Biol Fertil Soils (2012) 48:743–762
DOI 10.1007/s00374-012-0723-0

REVIEW

Soil enzymology: classical and molecular approaches

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Received: 11 April 2012 / Revised: 27 June 2012 / Accepted: 16 July 2012 / Published online: 15 August 2012

Abstract It is still problematic to use enzyme activities as indicators of soil functions because: (1) enzyme assays determine potential and not real enzyme activities; (2) the meaning of measured enzyme activities is not known; (3) the assumption that a single enzyme activity is an indicator of nutrient dynamics in soil neglects that the many enzyme activities are involved in such dynamic processes; (4) spatio-temporal variations in natural environments are not always considered when measuring enzyme activities; and (5) many direct and indirect effects make difficult the interpretation of the response of the enzyme activity to perturbations, changes in the soil management, changes in the plant cover of soil, etc. This is the first review discussing the links between enzyme-encoding genes and the relative enzyme activity of soil. By combining measurements of enzyme activity in soil with expression (transcriptomics and proteomics) of genes, encoding the relative enzymes may contribute to understanding the mode and timing of microbial communities' responses to substrate availability and persistence and stabilization of enzymes in the soil.

Keywords Soil enzyme · Genes encoding enzymes · Soil enzyme activity · Enzyme kinetics · Gene expression

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Introduction

Despite soil enzymology being one of the oldest fields of research in soil biochemistry (Dick and Burns 2011; Skujins 1978) with an extensive bibliography, as shown by several review chapters and entire books published on the topic (Burns 1978a, b; Burns and Dick 2002; Dick 2011a,b; Kiss et al. 1998) and by international conferences (Granada, Spain; Prague, The Czech Republic; Viterbo, Italy and Bad Nauheim, Germany), there are still problems in the interpretation of measurements of enzyme activities and in the use of these measurements for evaluating soil microbial functioning (Gianfreda and Ruggiero 2006; Nannipieri 1994; Nannipieri et al. 2002). Major development in soil enzymology occurred after 1950 (Skujins 1978), with researches on origin, production, stabilization and persistence of soil enzymes and on the role of enzyme activities in the soil–plant system. Hydrolase activities, such as polysaccharidase, urease, phosphatase and sulphatase activities, received more attention than oxidative enzyme activities, such as phenol oxidase and peroxidase activities (Burns 1978b; Ladd 1978; Sinsabaugh 2010) because hydrolysis of organic molecules was considered more important than the oxidation of organic matter for the release of plant nutrients and because accurate methods were initially

developed for the former rather than the latter enzyme activities. The development of soil enzymology was also due to the fact that soil enzyme assays are simple, accurate, cheap and generally based on short-term laboratory incubations.

It is well established that the measured enzyme activity in soil depends on the active enzymes having different locations (see below) and different origin (plant, microbial and faunal origin), with a multitude of microbial species as potential contributors (Burns 1982; Dick and Burns 2011; Nannipieri et al. 2002; Skujins 1978; Tabatabai 1994). The source and location are somewhat neglected when the measured enzyme activity is used to evaluate specific soil functions. Here, we shall discuss not only the classical approach in soil enzymology but also the molecular approach, which can unravel the origin of the measured enzymes, due to the detection of genes encoding the enzymes; this approach requires the classification of the target enzyme so as to choose the right primer for amplification of the gene encoding the target enzyme.

The aims of this review are:

1. To summarise the main findings and the present problems of classical soil enzymology and to propose future research needs with the hope of stimulating imaginative research for solving these problems. Most of the past literature is ignored because it was mainly published in books or in scientific journal before 1990s, and thus is not accessible by the electronic searches. Currently the main issues of classical soil enzymology are: (a) assays; (b) kinetics; (c) use of enzyme activities as indicators of soil functions; (d) location, state and role of extracellular stabilized enzymes in soil;
2. To discuss how detection and expression of genes encoding enzymes in soil can unravel the origin of enzymes contributing to measure enzyme activity in soil.

We shall use the term phosphomonoesterase instead of phosphatase because the latter term refers to several enzymes catalysing the hydrolysis of both esters and anhydrides of phosphoric acid (Nannipieri et al. 2011). Reviews are cited more than the original literature, and therefore, the reader is encouraged to consult cited books and reviews for a more complete knowledge of the discussed topics.

Classical soil enzymology

Assays Despite the availability of standardized methods to determine many enzyme activities in soil (Burns 1978b), there are still enzymes, such as nucleases and phytases, whose activity have not yet been determined in soil (Dick 2011a). Caution is required when using enzyme assays developed for homogenous solution for determining the respective enzyme activity in soil due to several problems such as poor solubility of the substrate, adsorption of substrates and reaction products by soil particles, interferences of soil components on the determination of reaction products or substrates, choice of the proper buffer, etc. (Burns 1978b; Dick 2011b; Nannipieri et al. 2011; Sinsabaugh 2010; Tabatabai 1994). As reviewed by Dick and Burns (2011) and Kiss et al. (1962), Hofmann G. and Hofmann E. made a great contribution to the development of enzyme assays in the 1950s by setting up methods for determining α-glucosidase, β-glucosidase, phosphomonoesterase and amylase activities. Unfortunately these, as well as other old methods, were insensitive or used inappropriate substrates. Emblematic is the case of the phosphomonoesterase assays, where the use of synthetic substrates, such as *n*-phenyl phosphate, replaced methods based on the release of phosphate, which can be easily adsorbed by soil particles. Later, it was shown that the use of phenol phosphate as a substrate was unsatisfactory because the reaction product, phenol, was also not quantitatively extracted from soil (Nannipieri et al. 2011). Thus, enzyme assays based on *p*-nitrophenyl phosphate as the substrate were developed that are more sensitive than the previous assay. In addition to assays determining acid and alkaline phosphomonoesterase activities, *p*-nitrophenyl derivates are used to determine other enzyme (glucosidases, galactosidases and arylsulfatases) activities in soil (Gianfreda and Ruggiero 2006; Tabatabai 1994). Since the released *p*-nitrophenol can sometimes be adsorbed by soil particles, calibration curves may need to be prepared by using different amounts of *p*-nitrophenol with the soil under study (Gianfreda and Ruggiero 2006). Another example of analytical problems in soil enzymology is the fixation of NH_4^+ (released in enzyme assays such as urease assays) by soils with NH_4^+-fixing clays (Nieder et al. 2011). An example of the interference of a soil component with a reaction product is the reaction of copper in soil with triphenylformazan, the reaction product of the dehydrogenase activity assay using tetrazolium salt as a substrate (Gianfreda and Ruggiero 2006). The present enzyme assays measure potential rather than real enzyme activities because the used optimal conditions, including the use of synthetic substrates, for the enzyme activity do not occur in situ (Burns 1978b; German et al. 2011; Gianfreda and Ruggiero 2006; Nannipieri et al. 2002; Tabatabai 1994). Recommendation to run assays in soils using optimal parameters as pH and substrate concentration aim at measuring not only the maximum potential soil enzyme activity (Dick 2011a) but also reproducible assay conditions allowing the comparison of enzyme activities among different studies. In addition, enzymes assays should be conducted at optimal pH values since this is a basic principle of enzymology. The choice of the buffer, which keeps constant the optimal pH value during the assays (Burns 1978b), in routine operational protocols depends on several factors such as the buffering capacity at the enzyme's optimal pH value and the absence of buffer inhibition or interference with the enzyme activity or

with soil constituents (Tabatabai 1994). Generally, enzyme assays are commonly chosen relying on published methods without preliminary verification of the suitability of the method and the buffer for the specific studied soil. Soil enzyme activity has been also measured using buffers at a pH value close to the soil pH value, which would then generate non-optimal but more realistic in situ conditions (Sinsabaugh et al. 2000; Li et al. 2009). Alternatively, soil enzyme activity has been measured in H_2O instead of buffer, aiming at assessing the enzyme activities under field conditions (Kandeler and Gerber 1988; Kandeler et al. 1996; Taylor et al. 2002; Dussault et al. 2008; Chaer et al. 2009). This approach has been criticized because the enzyme assays conducted in H_2O are less reproducible due to pH fluctuations during the enzyme assay, changes in the affinity of the enzyme for a given substrate and changes in the solubility of enzymes, substrates and cofactors (Burns 1978b; Tabatabai and Dick 2002; Turner 2010). However, the selection of a single pH optimal value for a given soil enzyme activity may be not strictly needed because the optimal pH of soil enzyme depends on the tested soils (Malcolm 1983; Turner 2010). Tabatabai and collaborators have set up several of the present and sensitive enzyme assays (Tabatabai 1994). The classical protocol used to set up an accurate soil enzyme assay included: (1) the determination of concentration changes of the reaction product or the substrate; (2) the use of an efficient extraction protocol for product or substrate from soil; (3) the choice of the buffer; (4) the monitoring of the effect of pH values on enzyme activity; (5) the study of the effect of time and temperature of incubation on enzyme activity; (6) the study of the effect of different amounts of soil on enzyme activity; (7) the study of the effect of different substrate and end-product concentrations on the enzyme activity; and (8) the choice of a proper control. Short (a few hours) incubation assays are generally preferred to long incubation assays so as to avoid the confusing effects of microbial growth and transformation of reaction products. Usually, shaking conditions are preferred to not shaking conditions because they permit a better contact between the enzyme and the substrate. In addition, the enzyme assays should be tested and validated with several soils with a broad spectrum of properties (Dick 2011b; Gianfreda and Ruggiero 2006; Tabatabai 1994). Fluorogenic substrates have been used to determine enzyme activities in soil since the hydrolysis of highly fluorescent compounds, such as 4-methylumbelliferone (MUF) and aminomethylcoumarin (AMC), to which substrates are conjugated, can be detected in soil (Deng et al. 2011). These enzyme assays can be used to determine activities of hydrolases but not those of oxidative enzymes in soil (Baldrian 2009). They are also usually more sensitive than the colorimetric methods (for example, MUF can be detected at concentrations of picomoles; Marinari et al. 2008). Another advantage is the use of the microplate format with the possibility of the simultaneous determination of different enzyme activities by using a small amount of soil. The major drawback of these methods is the quenching in the detection of MUF, which depends on the presence of soil phenolic compounds (Freeman et al. 1995). This can be overcome by determining the fluorescence of the standard in the soil. The fluorescence also depends on pH and temperature and thus it is important to keep these factors constant during the analysis (Deng et al. 2011). However, the use of small amounts of soil (less than 1 g) increases the variability of the measurements compared to the bench assays, which usually use 1 g of soil (Deng et al. 2011). According to Marx et al. (2001), to cope with the quenching and heterogeneity of soil, a microplate assay should include a minimum of three replicates at each substrate concentration (sample+buffer+substrate), a quenched standard (sample+buffer+4-MUF/7-AMC), a substrate control (sterile water+buffer+substrate) and a soil control (autoclaved soil+buffer+substrate). Comparisons between colorimetric and fluorogenic methods have given contradictory results. Acid phosphomonoesterase activity of soils with variable organic C content and pH values was higher when measured with MUF conjugates than with *p*-nitrophenylphosphate as substrates but the former enzyme activities were more variable (Drouillon and Merckx 2005). On the contrary, Berman et al. (1990) found higher acid phosphomonoesterase activities with *p*-nitrophenylphosphate and Christmas and Whitton (1998) did not find any significant difference in aquatic systems. Lower K_m values have been found with fluorogenic than with colorimetric assays probably because the former assays used more dilute soil slurries than the latter assays (Deng et al. 2011; Marx et al. 2001). However, β-glucosidase had a higher affinity toward 4-MUF-*β*-D-glucopyranoside substrate than toward *p*-nitrophenyl-*β*-D-glucoside when the respective K_a (the ratio between V_{max} and K_m) values were compared (Moscatelli et al. 2012). It is important to underline that kinetic constants of enzyme reactions with natural substrates are different when compared to those measured with synthetic substrates (Nannipieri and Gianfreda 1998). Sampling, handling and storage of soil are also important in soil enzymology. As it is discussed below, enzyme activities can show marked temporal and spatial variations and generally decrease with increasing soil depth. Soil sampling, of course, depends on the aims of research and representative samples should be taken if effects of different treatments, plant covers, etc. are studied. The sampling season should be also carefully considered if the study concerns monitoring enzyme activity during time or comparing enzyme activities among different treatments. Measurements should be carried out immediately after sampling (Gianfreda and Ruggiero 2006). If this is not possible, field moist soils should be stored at 4 °C for a short period (maximum 10–15 days; Gianfreda and Ruggiero 2006). However, as suggested by Valaskova and Baldrian (2006), freeze-drying can preserve enzyme activity of stored soil samples, air drying of soils can

change enzyme activities in an unpredictable way, depending on soil and the target enzyme (Rao et al. 2003). The enzyme activity associated with living cells is more affected by air-drying than the extracellular enzyme activity stabilized by soil colloids (Gianfreda and Ruggiero 2006). In conclusion, despite that several assays are available to measure some enzyme activities in soil, other enzyme activities can not be measured due to the lack of the proper assays. For example, no accurate methods are available to determine nuclease activities of soil and their determination is required so as to evaluate potential degradation of nucleic acids in soil. Other enzyme activities can be measured in soil extracts but not in soil (Sinsabaugh 2010). Despite their sensitivity, a small percentage (0.4–1.8 %) of published articles concerns MUF-based methods (Fig. 1), suggesting that the impact of these methods on soil enzymology is still scarce. More research involving soils with a broad range of properties and comparison with colorimetric enzyme assays is needed for MUF-based methods.

Kinetics Due to the high microbial diversity and the presence of animal and plant cells, it is reasonable to suppose that many different enzymes catalysing the same reaction are present in soil. However, the kinetics of soil enzyme activities determined by the present enzyme assays can be described by the Michaelis–Menten theory in soil slurries (batch studies) or soil columns (Nannipieri and Gianfreda 1998). The determined K_m and V_{max} values are probably the weighted average of the various constants of the many enzymes contributing to the determined activity but the weighting factor is unknown (Nannipieri and Gianfreda 1998). Two acid phosphomonoesterases catalysing the same reactions but with markedly different kinetic constants have been detected in soil extracts (Nannipieri et al. 1988). Kinetic constants of soil enzymes can be affected by management practices and soil factors, such as organic C content and texture. The type of transformation used for the calculation did not affect kinetic values of rhodanase, invertase and pyrophosphatase activities of soil whereas the Hanes–Woolf plot gave higher values than the Eadie–Hofstee and Linewear–Burk plots for phosphodiesterase, amidase, L-glutaminase and L-asparaginase activities (Nannipieri and Gianfreda 1998). Equations representing the kinetics of enzyme reactions in soil columns have been proposed by relating changes in both substrate and product concentrations with the flow rate (McLaren 1978). The rationale of this research was that soil cores represented less disturbed samples than soil slurries (Nannipieri and Gianfreda 1998). Both intracellular and stabilised extracellular enzymes of soil are present in a heterogeneous system and this may affect the kinetics of enzymes. Indeed microbial cells and extracellular enzymes are adsorbed by surface-reactive soil particles or entrapped in soil constituents, such as organic matter. In addition to changes in the conformation of enzyme molecules after their immobilization, steric limitations to the interaction of the substrate (especially those with high molecular weight) with the enzyme active site and different micro-environmental effects (for example, differences in pH values and substrate concentration between the surrounding of the immobilised enzyme and the soil aqueous phase) may affect the kinetics of adsorbed enzymes in soil (Nannipieri and Gianfreda 1998). Both K_m and V_{max} values of soil enzymes can be calculated by the Michaelis–Menten theory proposed for enzyme in the homogenous phase, despite the presence of many enzymes catalysing the same reactions and despite soil being a heterogeneous system. Equations developed for enzymes immobilised on solid supports are needed to correctly describe kinetics of intracellular enzymes of microbial cells or extracellular enzymes both adsorbed or entrapped by colloids in soil (Nannipieri and Gianfreda 1998). However, the apparent kinetic values may reflect the predominance of some enzymes and not reflect the activity of all active enzymes catalysing the same reaction.

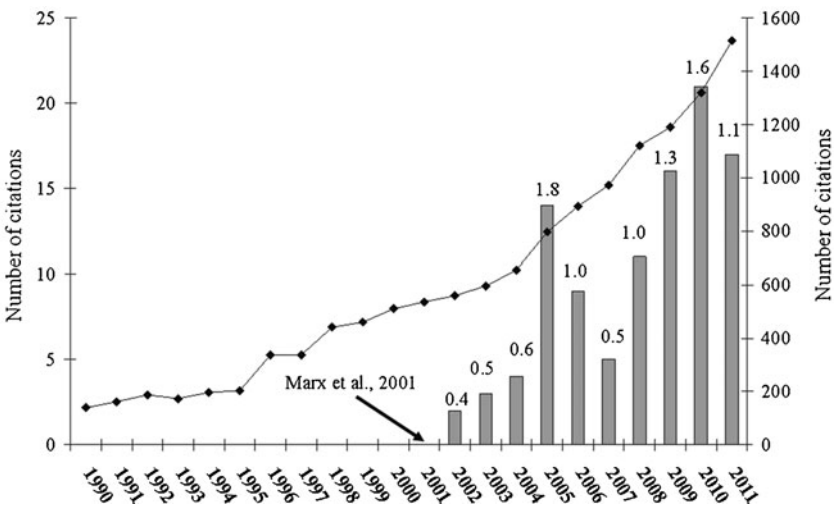

Fig. 1 Results of the search performed on Scopus on September 20, 2011 sorted by typing 'soil enzymes' in the title, abstract and keyword fields. *Filled bars* are the percentages of total number of enzyme articles using fluorogene substrates after Marx et al. (2001). The percentages are provided by *values above* each *bar*

Use of enzyme activities as indicators of soil functions The bibliography on the use of enzyme activities as indicators of soil functions is extensive, but as it has been already discussed (Burns 1978b; Skujins 1978; Gianfreda and Ruggiero 2006; Nannipieri 1994; Nannipieri et al. 1990, 2002), caution is required in this use for the following reasons: (1) present enzyme assays determine potential and not real enzyme activities (see above); (2) the meaning of measured enzyme activities is not known; (3) the many enzymes and reactions involved in a nutrient dynamics are neglected when a single enzyme activity is assumed to be an indicator of this dynamics; (4) spatio-temporal variations should be considered via in situ experiments; and (5) many direct and indirect effects make difficult the interpretation of the response of the enzyme activity to perturbations, changes in the soil management, changes in the plant cover of soil, etc.

Meaning of measurements A comparison between the measured potential enzyme activity and the real in situ enzyme activity may be possible, for example, in the case of urea hydrolysis. Indeed the hydrolysis of ^{15}N-enriched urea can be determined in different soil types and compared with urease activity of these soils.

The measured enzyme activity is due to activities from enzymes having different locations in soil (Burns 1982; Gianfreda and Ruggiero 2006; Nannipieri 1994). Activities of intra- or pericellular enzymes and esoenzymes (those attached to the outer cell membrane), and activities of extracellular enzyme stabilized by surface reactive particles or entrapped by humic substances are thought to be the most important. The former group of enzymes is arbitrarily defined as enzymes associated with living and active microbial cells. Activities of free extracellular enzymes and enzymes of dead cells and cell debris are considered to be short-lived and thus their contribution to the measured enzyme activity is supposed to be quantitatively not important (Burns 1982; Nannipieri et al. 2002). The extracellular stabilised enzyme activity has been termed 'abiontic' by Skujins (1976) from the Greek, where 'a-' means absence of a quality and 'biontic' means 'associated to life' (Dick and Burns 2011). To date, no methods are available to distinguish between the extracellular activity of stabilized enzymes from that of enzymes associated with active cells. Such separation is important because only the activity of enzymes associated with active microbial cells contributes to microbial activity whereas the stabilized extracellular enzyme activity is not related to microbial activity and can persist in soil under unfavourable conditions for soil microorganisms (Nannipieri et al. 2002). This methodological problem makes it difficult to understand the meaning of measurements of enzyme activities in soil that is their role in plant nutrition and soil fertility, oxidation of organic matter, metabolism of xenobiotics, etc. Both physical agents, such as irradiation (McLaren et al. 1957), and chemical compounds, such as antibiotics or inhibitory chemicals (phenol, acetone, thymol, chloroform, ether and toluene), have been used to distinguish the two soil enzyme activities (Dick and Burns 2011). These treatments should inhibit enzyme activity associated with active cells without affecting the activity of the extracellular stabilized enzymes (Burns 1978a; Gianfreda and Ruggiero 2006). However, all these treatments present drawbacks. Irradiation to inhibit microbial activity also inhibited enzyme activities such as urease activity (Dick and Burns 2011). Sterilization by microwave irradiation showed that phosphomonoesterase activity was less affected than microbial biomass (Speir et al. 1986). In addition Knight and Dick (2004) showed that it was problematic to standardize sterilization by microwave irradiation due to different interaction of microwaves with soil water content and changes of energy output of microwave ovens with manufacturer. Toluene, usually used in the assays to measure the activity due to the stabilised extracellular enzymes, can: (1) inhibit or stimulate the measured enzyme activity; and (2) increase the microbial cell permeability with overestimation of the intracellular enzyme activity (Gianfreda and Ruggiero 2006; Skujins 1978). An original and imaginative approach for determining the activity of the stabilised extracellular enzymes and the intracellular enzyme activity of soil was developed by Klose and Tabatabai (1999). It was assumed that present short-term enzyme assays only determine the stabilised extracellular enzyme activity, and that after the breakdown of microbial cells by chloroform fumigation both intracellular and the stabilised extracellular enzyme activities can be determined. Therefore, the former activity can be calculated by subtracting the enzyme activity before chloroform lysis from that after the lysis. The drawbacks of this method are: (1) chloroform fumigation does not lyse all microbial cells in soil (Nannipieri et al. 2002); (2) none has ever proved that short-term enzyme assays determine the intracellular enzyme activity of soil. Indeed, the stimulation of microbial growth in soil is often associated with the increases in hydrolase activities (Renella et al. 2007); and (3) protease activity of lysed cells hydrolyses enzymes during the fumigation period and thus there is the underestimation of the intracellular enzyme activity (Renella et al. 2002). Another approach, the physiological response method (Nannipieri et al. 1996a; 2002), correlates the enzyme activity with microbial biomass, both stimulated by glucose and a N source added to soil, when the two parameters are measured at different times. If both variables are significantly and positively correlated, and if their plotting with extrapolation of the microbial biomass to zero gives a positive intercept on the *y*-axis, this intercept is assumed to be the stabilised extracellular enzyme activity of soil (Nannipieri et al. 2002; McLaren and Pukite 1973). The drawbacks of this approach are: (1) not all soil microorganisms respond to glucose and the percentage of glucose-utilizing microorganisms depend on soil type,

management and pollution; and (2) constitutive but not inducible or repressible enzymes respond to microbial growth (Nannipieri et al. 1983, 2002). Also, enzyme-like reactions due to several components of soil (Boyd and Mortland 1990; Huang 1990; Ruggiero et al. 1996; Nannipieri et al. 2002) can contribute to the measured enzyme activities (Gianfreda and Ruggiero 2006; Nannipieri et al. 2002). Autoclaving or heating of soil has been proposed for measuring the enzyme-like activities since enzymes should be denatured by these treatments. However, two problems make this approach not valid: (a) autoclaving or heating can also modify soil components responsible for enzyme-like reactions, and (b) enzymes adsorbed by mineral soil components or entrapped by humic substances may retain their activities after autoclaving soil (Gianfreda and Ruggiero 2006; Nannipieri et al. 1996a, 2002; Stursova and Sinsabaugh 2008). In conclusion, there is no accurate method to measure the stabilised extracellular enzyme activity and the enzyme activity associated with active microbial cells in soil. No one has ever proved that the short-term enzyme assays only determine the stabilized extracellular enzyme activity. Both the fumigation and the physiological response method present drawbacks and future research by comparing both methods may be useful to evaluate their respective contribution to the measured enzyme activity of soil.

Use of enzyme activities to evaluate nutrient dynamics Single enzyme activities are often used as indicators of soil fertility, soil quality and soil microbial processes despite it is not clear what we are measuring. The measured enzyme activity is a potential activity and soil fertility, soil quality, and soil microbial processes are the expression of a multitude of enzyme reactions (Nannipieri 1994; Skujins 1978). A common conceptual mistake is to consider a single enzyme activity as an indicator of the rate of a whole metabolic process. Thus urease activity is often taken as an indicator of organic N mineralization although the enzyme is involved in urea hydrolysis and urea is not an important component of soil organic N, particularly when urea fertilisers are not used. Exopeptidases activities, such as glycine aminopeptidase or alanine aminopeptidase activities, have been assumed to represent organic N mineralization rate in soil (Allison and Vitousek 2005; Dong et al. 2007; Grandy et al. 2007). Nitrogen mineralization releases NH_4^+-N from organic N. By assuming that protein N is representative of organic N, the mineralization of protein N to NH_4^+-N involves several enzymes as reviewed by Ladd and Jackson (1982) and shown in Fig. 2. Therefore, it is conceptually wrong to assume that just the activity of an exopetidase represents the rate of organic N mineralization. An enzyme activity frequently used as indicator of organic C mineralization in soil is the β-glucosidase activity (Stott et al. 2010). Also, in this case, the assumption is conceptually wrong. The mineralization of plant residues mainly involves mineralization of cellulose and lignin, the main components of plant residues (Fig. 3). The former processes involve the action of several cellulases to produce cellobiose, which is hydrolysed by β-glucosidase activity to glucose, which is finally oxidised to CO_2 through glycolysis and citric acid cycle. The degradation of lignin, the limiting process of degradation of plant residues in soil because it is more recalcitrant than cellulose, involves the activity of several enzymes (lignin peroxidase, manganese peroxidase, versatile peroxidase and laccases) to produce phenols which are then oxidised to CO_2 through intracellular pathways (Theurl and Buscot 2010). As already discussed by Skujins (1978) and Nannipieri (1994), enzyme activities are substrate specific and cannot represent the rate of multienzymatic processes.

The ratio between the enzyme activity and microbial biomass has been also calculated to relate the contribution of the stabilised extracellular enzyme activity with that of the enzyme activity associated to microbial cells (Landi et al. 2000) but the stimulation or repression of the intracellular enzyme activity can affect the ratio and underestimate or overestimate the former contribution.

It has been proposed to measure several enzyme activities and to integrate them in an index. The first two proposed indices, the biological index of fertility and the enzyme activity number were based on dehydrogenase and catalase activities and on dehydrogenase, catalase, phosphomonoesterase, protease and amylase activities, respectively (Nannipieri et al. 2002). Therefore, the former index involves only oxidative enzyme activities whereas the latter involves both oxidative and hydrolase activities. Other indices are based on empirical relationships such as the relationship proposed by Trasar-Cepeda et al. (1998), which relates total N to microbial biomass C (MBC), N mineralization (Min N) and phosphomonoesterase (Phos.), β-glucosidase (β-gluc) and urease (Ure) activities: Total $N=(0.38\times10^{-3})$ MBC$+(1.4\times10^{-3})$ Min N$+(13.6\times10^{-3})$ Phos$+(8.9\times10^{-3})$ β-gluc$+(1.6\times10^{-3})$ Ure. The ratio between total N calculated by the relationship and that measured was equals to 1 for several native forest soils but not for Cu-polluted, lignite-mined and arable soils (Leiros et al. 1999).

Zornoza et al. (2007) developed two equations, one for Mollisol and the other for Entisol. The equation for the Mollisol calculated total N by the available P content (avail P), microbial biomass C (MBC), water-holding capacity (WHC) and phosphomonoesterase (Phos.), β-glucosidase (β-gluc) and urease (Ure) activities: Total N=0.44 avail. P +0.017 WHC+0.410 Phos−0.567 Ure+0.001 MBC+0.419 β-gluc−0.980. The equation for the Entisol calculated soil organic C by only considering two enzyme activities: Soil organic C content=4.247 Avail P+8.185 β-gluc+7.949 Ure+17.333. The correlation of the two equations depended on the annual rainfall (Zornoza et al. 2008).

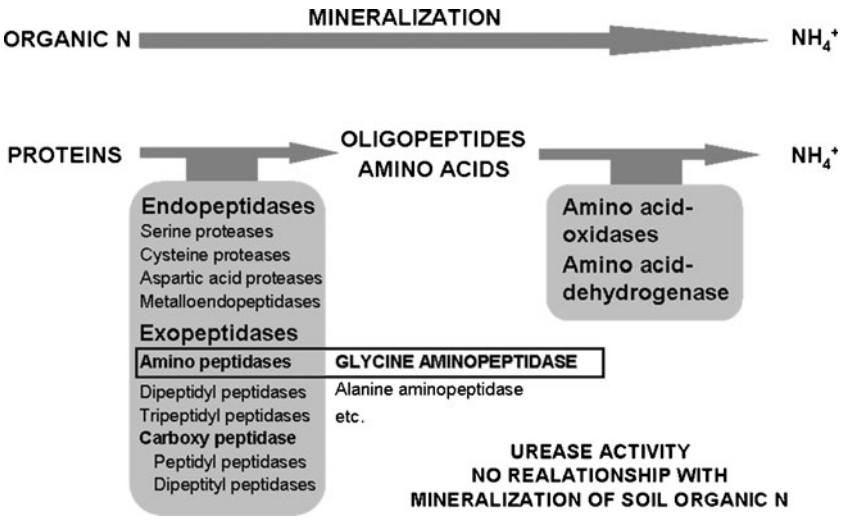

Fig. 2 Nitrogen mineralization in soil: The various enzymes involved in the transformation of protein N to ammonium-N and enzyme activities considered to represent the N mineralization rate in soil do not always closely relate to each other

The biological quality index, the ratio between the calculated total C content (TOC), and the determined TOC content, was proposed by Armas et al. (2007) to evaluate the quality of volcanic Andosols and Aridosoils. The equation for calculating TOC was: TOC=−2.924+0.037 HWSC−0.096 Cel+0.081 Dehy+0.0009 Res, where (Res) is soil respiration, HWSC is hot water-soluble C, Cel is cellulase activity and Dehy is dehydrogenase activity. The index developed by Chaer et al. (2009) for evaluating the quality of forest soils only considered phosphomonoesterase (Pho, unbuffered to measure enzyme activity at soil pH) activity and microbial biomass C; the ln TOC was equal to 1.236+0.276 ln Pho+0.289 ln MBC. The ratio between the calculated and the measured TOC content, but not the measured parameters, was sensitive to freeze–thaw and dry–wet cycles and to Cu pollution and pH changes. Sinha et al. (2009) developed the rhizosphere soil microbial index (RSMI) for rhizosphere soils sampled from trees growing in a coal mining ecosystem. The ratio between basal soil respiration and microbial biomass C, the ratio between microbial biomass C and the total soil organic C, and electrical conductivity, active microbial biomass C and dehydrogenase and phenol oxidase activities were the most sensitive parameters. These parameters were converted into a unitless score and finally the scores were integrated in the RSMI. Puglisi et al. (2006) proposed three indices of soil quality by measuring arylsulphatase, β-glucosidase, acid phosophomonoesterase, urease, invertase, dehydrogenase and phenoloxidase activities. These indices were developed by a data reduction technique (canonical discriminant analysis, CDA), which aggregates the ability of each enzyme activity in discriminating between soils subjected to different alteration events. The first index (AI 1) was developed by considering the seven enzyme activities; the second index (AI 2) was based on acid phosphomonoesterase, β-glucosidase, urease and invertase activities automatically selected by CDA as the most capable of discriminating between altered and non altered soils; the third (AI 3) index was developed by considering the most studied soil enzymes (acid phosphomonoesterase, β-glucosidase and urease) activities according to the bibliography and it was tested on several published data sets. The AI 3 index was capable of discriminating soils subjected to irrigation with brackish water, heavy metal contamination, intensive agricultural use, contamination by tannery and landfill effluents, mining activities and erosion (Puglisi et al. 2006). The

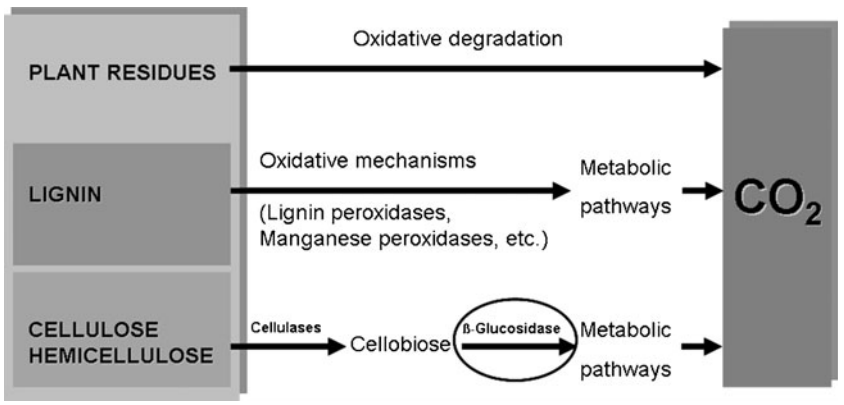

Fig. 3 Mineralization of the lingo-cellulose complex with the various enzymes involved in the oxidation of lignin and hydrolysis of cellulose. Both cellulose and hemicelluloses can also be degraded by oxidative reactions

above-mentioned indices are calculated from empirical relationships and the considered parameters are included without any biochemical rationality because the selected enzyme catalyse reactions involved in different processes. The index developed by Sinsabaugh et al. (1992) is the most reasonable from a conceptual point of view since it is based on the activities of six enzyme (β-1,4-glucosidase, β-1,4-endoglucanase or endocellulase, β-1,4-exoglucanase or exocellulase, β-xylosidase, phenol oxidase and peroxidase) involved in the lignocellulose degradation. This index was significantly correlated with the mass loss of white birch litter determined by the bag method in situ. Usually, most indices perform well when evaluating similar soils. However, absolute indices for soil quality are difficult to develop due to the intrinsic variability of microbiological properties and the several site-specific factors affecting soil enzyme activity (see below). Therefore, currently available indices of soil quality can be applied at the regional but not at the global scale (Bastida et al. 2008). Sun rays plots have been also used to show and compare different enzyme activities in a single plot but the information on the specific enzyme activity is lost (Nannipieri et al. 2002). In conclusion, the distinction between the stabilised extracellular enzyme activity and the intracellular enzyme activity is needed for relating enzyme activities to nutrient dynamics and other soil functions. Unfortunately, this is often ignored in soil enzymology and imaginative research is required to solve the problem. Single enzyme activities can not represent the rate of whole metabolic processes unless they catalyse the enzyme reaction, limiting the rate of the entire process. The approach by Sinsabaugh et al. (1992), integrating enzyme activities involved in the same process, is probably the correct one for using enzyme activities as indicators of organic matter degradation or nutrient transformation rates.

Spatio-temporal variability of enzyme activities and the rhizosphere effect The effects of soil properties on enzyme activities has been extensively studied (Burns 1978b; Gianfreda and Ruggiero 2006). Usually hydrolase activities are correlated with the organic matter content and microbial biomass but generally this does not occur for phenol oxidases activities (Sinsabaugh 2010). Enzyme activities have optimal pH value (Tabatabai 1994) and soil pH was positively correlated with phenol oxidase and peroxidise activities of soil (Sinsabaugh 2010).

Other natural factors, such as different geographical locations, different moistures and temperature and thus seasonal changes, etc, can affect enzyme activity contributing to the spatio-temporal variability of soil enzyme activities (Burns 1978a, b; Gianfreda and Ruggiero 2006). Usually, enzyme activities fluctuate with seasons, decreasing in summer and winter with moisture and temperature, respectively, being the limiting factors for biological (fauna, microorganism and plant roots) activities (Burns 1978a, b). Both phenol oxidase and peroxidase activities showed higher spatio temporal variations than hydrolase activities probably due to their higher turnover rates as the result of their higher molecular weight, with greater chances to be inactivated by environmental factors than hydrolases, and their degradation, due to the attacks by reactive oxidation products (Sinsabaugh et al. 2008; Sinsabaugh 2010). Soil, being a heterogeneous system, can present strong variability in value of properties and this variability can be evaluated by geostatistics (Decker et al. 1999; Snajdr et al. 2008). The comparison of spatial variability of urease, acid phosphomonoesterase and casein-hydrolysing activities and organic C and total N contents of a surface soil under grass–legume association showed that urease activity was the most variable parameter and total N content the least variable parameter (Bonmati et al. 1991). Dehydrogenase, urease, glutaminase and β-glucosidase activities showed little or no spatial distribution whereas alkaline phosphomonoesterase activity depended on the spatial distribution of organic C and inorganic P contents of soil (Bergstrom et al. 1998). On the contrary, all these enzyme activities were influenced by the crop when enzyme activities of samples taken from soil that was strip-cropped to corn (*Zea mays* L.) and soybean (*Glycine max* L. Merr.) and from between crop rows were compared. Enzyme activities of rhizosphere soils are generally higher than those of the bulk soil, like microbial biomass and microbial activity (Badalucco and Nannipieri 2007), because the rhizosphere soil is an environment richer in organic C substrates than the bulk soil. The higher enzyme activity of the rhizosphere than bulk soil may depend not only on the stimulation of microbial activity by rhizodeposition but also on the release of enzymes by roots or by lysis of root cells. For example, acid phosphomonoesterases are released under P-deficient conditions by epidermal cell of tip roots of white lupin (Badalucco and Nannipieri 2007). The pivotal study by Tarafadr and Jungk (1987) showed that both acid and alkaline phosphomonoesterase activities increased from bulk to rhizosphere soil of young clover (*Trifolium alexandrium*) and wheat (*Triticum aestivum*) as did both fungal and bacterial counts and inorganic P content whereas both total and organic P decreased. Phosphomonoesterases activities were present in microbial cells as well as in cell membrane fragments when rhizosphere soils were treated cytochemical techniques and examined by electron microscopy (Ladd et al. 1996). Soil properties can also vary within the profile and generally enzyme activities, like microbial biomass and organic matter content, decrease with soil depth (Bergstrom et al. 1998; Gianfreda and Ruggiero 2006).

Effects of plant cover, tillage, fertilization, pollution, climate changes and fire on enzyme activities As already mentioned, enzyme activities have been studied to evaluate the

response of soil functions to any perturbation (pollution, fire, etc.) on soil or to changes in environmental factors or management practice. Since it is not possible to cover the extensive bibliography we shall discuss the main mechanisms underlying these studies. Effects can be contradictory depending on soil, crop, enzyme activity, perturbation (pollution, fire, etc.), environmental factors and management practices (e.g. tillage, type of crop, fertilization, etc.; Gianfreda and Ruggiero 2006). Indeed, direct and indirect effects can make difficult the interpretation of enzyme activities in these studies and besides the target enzyme activity may be associated with several processes. For example, phenol oxidase activity can be involved in the synthesis of secondary compounds, decomposition, defence and humification (Sinsabaugh 2010). Land management can change soil properties with effects on the composition of microflora, and thus on effects on enzyme activities. Usually, composition of microbial communities depends on soil pH (Rousk et al. 2011; Fierer and Jackson 2006; Lauber et al. 2008). The conversion from a native grassland soil to a cropped soil usually decreases soil organic matter content and microbial biomass and thus also enzyme activities (Nannipieri 1994). However, tillage effects depend on the enzyme activity and season of soil sampling (Gianfreda and Ruggiero 2006). For example, organic matter degradation by soil microflora caused by soil tillage was associated to stimulation of dehydrogenase activity. No-tillage usually increases enzyme activities, microbial biomass and soil organic matter content of the surface soil (Gianfreda and Ruggiero 2006). Generally, monoculture degrades soil physical properties with negative effects on microbial activities including enzyme activities whereas crop rotations can stimulate microbial activities of soil due to the increased C inputs to soil with respect to monoculture (Gianfreda and Ruggiero 2006). Crops with higher root developments than other can stimulate enzyme activities by the rhizosphere effect. As examples of the effect of fertilization on soil enzyme activities, we focus here on the effects of N compounds on urease activity and those of inorganic P on phosphomonoesterase activities of soil. Both examples show how fertilizers can control the microbial synthesis of enzymes but not the activity of the extracellular enzymes stabilised in soil. Urease activity of soil can be decreased by both NH_4^+ and NO_3^- due to repression of the microbial synthesis of the enzyme by the N products produced by the microbial metabolism of the two inorganic N forms (McCarty et al. 1992). Inorganic P can repress phosphomonoesterase activities in both plant and microbial cells. However, the negative effect of inorganic P on phosphomonoesterase activities of soil can be partially detected because the measured enzyme activity also depends on the stabilised extracellular phosphomonoesterase activities, which are not related to microbial activity (Nannipieri 1994). Organic amendments usually increase enzyme activities of soil by stimulating microbial growth if toxicants such as heavy metals are not present in the amendment or if present the stimulatory effect of the organic component of the amendment masks the inhibitory effect of the pollutant (Nannipieri 1994; Gianfreda and Ruggiero 2006). The increase in enzyme activity may also be due to the increase in the activity of the extracellular enzyme–organo complexes because they are added with the amendment or the added organic moieties stabilise extracellular enzymes produced by the stimulated microbial activity. The positive effects usually decrease with repeated additions (Gianfreda and Ruggiero 2006). Climate changes, such as the increase in temperature and atmospheric CO_2 concentration and more frequent drying and wetting cycles, can affect organic matter turnover and composition and activities of microbial communities with effects on both the extracellular stabilised enzyme activities and enzyme activities associated to active microbial cells. As reviewed by von Lutzow and Kogel-Knabner (2009), different pools of the organic matter can have different sensitivity to the increase in temperature and thus these pools can show different degradation rates. It is reasonable to hypothesize the same for the different enzyme-organic complexes, whose presence and role will be discussed below. The increased atmospheric CO_2 concentration stimulates the photosynthetic activity of C_3 plants with increases in rhizodeposition and consequent increases in microbial activity of the rhizosphere soil (Drigo et al. 2008; Nannipieri 2011). Xylanase, invertase, alkaline phosphomonoesterase, arylsulphatase and casein-hydrolying activities of the 0- to −5-cm soil layer under shortgrass steppe were increased but not microbial biomass under elevated atmospheric CO_2 concentration (Kandeler et al. 2006) probably due to the stimulation of microbial activity of soil by the increased rhizodeposition. In the same site, Moscatelli et al. (2005) and Lagomarsino et al. (2008) showed that the enhancement of enzyme activities were related to the increased nutrient acquisition by plant. When the transient elevated CO_2 concentration was removed, microbial biomass N increased probably due to the decreased competition of roots with soil microflora for N. Higher enzyme activities measured in treated soils over the following months was possibly due to mineralization of the rhizodeposition. Sinsabaugh (2010) reported no effects of elevated CO_2 on phenol oxidase and peroxidase activities of soil under aspen, birch, sugar maple and sweetgum whereas phenol oxidase activity of soil under loblolly pine declined. The increase in C storage in soil can counteract the increase in the atmospheric CO_2 concentration. The ratio between β-glucosidase activity and the total soil organic C (SOC) content showed trends in the C storage of soil with ratio higher than 17 g glucose equivalent (as estimated by the β-glucosidase assay) kg^{-1} SOC h^{-1} whereas values lower than 17 g glucose equivalents kg^{-1} SOC h^{-1} indicated a decrease (Stott et al. 2010). The interpretation of the effects of pollutants on soil enzyme activities is problematic due the presence of both

direct and indirect effects (Nannipieri 1994; Schaffer 1993). In addition to direct effects on the enzyme activities, organic pollutants can have indirect effects, such as the growth of resistant microorganisms using the organic pollutant and/or debris of killed sensitive microorganisms as an energy source, changes in the microbial synthesis of the target enzymes, etc. All these indirect effects can mask or overestimate any direct inhibitory effect on the target enzyme (Nannipieri 1994; Schaffer 1993). Heavy metals, unlike organic pollutants, are not degraded but their availability can change due to changes in their speciation in soil (Landi et al. 2000). Also, there are both direct and indirect effects such as the growth of resistant microorganisms using debris of killed sensitive microorganisms, changes in the microbial synthesis of the target enzymes, stimulation of microbial processes inactivating the heavy metal, etc. These indirect effects can mask direct inhibitory effects on the target enzyme. It is possible to calculate the ecological dose 50 % (ED_{50}) that is the concentration of the heavy metal inhibiting the enzyme activity. Speir et al. (1995, 1999) proposed two kinetic models, whereas Haastra et al. (1985) proposed the sigmoidal dose–response model. Moreno et al. (2001) showed that one of the two models proposed by Speir et al. (1995, 1999) was the best to calculate the ED_{50} of ATP content and urease and dehydrogenase activities of two Cd polluted soils. The ED_{50} values can be used to rank the sensitivity of different enzyme activities to the heavy metals in soil (Coppolecchia et al. 2011). Enzyme assays have been set up using non-contaminated soils. Therefore, when evaluating the effects of heavy metals on soil enzyme activities, effects of buffers on the metal speciation and the interactive effects among metal species, enzymes and substrates should be evaluated. Indeed changes in the metal form can occur during the assays affecting the enzyme reaction. To date no specific studies have addressed this specific aspect.

Fire decrease soil organic matter, microbial biomass and thus enzyme activities and produce recalcitrant organic fractions, such as charcoal. Generally, there is a recovery of enzyme activities after burning, depending on the intensity and frequency of the fire and on the enzyme activity; chitinase activity of soil increased after prescribed fire of hardwood forest whereas acid phosphomonoesterase activity was markedly reduced (Gianfreda and Ruggiero 2006). In conclusion, interpretation of measurements of enzyme activities subjected to any perturbation (pollution, fire, etc.) or to changes in environmental factors or management (tillage, type of crop, fertilization, etc.) of soil is difficult due to the presence of direct and indirect effects on the target enzyme activity. In addition, results can be contradictory depending on soil and crop type, used enzyme assay, and perturbation and management type (Gianfreda and Ruggiero 2006). Another problem about using enzyme activities for understanding the response of soil function to any effect is that the target enzyme activity may be associated with several processes. For example, phenol oxidase activity can be involved in synthesis of secondary compounds, decomposition, defence and humification (Sinsabaugh 2010). The development of methods distinguishing stabilised extracellular enzyme activity and enzyme activity associated with active microbial cell in soil is needed to have further insights on these effects.

Location and role of the extracellular stabilised enzyme activity On average only 4 % of the total organic N of soil is present as microbial biomass and the rest is present as extracellular organic N, of which 30–50 % is released as amino acid N after acid hydrolysis. It is assumed that the amount of extracellular proteins stabilised by their interactions with surface-reactive soil particles is much higher than that of the intracellular or extracellular proteins associated with active microbial cells (Nannipieri 2006). The location and properties of the stabilised extracellular proteins enzymes have been studied by: (1) using ultracytochemical test to visualize the enzyme location by electron microscopy; however, this approach can not be used to localise enzymes on electron-dense (for example, clays) particles or soil components, such as humic molecules, which react with OsO_4 (Ladd et al. 1996); therefore, new procedures are needed to visualize extracellular enzymes on surface-reactive particles; (2) monitoring enzymes in soil fractions; (3) preparing synthetic enzyme–mineral (enzyme–clay complexes) and enzyme–organo complexes and characterising their properties, and (4) extracting naturally occurring enzymes from soil followed by their purification and characterization.

Enzyme activities of soil fractions This approach has contributed little to solve the problem of location of enzyme activities in the soil matrix (Ladd et al. 1996). Soil has been dispersed and fractionated with different procedures based on both particle size and particle density and thus the comparison among different studies is problematic (Gianfreda and Ruggiero 2006; Ladd et al. 1996). The fractionation procedures should be soft so as to avoid the lysis of cells. The use of low-energy sonication showed an enrichment of xylanase activity with sand fraction whereas urease and invertase activities were associated with silt- and clay-sized fractions (Stemmer et al. 1998). Urease and xylanase activities of the different particle-size fractions responded differently to different rates of organic and inorganic amendments (Kandeler et al. 1999a). Tillage affected differently enzyme activities of particle-size fractions with protease activity being the highest in the coarse and clay fractions and invertase activity being the highest in the silt fraction when soil was subjected to reduced and minimum tillage. Alkaline phosphomonoesterase activity was always the highest in the silt or clay fraction regardless of the tillage system and xylanase activity was the highest in the coarse

sand fraction under conventional tillage (Kandeler et al. 1999b). Both arylsulfatase and acid phosphomonoesterase activities were reduced in all aggregate size fractions by cultivation (Gupta and Germida 1988). Urease activity was the highest in the clay fraction, alkaline phosphomonoesterase and arylsulfatase activities were the highest in the silt-sized and clay particles, and xylanase activities were equally distributed among the different soil particles of heavy metal polluted soil (Kandeler et al. 2000). It was suggested that distribution of enzyme activities among the different-sized particles does not depend on management, pollution and type of soil (Kandeler et al. 2000). Light and heavy fractions of alpine and forest soils showed different chemical composition as revealed by pyrolysis–gas chromatography/mass spectrometry, with predominance of plant components, such as lignin, in the former fraction and predominance of microbial products, such as polysaccharides, in the latter fraction (Grandy et al. 2007). Generally, enzyme activities did not correlate with chemical structure of both fractions probably because of the different time scales over which enzyme activities and organic chemistry change. Despite most of the Cu and hydrolase activities being associated with the silt and clay fractions of a soil from a wood preservation site treated with or without organic and inorganic amendments, the decrease in the labile Cu content of the fraction increased the hydrolase activity (Lagomarsino et al. 2011). Only a few studies have compared composition of microbial communities among different-sized particles with the distribution of enzyme activities so as to understand the link between the measured enzyme activities and microbial abundance and diversity. Bacterial diversity and abundance, as shown by 16 S rRNA-based analysis, increased by decreasing the particle size (Gerzabek et al. 2002). In conclusion, these studies can help to understand the location of enzyme activities in the soil matrix if the procedures used to prepare soil fractions do not cause artefacts, such as lysis of cells and adsorption of enzymes to soil particles during soil fractionation. Also, here, methods separating extracellular stabilised enzyme activities from enzyme activities associated to microbial cells would improve the meaning of these studies.

Characterization of enzymes extracted from soil The characterization of naturally occurring organo-, mineral- or organo–mineral complexes requires: (1) their extraction from soil in high yields; (2) avoiding lysis of soil microbial, animal and plant cells with the consequent release of intracellular enzymes; and (3) preventing the formation of artefacts during the extraction (Nannipieri et al. 1996b). Several solutions have been used to extract enzymes from soil (Fornasier et al. 2011; Nannipieri et al. 1996b; Tabatabai and Fu 1992). The choice of the extractant depends on the target enzyme, the enzyme location in the soil matrix, the enzyme fraction (free or stabilized) and the type of enzyme complex (enzyme–clays, enzyme–organo, enzyme–organo–mineral; Fornasier et al. 2011). Usually, salt solutions such as phosphate, acetate, citrate, tris(hydroxymethyl)-aminomethane (tris), tris–borate, borate, etc. give low extraction yields (Nannipieri et al. 1996b; Tabatabai and Fu 1992). By considering that high yields could only be obtained by extractants normally employed in Humic Chemistry and that high alkaline NaOH solutions could not be used due to lysis of soil organisms, Nannipieri et al. (1974) employed 0.1 M sodium pyrophosphate at pH 7.1. This extractant was proposed by Bremner and Lee (1949) to extract organic matter from soil under mild conditions. This mild extraction did not lyse soil ureolytic microorganisms. According to Ruggiero and Radogna (1984) pyrophosphate was more efficient than phosphate–EDTA in extracting laccase from soil but Mayaudon (1986) found the opposite when moist soils were studied. Air-drying of soil should be avoided in studies on extraction of enzymes since air-drying of soil can partly kill soil microbial cells with release of intracellular enzymes (Nannipieri et al. 1996b). Shcherbakova et al. (1981) found that the efficiency of pyrophosphate and that of phosphate–EDTA–urea–NaCl solution depended on the target enzyme. Procedures using ammonium sulphate or protamine sulphate precipitation and chromatography by Sephadex permeation gel, polyvinylpyrrolidone resin, or CM-cellulose at pH 4 with acetate buffer or DEAE-cellulose at pH 8 with Tris–HCl buffer, commonly used for enzyme purification, were unsuccessful due to the great excess of humic and mineral compounds in the pyrophosphate soil extract (Ceccanti et al. 1978). An effective purification of the pyrophosphate extract was obtained by exhaustive ultrafiltration on Amicon molecular cut-off membranes PM-10 (10,000-Da cutoff) using 0.1 M pyrophosphate at pH 7.1 as the dialyzing medium. This procedure efficiently eliminated humic and inorganic salt–metal impurities. The material retained by the PM-10 membrane was further separated by ultrafiltration on XM-100 (100,000-Da cutoff) membrane in two fractions with a molecular weights higher (fraction A_I) and lower (fraction A_{II}) than 100,000. The ultrafiltration also eliminated some inhibitors of the extracted urease. The three fractions were characterised in terms of isoelectric point range (trends to more acid values from fraction A_I to fraction R), humification (trends to more humified materials from fraction R to fraction A_I) and age, as determined by the change in the conformation from L to D form of the terminal amino acids with ageing (trends to more old material from fraction R to fraction A_I; Ceccanti and Masciandaro 2003; Kimber et al. 1990). The A_I and A_{II} fractions were chromatographed by Sephadex gel giving five urease active fractions (three from the A_I fraction and two from the A_{II} fraction) with different molecular weight (Ceccanti et al. 1978). Also in this case, the elution by pyrophosphate eliminated inhibitors of urease. The re-

chromatography of each peak gave the same peak indicating that they were real enzyme complexes and not the results of artefacts; the C/N ratio and the colour indicated that they were humus–urease complexes (Ceccanti et al. 1978). The fractions with higher isoelectric points were more humified (condensed) than those with lower isoelectric points (Ceccanti et al. 1986). Gel chromatography of A_I and A_{II} fractions gave three peaks and 1 peak with acid phosphomonoesterase activity, respectively (Nannipieri et al. 1985). Both humus–urease and humus–acid phosphomonoesetrase complexes of higher molecular weight were more resistant to thermal denaturation and proteolysis than the respective lower molecular weight complexes (Ceccanti et al. 1978; Nannipieri et al. 1988). It was suggested that higher molecular weight humus–enzyme complexes have more chances to possess the molecular structure proposed by Burns et al. (1972) than low molecular weight humus–enzyme complexes (Nannipieri et al. 1996b). According to Burns et al. (1972), hydrolases are protected in organo–enzyme complexes by a network of organic molecules with pores permitting the passage of low molecular substrates but not that of high molecular weight compounds, such as proteases. This model can only work for hydrolases acting on low molecular weight substrates but not for those hydrolysing high molecular weight substrates (Ladd and Butler 1975). Proteases are suitable enzymes for testing this molecular model because there are assays using low- and high-molecular weight substrates (Ladd and Butler 1975). Sodium pyrophosphate was able to extract *N*-benzoyl-L-argininamide (BAA)- and *N*-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL)-hydrolysing activities and casein-hydrolysing activities, that is proteases acting toward low- and high-molecular weight substrates, respectively (Bonmati et al. 1999). More than 70 % of the BAA- and ZPL-hydrolyzing activities were present in the humus–protease complexes with nominal molecular weight higher than 10,000 Da (A_I–A_{II} fractions) and at least 30 % of these activities belonged to complexes with nominal molecular weight higher than 100,000 Da (A_I fraction). On the contrary, casein-hydrolysing activities showed a more even distribution among these fractions (Bonmati et al. 2009). Analysis by pyrolysis–gas chromatography–mass spectrometry (Py–GC) showed that BAA-hydrolysing activities were associated with the condensed humic matter, ZPL-hydrolysing activities were associated with less condensed organic matter, and casein-hydrolysing activities were present as glycoproteins, which are more resistant than proteins to thermal and proteolytic denaturation (Nannipieri et al. 1996b). Incubation at 65 °C showed that casein-hydrolysing activities were more resistant than the other two protease activities to thermal denaturation. Therefore, the presence of extracellular glycoproteins may also explain the resistance of stabilised extracellular enzyme activities in soil. Other evidences of the presence of glycoproteins in soil are the increases of the activity of extracted malathion esterase after treatment with hyaluronidase, acting on the *N*-acetylhexoseamine-tyrosine bond (Satyanaryana and Getzin 1973) and the proteolysis of extracted diphenol oxidase when treated with lysozima and pronase but not when treated with only pronase (Mayaudon 1986). Several enzymes (laccase, polyphenol oxidase, phosphomonoesterase, phosphodiesterase, arylsulfatase, cellulose, xylanase, β-glucosidase, invertase and protease) extracted from soil by phosphate-EDTA at pH 8.1 were suggested to be fungal glycoproteins, associated with lipolysaccharides of bacterial origin linked by Ca ions to humic molecules (Mayaudon 1986). In conclusion, studies on enzymes extracted from soil seems to indicate that both the presence of glycoproteins as well as the entrapment of enzymes in the network of humic molecules is responsible for the resistance to thermal denaturation and proteolytic degradation of the extracellular enzymes stabilised in soil. The possibility that a rearrangement of these complexes can occur during the extraction from soil is not excluded.

Model studies involving complexes between pure enzymes and soil components Model studies have involved the preparation of complexes with pure enzymes and free or coated clays, partially or completely covered by Fe–Al (hydro)-oxides of aluminium and iron), organic matter or clay–humic moieties (Gianfreda and Rao 2011). The aim of these studies was to mimic the formation of natural extracellular enzyme complexes in soil. Usually, immobilised enzymes are more resistant to thermal denaturation and proteolysis than free enzymes (Burns 1982; Gianfreda and Ruggiero 2006; Gianfreda and Rao 2011; Ladd and Butler 1975; Nannipieri 1994; Nannipieri et al. 1996b, 2002; Nielsen et al. 2006; Quiquampoix et al. 2002; Stotzky 1986). However, decreases or no effect on the stability of free enzymes immobilised by clays, other inorganic soil components or humic molecules have been also observed since the behaviour of the immobilised enzymes depends on the number and type of chemical bonds between the enzyme and the sorbent, the binding mode, the chemical and physical properties of the sorbent phase, the changes in the molecular conformation of the enzyme, the chemical conditions (e.g. charges, hydrophobic or hydrophilic) of the microenvironment surrounding the immobilized enzyme and the H_2O partition in the multiphase systems (Gianfreda and Rao 2011). The increased stability of the immobilised enzyme is generally due to: (1) the reduction of the molecular mobility making the immobilized enzyme more resistant to thermal and pH denaturation; and (2) the reduced accessibility of microorganisms or proteases making the immobilized enzyme more resistant to proteolysis (Gianfreda and Rao 2011; Nannipieri et al. 1996a, b). In addition, enzyme adsorption onto the sorbent phases can modify the kinetic properties of the immobilized enzyme, as above discussed.

The bibliography on the adsorption of proteins by clay minerals and on the properties of the relative complexes is extensive. According to Stotzky (1986) proteins are rapidly adsorbed by clay minerals and only a small amount of protein can be desorbed by water washing; the proteins that cannot be desorbed are termed 'bound proteins'. Protein adsorption by clay minerals depends on several clay properties such as surface area, cation-exchange capacity, charge density, saturating cation and degree of clay swelling (Boyd and Mortland 1990; Nielsen et al. 2006; Stotzky 1986). Important also are protein properties, such as isoelectric point, protein structure and conformation and hydrophobicity and hydrophilicity of the molecule. The ion exchange mechanism is important because the highest amount of proteins is adsorbed in the range of the isoelectric point of the protein; therefore, the proteins adsorption by clays is pH dependent. However, protein adsorption by clay minerals can also involve hydrophobic effects, van der Waal forces and hydrogen bonding (Quiquampoix et al. 2002). Model studies about the interactions between enzymes and humic molecules involves two approaches: (1) interactions between extracted humic molecules and the pure enzyme, and (2) preparation of the so-called enzyme humic-like complexes (Burns 1986; Nannipieri et al. 1996b; Nielsen et al. 2006). The former studies are scarce. However, if the enzyme is adsorbed on the external humic surface by electrostatic interactions or weak bonds, it is not resistant to thermal denaturation or proteolysis whereas such resistance is acquired when enzymes are entrapped in the humic network (Nannipieri et al. 1996b). Tyrosinase formed complexes with humic acids when both were flocculated with Ca^{2+} and carboxyl and phenolic groups of the humic acids were involved in the complex formation (Ruggiero and Radogna 1988).

The so called synthetic humic–enzyme complexes are prepared by entrapping the enzyme during the oxidative coupling of phenolic compounds promoted by enzyme or inorganic catalysis (Burns 1986; Ladd and Butler 1975; Nannipieri 2006; Nielsen et al. 2006). It was hypothesised that oxidative coupling of phenols with formation of radicals and quinones may be involved in soil humification process (Stevenson and Cole 1986), but nowadays this model is no longer considered valid (Schmidt et al. 2011). The resulting synthetic humus–enzyme complexes have chemical and physical properties similar to those of the naturally occurring humic substances (Burns 1986). Generally the immobilised enzyme is resistant to thermal denaturation and proteolytic degradation and there is a shift in pH optima toward alkaline values due to the polyanionic moiety surrounding the immobilized enzyme (Burns 1986; Ladd and Butler 1975; Nannipieri et al. 1996b). Lysine and other basic amino acids of trypsin, pronase, subtilisin, papain, carboxypeptidase A, urease and acid phosphomonoesterase were supposed to be involved in the bonds with the quinone units (Rowell 1974; Nannipieri et al. 1996b).

In conclusion, these model studies have given insights into the interactions between enzymes and surface-reactive soil components. Adsorption onto clay minerals can make the immobilized enzyme more resistant to denaturation but not the adsorption on humic substances and only the entrapment of the enzyme in the humic substances stabilizes the enzyme molecule (Burns 1986; Gianfreda and Rao 2011; Nannipieri et al. 1996a, b; Nielsen et al. 2006). Formation of the organo–enzyme complexes with humic acids is questionable since they are extracted with harsh procedures; probably it is more meaningful to study the interactions between enzymes and water-soluble rather than alkaline soluble humic substances. Finally, the synthetic humus–enzyme complexes are prepared through oxidative coupling of phenols, a process not anymore considered to be involved in the humification process.

Role of extracellular stabilised enzymes in soil microbial ecology Burns (1983) suggested an innovative hypothesis about the role of the stabilised extracellular enzyme activities in soil microbial ecology. Their presence is important for microbial life in soil. The hydrolysis of high molecular weight substrates, such as cellulose and proteins, is important for nutrient cycling and it requires the release of extracellular enzymes by active soil microorganisms. The prolonged release of such extracellular enzymes by soil microorganisms is not feasible due to the low chances of success since: (1) the substrate may not be present in the surroundings of the immobilized cell; (2) the released extracellular enzyme may be degraded by a microbial cell that has not released the enzyme or adsorbed by a surface-reactive soil particle; (3) the released extracellular enzyme may be successful if it reaches the substrate and finds the optimal conditions for the enzymatic reaction; and (4) the formed products should be taken up by the microorganism releasing the enzyme and not by an opportunistic one.

The presence of the stabilised extracellular enzyme can perform the reaction and release the reaction products, which can trigger the synthesis of the enzyme by soil microorganisms.

Allison and Vitousek (2005) proposed the economic theory as conceptual model for evaluating the conditions of enzyme production by soil microbial communities. In this model, enzyme production and release costs are outweighed by the nutrient acquisition from complex substrates. It is difficult to mimic what it is occurring at the micro environmental scale in soil, in terms of nutrient solubility, microhabitat characteristics and presence of active extracellular enzymes releasing nutrients independently on the microbial activity. Renella et al. (2007) reported that acid phosphomonoesterase and protease production were significantly

higher in soils under forest or set aside management, alkaline phosphomonoesterase and phosphodiesterase production were generally higher in the neutral and alkaline soils whereas urease production showed no obvious relationships with soil physicochemical properties. The economic model should be evaluated in the light of the large complexity of soil microbial communities, where specific ecological relationships (e.g. symbiosis and mutualism) and shared metabolic pathways among microbial species may profoundly influence enzyme production within microbial communities. For example, Renella et al. (2006) reported changes in the bacterial communities coincident with acid and alkaline phosphomonoesterase production in soils during plant litter decomposition.

The contribution of bio-molecular techniques

The literature on the use of molecular techniques is extensive and mainly concerns the determination of microbial diversity in soil. Although nowadays the determination of unculturable microorganisms is possible, the determination of rare microbial species in soil is still a problem (Delmont et al. 2011; Elshahed et al. 2008). Several thousand microbial species can inhabit 1 g of soil (Torsvik et al. 1996) and implementation of the soil metagenomic approach may contribute to delineate the real complexity of the soil microbial communities (Vogel et al. 2009). Sequencing of DNA extracted from soil can also permit detection of genes encoding both known and unknown enzymes (Vogel et al. 2009) and thus soil metagenomic studies can also lead to advances in soil enzymology in the future. Since soil metagenomic research is still in the pioneering period, here we will mainly discuss studies comparing genes encoding enzymes with the respective soil enzyme activity.

Detection of enzyme-encoding genes

While the literature on the gene detection in soil is still poor mainly because protocols of RNA extraction have lagged behind those of DNA extraction due to problems such as RNA degradation by RNase, contamination and instability of mRNA, which represents a small percentage of total RNA (Trayhurn 1996). Recently these problems have been overcome and there are several protocols co-extracting RNA and DNA from soil. The extracted mRNA can be separated from the other RNAs and analyzed by several techniques such as microarrays, Northern blotting, ribonuclease protection assay, and reverse-transcription PCR (Pietramellara et al. 2011).

Though detection of the presence of selected of genes in soil is now possible, only few studies have focused on the relation between gene abundance and enzyme activity in soil. Chitinase (E.C. 3.2-1.14) is a glycoside hydrolase and its activity in soil was the first to be compared with the respective enzyme-encoding genes. These hydrolases include several families of enzymes such as cellulases, xylanases, etc., and their classification is based con the catalytic domain of the enzyme (Henrissat 1991; Henrissat and Bairoch 1993; Suzuki et al. 1999). Chitinases are included in families 18 (bacteria, fungi, virus, animals and some plant enzymes) and 19 (plant and *Streptomyces griseus* enzymes). Metcalfe et al. (2002) showed that chitinase activity of a brown forest soil, measured either by weight loss of chitin or by an assay using 4-methylumbelliferyl-$(GlucNAc)_2$, was higher in soils treated with sludge. Ten DNA libraries constructed from DNA extracted from bags and amplified by degenerated primers targeting a gene fragment from family 18, were dominated by actinobacterium-like chitinase sequences. The outcome from this milestone paper was that sludge application to soil increased enzyme activity but decreased diversity of chitinase of family 18. By using selective inhibitors of bacterial and fungal growth, Watanabe and Hayano (1994) showed that bacteria were mainly responsible of benzyloxycarbonyl-L-phenylalanyl-L-leucine (ZFL) and casein hydrolyzing activities of soil and among the cultured bacteria, *Bacillus* spp. were those showing the highest activities of the two proteases. The use of protease inhibitors on the enzymes extracted from soil with 0.1 M phosphate showed the presence of a serine protease (synthesized by *sub* genes) and a metalloprotease (Kamimura and Hayano 2000). The serine proteases were also mainly responsible for casein-hydrolysing activities of soil (Watanabe et al. 2003). This was confirmed by Mrkonjic Fuka et al. (2008a), who showed that the abundance of *sub* and *npr* (encoding neutral metalloproteases) genes was positively correlated with casein-hydrolysing activities in a sandy soil but not in a clay soil probably because enzyme activity of the latter soil mainly depended on extracellular proteases adsorbed by clays. The abundance of *sub*-containing bacteria decreased with soil depth (Mrkonjic Fuka et al. 2008b) and the abundance of *npr*-containing bacteria was affected by both soil type and season (Mrkonjic Fuka et al. 2008c). The composition of both *npr* and *apr* (encoding neutral-metalloprotease) bacterial communities of rhizosphere and bulk soil fertilized with organic or inorganic fertilizers affected the ZFL-hydrolyzing activities of soil (Sakurai et al. 2007). The plant vegetation stage affected more the abundance of alkaline and neutral metalloprotease (*apr* and *npr*) and chitinase (*chi*A) of bacterial communities in the rhizosphere soil than the plant genotype (Gschwendtner et al. 2010).

The biochemistry of lignin degradation is rather complex involving different enzymes such as lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases. In soil microbiology, laccase-encoding genes have been mainly used for studying structural and functional diversity

of fungi (Theurl and Buscot 2010). Diversity of fungal laccase-encoding genes was greater in the surface than in the deeper soil layers and there was a large variability in the surface soil (Luis et al. 2004, 2005). The presence of laccase-encoding genes of basidiomycetes (DNA was extracted, amplified and cloned with final sequencing) changed during different season whereas laccase activity of phosphate extracts of soil remained constant through the year (Kellner et al. 2009).

In some cases, the lack of suitable enzyme assays for specific enzyme activities also limits the comparison of enzyme-encoding genes and the respective enzyme activity in soil. These studies can give insights on the origin of enzymes in soil. The presence of a few studies is also related to the lack of assays for determining the target enzyme activity in soil. For example, culturable bacteria with phytase (β-propeller phytase)-encoding genes have been isolated (Jorquera et al. 2011) but there is not an accurate method for determining phytase activity of soil because the method involves the determination of the released phosphate and it is well established that phosphate can be easily adsorbed in soil and released not only by phytase but also by other enzyme.

Another problem in studies on enzyme-encoding genes in soil is the presence of unknown enzymes catalysing some target enzyme reaction. As already shown, the metagenomic approach can help to solve this problem. However, when the research involves the amplification of the extracted DNA, it may be useful to use degenerate primers. In this case, the amplified selected region should be sufficiently long to cover substrate specificity but the degree of degeneracy should be a compromise for maximizing coverage without missing specificity. By using these degenerated primers, the sequence alignment showed three known dioxygenase and seven dioxygenase genes not reported before when was DNA extracted from polychlorinated biphenyl-contaminated soil, amplified and sequenced (Iwai et al. 2010).

Contribution of transcriptomic and proteomic to soil enzymology Soil proteomics can be still considered in its infancy because extraction of expressed proteins is problematic not only for protein adsorption by surface reactive soil particles but also for their low concentrations compared to the overall soil protein concentration (Nannipieri 2006). In addition, different methods have been used to extract proteins from soil (Nannipieri 2006; Schneider et al. 2012), and the comparison among these methods is needed so as to understand how the used method can affect soil proteome. However, extraction and identification of enzymes produced by soil microbial communities may be possible, and combing these studies with transcriptomics studies and measurements of the respective soil enzyme activities may disclose the adaptive mechanisms of enzyme synthesis, the recovery and identification of the synthesized enzymes, their relative contribution to the specific soil activity and the presence of the active microbial species.

Cañizares et al. (2012) related the first linking the detection and expression of bacterial β-glucosidase encoding genes (βgluF2/βgluR4 primers) with the relative enzyme activity of a Mediterranean soil under different long-term management practicesand found that these genes were over expressed (the ratio between β-glucosidase transcripts and gene copies) in the tilled soils probably as a response of bacteria to stress. However, only 50 % of the amino acid sequences were matched by the retrieved database sequences, indicating the presence of soil bacteria with unknown β-glucosidases. Schulze et al. (2005) probably carried out the most complex approach for characterizing the metaproteome from dissolved organic matter and clay minerals of a forest soil by multidimensional mass spectrometry. Proteins, but not enzymes, identified in the soil solution were mainly of intracellular origin, whereas both bacterial and fungal cellulases and laccases were mainly associated to soil particle surfaces. However, despite the ever-increasing soil proteomic literature (Keller and Hettich 2009), the identification of extracellular enzymes in soil is quite rare. This may be either due to fast enzyme turnover, low yields in the extraction of the newly expressed enzymes because of their adsorption by several surface-reactive soil particles, and unknown modifications of proteins in the soil environment, which make them not detectable by mass spectrometry (Giagnoni et al. 2011). Recently enzymes have been detected in the proteome extracted from litter during degradation (Schneider et al. 2012).

In conclusion, both soil transcriptomic and proteomic related to enzyme activity of soil are still in the infancy period probably because there are still methodological problems in soil proteomics. As it occurs for metagenomic DNA (Bakken and Frostegård 2006), the different methods used to extract RNA and proteins from soil do not provide a uniform and unbiased subsample. However, while soil metagenomics is greatly progressing revealing the large microbial diversity of soils and the relevant enzymatic potential, it is also clear that only integrated genomic and proteomic approaches can elucidate the actual levels of expression of the biocatalytic potential of soil microbial communities, and relate it to soil and environmental factors.

Conclusions and future research needs

Enzyme assays are generally simple, rapid and accurate and this may explain the extensive bibliography of soil enzymology. Most of this bibliography is nowadays ignored, probably because published in books or scientific journals before 1990s, and thus are not accessible by the electronic searches.

Several research problems and future research needs are included in the concluding sentences at the end of headings and subheadings. Among these, very important is to remark the problems in using enzyme activities as indicators of soil functions because: (1) present enzyme assays determine potential and not real enzyme activities and the relationship between the two type of activities is not known; (2) the meaning of measured enzyme activities is not known; (3) the assumption that single enzyme activity is an indicator of nutrient dynamics does not consider that many enzymes are involved in such dynamics; (4) spatio-temporal variations are not always considered in situ experiments; and (5) many direct and indirect effects make difficult the interpretation of the response of the enzyme activity to perturbations, changes in the soil management, changes in the plant cover of soil, etc. Future research in using soil enzyme activities as indicators of soil functions should carefully consider what was already debated and enzyme assays should distinguish the stabilised extracellular enzyme activity from the intracellular enzyme activity.

The future direction of enzyme research in soil is surely towards combined proteogenomic studies (Nannipieri 2006), which will provide new insights on the origin of the measured enzymes. In fact, the metagenomic approach can disclose potential genes coding for enzymes catalysing target reactions, whereas only transcriptomics and proteomics can assess the actual levels of enzyme expression and indicate which enzymes can be used as ecological soil indicators.

The classical soil biochemical measurements and the 'omic' approaches can provide complementary information on microbial processes and plant-microbe interactions in soils at comparable costs although more labour intensive.

Acknowledgment The research leading to these results has received funds from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013 under REA agreement n° 289949 (TRAINBIODIVERSE) and by Ente Cassa di Risparmio (Proteomic approach to better understanding soil functionality and interactions between soil and plant; no. 2009.41).

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