soybean (T2) and wheat (T3) were n = 10 and n = 8, respectively.

In turn, FDA hydrolytic activity strongly correlated with OC ($r = -0.58$; $P < 0.001$) and TN ($r = 0.787$; $P < 0.001$) (Fig. 2), water-filled pore space ($r = -0.658$; $P < 0.001$). These results suggest that FDA hydrolytic activities integrate information from microbial status (namely, fungal) and soil physico-chemical conditions [Aon et al., unpublished results].

Synthetically, enzymatic activities that hydrolyze FDA may function as a sensitive estimator of fungal biomass (saprotrophic and mycorrhizal), as could be judged through comparison with culture-dependent and -independent methods.

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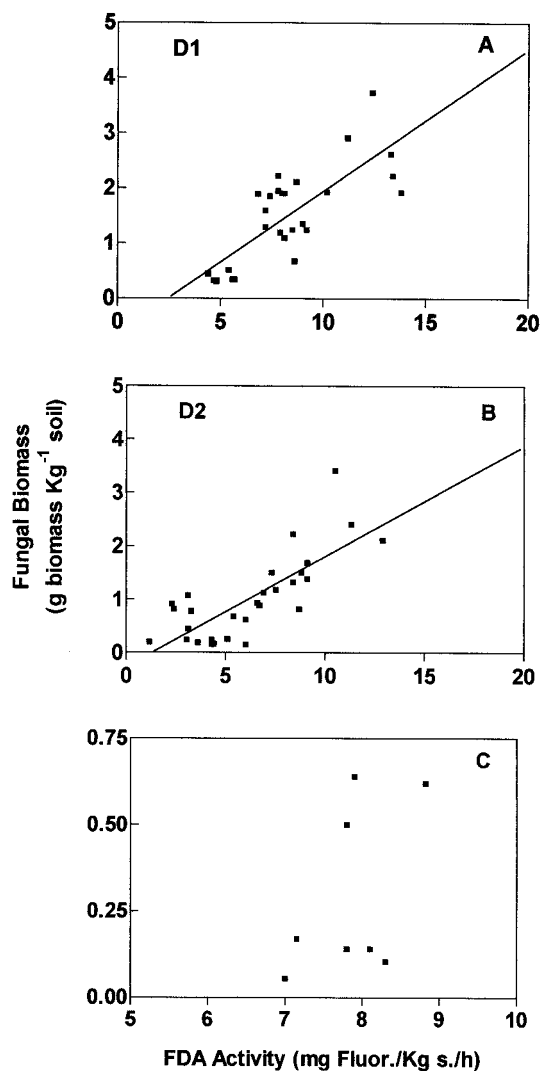

Fig. 1. Regression analysis and scatter plots of saprotrophic fungal biomass estimated through plate counting in two depths: D1 (5–10 cm) (A), D2 (15–20 cm) (B), or 18:2ω6 lipid marker (C) as a function of FDA hydrolytic activity. Correlation and regression analysis as well as the fungal biomass and enzymatic activity were determined as described under Materials and Methods. In panel A, the equation relating fungal biomass and FDA hydrolytic activity is: $Y = 0.257 \pm 0.040 X - 0.623 \pm 0.349$, whereas in panel B the equation is: $Y = 0.208 \pm 0.033 \times - 0.271 \pm 0.225$. Plotted are the values obtained from four sampling times (A, B: T0-T3; see Materials and Methods) or one sampling time (C, T2; see Materials and Methods) during soybean crop.

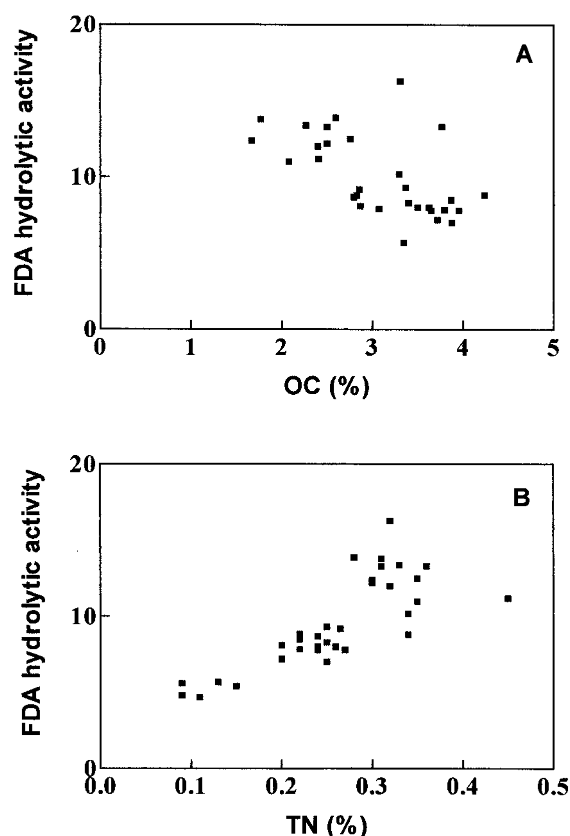

Fig. 2. Scatter plots of the FDA hydrolytic activity as a function of organic carbon (OC, A), and total nitrogen (TN, B) content of soil. The enzymatic activities and physico-chemical variables were determined as described under Materials and Methods. Plotted are the values obtained from three sampling times during the soybean and wheat crops (T0–T3; see Materials and Methods). Statistical analysis was performed as described under Materials and Methods.

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