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Biosynthesis and degradation of glycerides in external mycelium of *Glomus mosseae*

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Abstract The activities of enzymes involved in the glyceride metabolism of *Glomus mosseae* external mycelium are reported. Total mycelial homogenates were incubated with radiolabeled triolein and palmitic acid for various times under different conditions. The results obtained demonstrate the capacity of *G. mosseae* external mycelium to synthesize and hydrolyze its own acylglycerides. Neutral lipid biosynthesis progressively increased along with root colonization. Incorporation of [^{14}C]-palmitate was mainly into triacylglycerols and as a minor fraction into diacylglycerols. The activity of palmitoyl-CoA ligase in external mycelium also increased in parallel with mycorrhiza development. The hydrolysis of triacylglycerols was very low at the beginning of colonization and then increased. However, lipase activity was lower than that of acyl-CoA ligase even at late stages of colonization. Thus, triacylglycerol biosynthesis apparently prevails over degradation during *G. mosseae* mycelium development in the period examined.

Keywords Acyl-CoA ligase · Acylglycerol synthetase · External mycelium · Lipase activity · Triacylglycerols

Introduction

The external mycelium of arbuscular mycorrhizal fungi (AMF) plays an important role in plants. Hyphae that grow from roots into the soil matrix are the functional organs responsible for nutrient uptake and translocation. However, much remains to be learned about their growth and distribution (Sylvia 1992). Also, little is known about the metabolism of carbon compounds in the external mycelium because AMF cannot be grown in pure culture in the absence of a host.

The triacylglycerol fraction is the major lipid class both in spores and in the external mycelium of such fungi. It represents about 50% of the total organic matter of spores (Gaspar et al. 1994a). It has been shown that triacylglycerols decrease to nearly one-fourth of their initial concentration by 10 days after germination (Gaspar et al. 1994b). Such degradation implies the action of hydrolytic enzymes of the lipase type and this was confirmed when an active lipase was isolated from spores and then purified (Gaspar et al. 1997b). During external mycelium development, we observed an increase in triacylglycerol concentration (Gaspar et al. 1997a) which reached its maximum in the hyphae obtained from roots 3 months after colonization. Such accumulation of triacylglycerols in the external mycelium could be the result of either considerable biosynthetic activity or uptake from the soil or root. Results supporting the idea that lipids are translocated from intra- to extraradical mycelium were obtained by Pfeffer et al. (1999) in experiments with intact mycorrhiza. These earlier studies suggest that AMF have an active lipid metabolism throughout their life cycle.

In this present work, we studied the activity of enzymes involved in the glyceride metabolism of *Glomus mosseae* external mycelium. We report variations in enzyme activities during mycelium growth and possible pathways of triacylglycerol metabolism.

Materials and methods

Biological material and growth conditions

Cucumis sativus cv palmetto seeds were surface sterilized in 10% NaOCl for 15 min with continuous stirring, germinated on moistened sand, and transplanted to 500-ml open pots of a steamed 2:3 (v/v) soil:vermiculite mixture. The AMF inoculum consisted of 5 g of rhizosphere soil from a *Sorghum vulgare* Pers pot culture with *G. mosseae* (Nicolson & Gerdemann) (Gerdemann & Trappe) isolated from soil sampled in San Bernardo, Pcia. Buenos Aires, Argentina (SB2 germ plasm collection at Instituto Spegazzini).

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This contained spores, extramatrical mycelium and colonized root fragments. This inoculum was introduced into nine pots with three plants each. Cucumber plants were watered from below using a capillary system and fed once a week with a nutrient solution (Hewitt 1952) lacking phosphate. Plants were kept in a controlled-climate glasshouse under 14 h of light per day with the natural light augmented by halogen lamps (250 mmol m^{-2} s^{-1} photon flux density; lamps were switched on when natural light intensity decreased below this threshold). Root length colonized was measured as described in a previous study (Gaspar et al. 1997a).

External mycelia were isolated from roots of 45-, 60- and 90-day-old cucumbers colonized by *G. mosseae*. Roots from each of three replicate groups of pots collected at each sampling time were used. The roots were disinfected with 2% chloramine T plus 200 ppm streptomycin for 20 min, drained and rinsed three times with sterilized water. The external mycelium was collected manually with forceps under a dissecting microscope. Microscopic inspection of the samples was performed to check for microbial contamination. Samples were maintained at –20°C until use.

Preparation of external mycelium homogenate

The external mycelia (5–35 mg) were frozen in liquid nitrogen and finely pulverized in a mortar. The resulting powder was homogenized using a 0.2-ml glass potter (Wheaton, USA) in solutions containing an appropriate buffer for each assay. This total homogenate was used immediately for assays of enzyme activities. Preliminary to any enzymatic assays, total protein concentration of the mycelium homogenate was determined colorimetricaly (Lowry et al. 1951). Total lipids were extracted from other portions of mycelium powder and quantified as described previously (Gaspar et al. 1997a).

Chemicals

1-^{14}C-palmitic acid (57.0 mCi/mmol) and 1-^{14}C-triolein (112 mCi/mmol), both 99% radiochemically pure, were purchased from New England Nuclear Corp. (Boston, Mass.). Cofactors used for enzymatic reactions were provided by Sigma Chemical Co. (St. Louis, Mo.). All chemicals and solvents were of analytical grade.

Biosynthesis of acylglycerols

Five nmol of 1-^{14}C-labeled palmitic acid (0.285 µCi/tube) and 40 nmol of unlabeled palmitic acid were dissolved in 20 µl of propyleneglycol and incubated with 160 µg of total mycelia protein in a shaking bath at 32°C for 2, 4, 7 and 15 h. The incubation solution contained 50 mM potassium phosphate buffer pH 7.4, 20 mM D, L-α-glycerophosphate sodium salt, 2 mM $MgCl_2$, 5 mM N acetyl-L-cysteine, 5 mM ATP (sodium salt) and 0.2 mM CoASH (lithium salt) (Tietz 1969; González Baró and Pollero 1993) in a final volume of 500 µl.

In order to measure the incorporation of labeled palmitate into lipids, 2 ml of $CHCl_3$:CH_3OH (2:1 v/v) was added to the mixture at the end of the incubation period and total lipids were extracted following the procedure of Folch et al. (1957). The different lipid classes were separated by thin layer chromatography (TLC) on high performance TLC Silicagel 60 plates (Merck, Darmstadt, Germany) with hexane/ether/acetic acid (80:20:1.5 by vol). The radioactivity associated with each spot was quantified by proportional scanning counting with a Berthold LB2723 Dünnschicht Scanner II apparatus. Appropriate standards, run simultaneously, were visualized by exposure to iodine vapors. Assay mixtures without enzyme preparations were incubated and processed simultaneously as controls. The biosynthetic activity was expressed as % of synthesized acylglycerols h^{-1} mg^{-1} total proteins. The sum of radioactivities in triacylglycerols, diacylglycerols and free fatty acids is 100%.

Fatty acyl coenzyme A ligase assays

Basically, the procedure used was a modification of that applied by Singh et al. (1988). The reaction mixture, in a total volume of 0.5 ml, contained 5 nmol of 1-^{14}C-labeled palmitic acid (0.285 µCi/tube) and 40 nmol of unlabeled palmitic acid which were added dissolved in propyleneglycol (20 µl/tube); 10 mM ATP; 50 µM CoASH; 5 mM $MgCl_2$; 0.5 mM dithiothreitol; 50 mM KCl; 0.05% (w/v) Triton X-100 and 30 mM 3-(*N*-morpholino) propane sulfonic acid pH 7.8. The reaction was started by the addition of mycelia homogenate equivalent to 200 µg of proteins. The incubations were performed at 30°C with continuous stirring for 1, 2 and 4 h and were stopped by the addition of 2.5 ml Dole's reagent (Dole 1956) (isopropyl alcohol/heptane/2 M H_2SO_4 40:10:1). The denatured protein was removed by centrifugation. Water (0.9 ml) and n-heptane (1.6 ml) were added to the supernatant fraction and mixed on a vortex mixer. The lower (aqueous) phase which contained the reaction product (palmitoylCoA) was washed three times with 1.6 ml portions of heptane and 500 µl of the lower layer was dissolved in Aquasol scintillation (New England Nuclear) and mixed vigorously. The radioactivity was determined by liquid scintillation counting (LSC) in an LKB Wallac 1219 Rackbeta. The activity was expressed as nmol palmitoyl CoA h^{-1} mg^{-1} of proteins.

Lipase activity assays

Assays for the lipase activity determination were performed as described by Gaspar et al. (1997b) with minor modifications. The basic assay mixture contained 1 µl of labeled triolein (0.1 µCi/tube), 30 µg unlabeled triolein, 0.3% v/v Triton X-100, and 50 mM Tris HCl buffer pH 8; the reaction mixtures were emulsified by sonication using a Heat Systems-Ultrasonics, Inc. model: W220-F sonicator at 4°C for 1 min. Emulsions were always prepared immediately before use. The assay mixtures for lipase activity contained 130 µl substrate emulsion, 200 µg of total mycelia homogenate proteins and bidistilled water made up to a final volume of 500 µl. These were incubated at 30°C with continuous stirring for 1, 2, 3 and 4 h. The incubations were stopped by immersing the tubes in a boiling water bath for 10 min. The products of hydrolysis and the remaining substrate were extracted with $CHCl_3$:CH_3OH (2:1, v/v) and separated by TLC as described previously. Lipids were identified by comparison with standards run on the same plate. Radioactivity found in different products of hydrolysis was detected on the chromatographic plates by proportional scanning counting and quantified. The lipase activity was expressed as µmol triacylglycerols hydrolyzed h^{-1} mg^{-1} protein.

Results

Variation in the total lipid and protein content of external mycelium isolated from cucumber roots with time after colonization is shown in Table 1. Within the first 60 days of inoculation, the percentage of proteins was higher than that of lipids, whereas after 60 days lipids increased markedly and total proteins diminished to about one-fifth of their initial value.

External mycelia homogenates were incubated with [1-^{14}C]-palmitic acid in the presence of α-glycerophosphate to examine the synthesis of acylglycerides. Neutral lipid biosynthesis increased gradually up to 4 h of incubation (Fig. 1A), after which the rate of increase slowed. Figure 1B shows that the biosynthesis capacity of these lipid classes progressively increased with time after inoculation. The incorporation of [^{14}C]-palmitate was

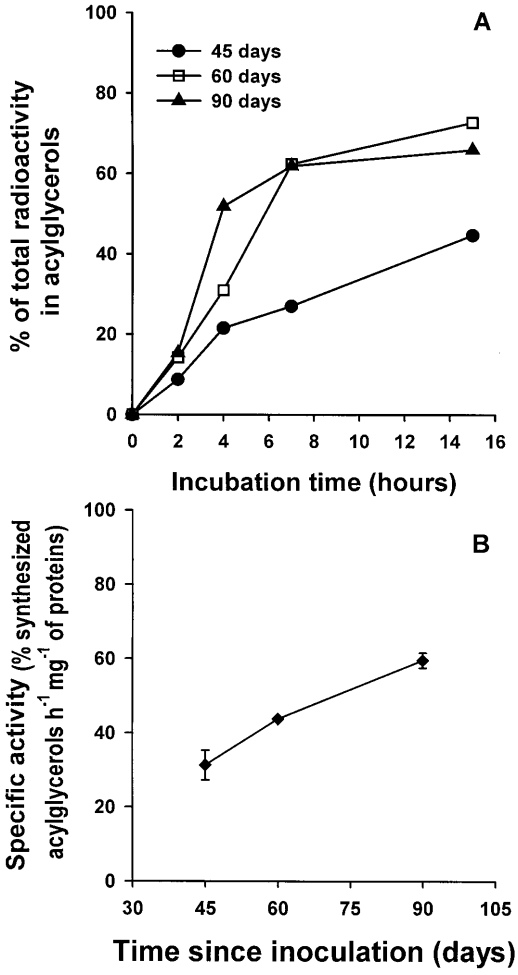

Fig. 1 **A** Time course of acylglycerol synthesis in total mycelium homogenates isolated from cucumber roots at different times after inoculation. The sum of radioactivity in triacylglycerols, diacylglycerols and free fatty acids is 100%. **B** Change in acylglycerol synthetase specific activity in external mycelium with time after inoculation; *bars* SD

Table 1 Total protein and lipid content (μg/mg mycelium) of external mycelium isolated after 45, 60, and 90 days from cucumber roots colonized by *Glomus mosseae*. The data are the means of three independent experiments (separate plantings) ± SD

Days	Proteins	Lipids
45	26.0±5.1	1.6±0.1
60	31.4±0.9	2.5±0.2
90	5.8±0.8	27.8±0.9

found mainly in triacylglycerols and as a minor fraction in diacylglycerols (Table 2).

The activity of palmitoyl-CoA ligase in the external mycelium also increased in parallel to mycorrhiza development. The peak of specific activity was obtained in external mycelia isolated from roots 60 and 90 days after inoculation (Fig. 2).

[1-^{14}C]-Triolein, used as substrate for lipase activity assays, was hydrolyzed, yielding free fatty acids, diacyl-

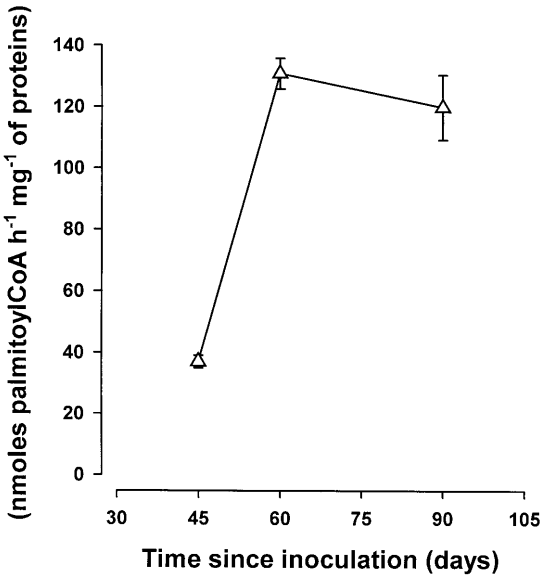

Fig. 2 Change in palmitoyl-CoA ligase activity in external mycelium with time after inoculation; *bars* SD

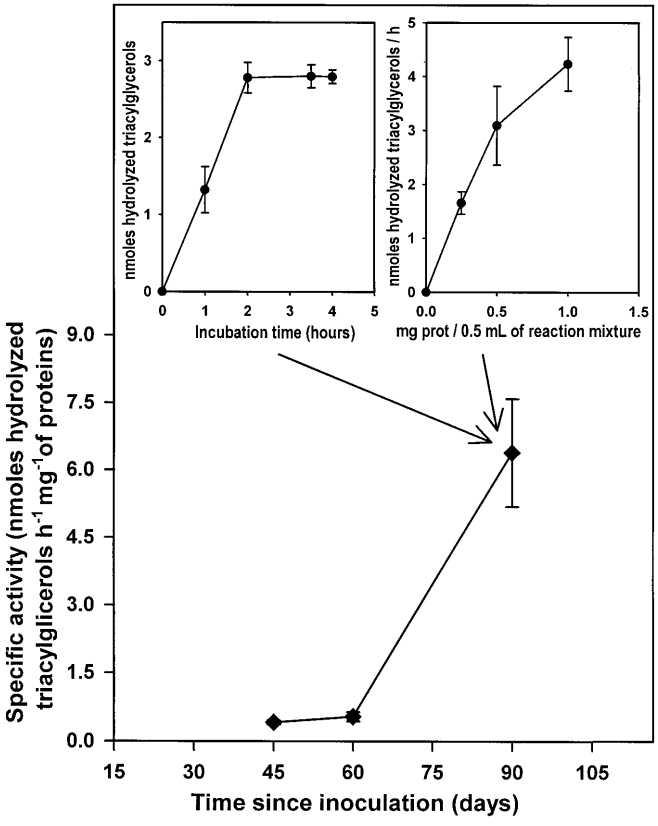

Fig. 3 Change in triacylglycerol lipase activity in external mycelium with time after inoculation of cucumber. The insets show the effects of protein concentration and incubation time on triacylglycerol hydrolysis in total mycelium homogenates isolated from roots of 90-day-old cucumber colonized by *Glomus mosseae*; *bars* SD

Table 2. Incorporation of [1-^{14}C]-palmitate into neutral lipids in external mycelium of *G. mosseae* collected from cucumber roots 45, 60 and 90 days after inoculation. The data (%) are the means of three independent experiments ± SD

Days	Fatty acids	Acyglycerols	
		Triacylglycerols	Diacylglycerols
45	73.02±11.50	18.43±5.01	8.55±2.33
60	37.09±7.44	45.57±10.20	17.34±5.41
90	38.14±8.31	45.76±9.78	16.10±4.09

glycerols and monoacylglycerols. The results obtained are shown in Fig. 3. The triacylglycerol lipase activity in the external mycelium was found to be very low 45 days after inoculation, but then increased with time as in the other enzymatic systems. This increment was dramatic between 60 and 90 days. Figure 3 also shows that triacylglycerol hydrolysis in the external mycelium isolated 90 days after inoculation was linear at least for 2 h of incubation, and was linearly proportional to the amount of the mycelium protein at least up to 0.5 mg protein/0.5 ml of reaction mixture.

Discussion

The most conspicuous feature of AMF is the accumulation of triacylglycerols either in spores (Gaspar et al. 1994a) or in external mycelium during late stages of colonization (Gaspar et al. 1997a). In our investigations, which focused on lipid metabolism, we detected the activities of triacylglycerol lipase and acylglycerol synthetase, two key enzyme systems implicated in degradation and in triacylglycerol synthesis, respectively. In addition to the detection of enzymatic activities, we also found that these enzymes showed differences in their activities with time after inoculation.

The acylglyceride biosynthetic activity detected in vitro in the external mycelium of *G. mosseae* suggests that this fungus is capable of synthesizing its own triacylglycerols. This system of biosynthesis involves the actions of an acyl-CoA ligase, which activates fatty acyl chains, and of acyltransferases and phosphatidate phosphohydrolase, which perform the glycerol esterification. We detected and quantified the activity of an acyl-CoA ligase on palmitic acid. Using this substrate, we also demonstrated the existence in the external mycelium of an activation-transference system which catalyzes the esterification of the glycerol 3-phosphate by activated fatty acyls. The results show high activity of the acyltransferase and an even higher acyl-CoA activity. The occurrence of this glyceride biosynthetic activity and its increment with time after inoculation could explain the high triacylglycerol concentration previously observed in vivo in the external mycelium at 90 days after inoculation (Gaspar et al. 1997a). Recently, on the basis of labeling experiments with intact mycorrhiza, Pfeffer et al. (1999) proposed that lipids are synthesized by the fungus within the root and are stored or exported in this form to the extraradical mycelium, where they are stored or metabolized. Nevertheless, our results from in vitro assays with the isolated external mycelium clearly indicate the occurrence of an enzyme system for acylglyceride synthesis.

Although we have demonstrated the occurrence of key enzymes in the pathway of triacylglycerol synthesis, we did not determine whether the precursors or the intermediates for this synthesis are provided by host metabolites or by the fungus itself. Glycerol 3-phosphate may be produced by a reduction of dihydroxyacetone phosphate, an intermediate in glycolysis, or by the phosphorylation of glycerol by the enzyme glycerol kinase in the presence of ATP. The presence of glycerol kinase in AMF was recently reported by Harrier et al (1998). Mc Donald and Lewis (1978) and Saito (1995) found active enzymes of the glycolytic pathway in internal hyphae by means of histochemical studies. Recently, by glucose-labeling experiments, we too found evidence of that catabolic pathway in AMF external mycelium (results not shown). Therefore, the glycerol 3-phosphate necessary for glyceride synthesis in *G. mosseae* could arise by any of the above-mentioned mechanisms. In addition, the acyl groups necessary for the acylglycerol synthesis would be provided by acetate through the glycolytic pathway.

We have also detected lipolytic activity in the external mycelium of *G. mosseae* by using the substrate triolein, which is the major molecular species in the triacylglycerols of arbuscular mycorrhizae (Gaspar et al. 1994a). The hydrolytic activity on triacylglycerols is low initially but increases with external mycelium development. Enzymes that catabolize lipids are essential not only for the turnover of these molecules but also for providing their breakdown products for the synthesis of other cell constituents or for energy. The increase of the external mycelium and its phospholipid content (Tunlid and White 1992) suggests that the hydrolytic activity of triacylglycerols in the mycelium contributes to the provision of carbon skeletons and energy necessary for phospholipid synthesis and membrane formation. A similar carbon transfer from spore triacylglycerols to phospholipids has been reported during germ tube development (Gaspar et al. 1994b). It is also possible that most of the phospholipid carbons arise from glucose degradation in the mycelium. Despite the increment in lipolytic activity of the external mycelium isolated 90 days after inoculation, the lipase specific activity is 17-fold lower than that found for the enzymes involved in biosynthesis, such as acyl-CoA ligase, at the same time. Thus, triacylglycerol biosynthesis seems to prevail over degradation. On the other hand, the lipolytic activity in mycelium is also lower than that reported for spores in previous studies (Gaspar et al. 1997a). This might indicate a lower requirement for energy and carbon during mycelium development than during spore germination.

In conclusion, we found strong evidence that *G. mosseae* external mycelium can synthesize its own triacyl-

glycerols. This anabolic activity is important from the beginning of colonization, but catabolism becomes notable at later stages. Although triacylglycerols may be degraded in part or converted into phospholipids, they should be kept at high concentrations in the external mycelium for later transfer to spores, where they play a major role during germination.

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References

Dole VP (1956) A relation between non esterified fatty acids in plasma and the metabolism of glucose. J Clin Invest 35:350–357

Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Gaspar ML, Pollero RJ, Cabello MN (1994a) *Glomus antarcticum:* the lipids and fatty acid composition. Mycotaxon 60:129–136

Gaspar ML, Pollero RJ, Cabello MN (1994b) Triacylglycerol consumption during spore germination of vesicular-arbuscular mycorrhizal fungi. J Am Oil Chem Soc 71:449–452

Gaspar ML, Pollero RJ, Cabello MN (1997a) Variations in lipid composition of alfalfa roots during colonization with the arbuscular mycorrhizal fungus *Glomus versiforme*. Mycologia 89:37–42

Gaspar ML, Pollero RJ, Cabello MN (1997b) Partial purification and characterization of a lipolytic enzyme from spores of the arbuscular mycorrhizal fungus *Glomus versiforme*. Mycologia 89:610–614

González-Baró MR, Pollero R (1993) Palmitic acid metabolism in the hepatopancreas of the freshwater shrimp *Macrobrachium borelii*. Comp Biochem Physiol 106:71–75

Harrier LA, Wright F, Hookes JE (1998) Isolation of the 3-phosphoglycerate kinase gene of the arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe. Curr Genet 34:386–392

Hewitt E J (1952) Sand and water culture methods used in the study of plant nutrition. Commonwealth Agriculture Bureau, Technical Communication No. 22

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

McDonald RM, Lewis M (1978) The occurrence of some acid phosphatases and dehydrogenases in the vesicular-arbuscular fungus *Glomus mosseae*. New Phytol 80:135–141

Pfeffer Ph E, Douds Days Days, Bécard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120:587–598

Saito M (1995) Enzyme activities of the internal hyphae and germinated spores of an arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. New Phytol 129:425–431

Singh I, Bhushan A, Relan N, Hashimoto K (1988) Acyl-CoA ligases from rat brain microsomes: an inmunochemical study. Biochim Biophys Acta 963:509–514

Sylvia DM (1992) Quantification of external hyphae of vesicular-arbuscular mycorrhizal fungi. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology. Academic, New York, pp 53–65

Tietz A (1969) Studies on the biosynthesis of diglycerides and triglycerides in cell-free preparations of the fat body of the locust *Locusta migratoria*. Israel J Med Sci 5:1007–1017

Tunlid A, White DC (1992) Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In: Stotzky G, Bollag JM (eds) Soil biochemistry. Dekker, New York, pp 229–262