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Review

Measurement of asymbiotic N_2 fixation in Australian agriculture

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ABSTRACT

A wide range of bacteria capable of nitrogen fixation (free-living and associative) can be found in all agricultural soils across Australia, however measurement of their effectiveness in N_2 fixation has proved to be problematic because rates are low compared to symbiotic systems and quantitative methodologies barely adequate. It is generally believed that associative N_2 fixation rates may be greater than free-living N_2 fixation rates in ecosystems where grasses (including cereals) dominate, although this has not been unequivocally proven. Conditions promoting asymbiotic N_2 fixation are reduced availability of oxygen, high temperature and soil water, and large amounts of microbially available C in the soil. The most direct measure of N_2 fixation, incorporation of $^{15}N_2$, has rarely been used in undisturbed systems, and we can find no examples of its field application in Australia. Nitrogen balance calculations, based on long-term changes in total soil N of systems and crop N removal, have been used to infer asymbiotic N_2 fixation, but do not measure it directly. Such N balance studies can thus only give an indication of potential asymbiotic N_2 fixation over long periods of time, but cannot confirm it. There are no robust N balances published for Australian ecosystems. The acetylene reduction assay for nitrogenase activity has been used in Australia to study responses of both free-living and associative N_2 fixation systems to regulating factors. These studies have highlighted the importance of C supply, high soil water content and temperature in increasing asymbiotic N_2 fixation in soils. However significant methodological limitations do not allow field scale quantification using this assay. On balance we would concur with the authors of several earlier global reviews of this topic and conclude that (in Australia) contributions of nitrogen to crop growth from asymbiotic N_2 fixation are likely to be <10 kg N ha^{-1} y^{-1} and generally not of agronomic significance under low rainfall conditions. In tropical environments where higher rainfall and temperatures coincide, rates are likely to be greater if soil mineral N is low and carbon substrates are available for N_2 fixing microorganisms. If asymbiotic N_2 fixation is to be encouraged or profitably managed, there is a need for more reliable field measurement and a combination of methodologies including ^{15}N might provide more definitive quantitative indications.

1. Introduction

At least 90 genera of specialised microorganisms (diazotrophs) are known to have the enzyme complex nitrogenase and can 'fix' atmospheric N_2 into NH_3. These organisms exist as free-living entities (free-living N_2 fixation), in loose associations with lichens, cycads and some higher plants (associative N_2 fixation, e.g. rice and sugarcane), or in complex symbioses where host plants provide the needed carbon (energy) source, and the plants sequester most of the biologically fixed N for their own needs (symbiotic N_2 fixation, e.g. legumes and actinorhizal plants). The legume and *Azolla* (in flooded rice) symbioses are of substantial economic importance in agriculture globally. Investigations into endo or epiphytic N_2 fixing associations between grass species and heterotrophic or cyanobacteria are yet to provide unequivocal evidence for truly symbiotic N_2 fixation in cereals (James, 2000). N_2 fixing symbioses are characterised by significant net transfer of photosynthetically fixed plant C to the micro-symbiont and significant net transfer of biologically fixed N to the host plant. Although associative N_2 fixing systems are thought to effect useful N contributions in some ecosystems (Boddey et al., 1995b), problems with methodology and interpretation often confound unequivocal quantitative estimation (Giller and Merckx, 2003).

The suggested quantities of N_2 fixed vary depending on the N_2 fixing system in question, but in general free-living and associative N_2 fixers would fix very much less N than symbiotic systems where host plants directly supply the micro-symbiont with energy and protect the nitrogenase enzyme from deactivation by oxygen (Bergersen, 1991). Obligate and facultative anaerobes only fix N_2 in the absence of oxygen (e.g. *Clostridium*, *Desulfovibrio*, *Klebsiella*,

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Enterobacter and *Bacillus*). Under aerobic conditions several strategies are used to protect nitrogenase. Microaerophilic bacteria (e.g. *Azospirillum*) will only fix N in low oxygen environments such as that in the centre of an actively respiring colony. Aerobic bacteria that have respiration rates 10–50 times greater than other organisms can lower intracellular O_2 concentration to a point where N_2 fixation occurs (e.g. *Azotobacter*). Protection of nitrogenase is also afforded by surrounding cells with polysaccharides (e.g. *Derxia*) or by confining N_2 fixation to heterocysts (e.g. *Anabaena* and *Nostoc*). Some of these mechanisms for lowering the oxygen concentration around cells come at a high C cost (e.g. polysaccharide production in *Derxia* (Hill, 1971) and high respiration rates in *Azotobacter*), and this reduces bacterial growth and therefore limits N_2 fixation. However, where such organisms are living as endophytes or in plant rhizospheres where O_2 concentration is likely to be lowered by plant respiration, growth rates of N_2 fixing bacteria may be higher. In the absence of any protection from atmospheric oxygen, nitrogenase activity is very low. These mechanisms are important to consider when assessing the likelihood of significant asymbiotic N_2 fixation in a given environment.

The efficiency of N_2 fixation by free-living diazotrophs, expressed in terms of energy and carbon cost (20–100 g C g^{-1} N fixed, Zuberer, 1999), is about one-tenth of that in legume symbioses (2–8 g C g^{-1} N fixed, Silvester and Musgrave, 1991). Associative N_2 fixing systems, where diazotrophs are associated with plant rhizospheres or aerial tissues (Kennedy and Islam, 2001), are thought to fall between free-living and symbiotic systems in terms of the quantities of N_2 fixed (Dart, 1986). However, evidence for significant broad-scale associative N_2 fixation is weak, even where plants have been inoculated specifically for this purpose. In most cases growth stimulation from such inoculants has not been due only to N_2 fixation, but often to other growth promoting substances and processes (Vessey, 2003; Kennedy et al., 2004; Dobbelaere and Okon, 2007), with the noteworthy exceptions of sugarcane (Reis et al., 2007), a few other C4 grasses (Reinhold-Hurek and Hurek, 1998) and rice (James, 2000). Nevertheless future prospects for increased contributions from N_2 fixing endophytes appear positive (see Triplett, 2007).

Biological nitrogen fixation was a key component in the successful development of the ley farming system in Australia through the middle of the last century (Perry, 1992), and the widespread adoption of N_2 fixing crop legumes through the 1980s and 1990s which became a key element of Australian grain production systems (Fillery, 2001). More recently in Australia, cereal crop production has intensified, crop legume sowings have decreased, and N fertiliser use has substantially increased (Angus, 2001). While the extent and contributions of symbiotic N_2 fixation in legumes to Australian agriculture have been reviewed a number of times (e.g. Unkovich et al., 1997; Peoples et al., 2001) the significance of non-symbiotic N_2 fixation to Australian agriculture has not been presented, although a recent study reported the potential role of asymbiotic N_2 fixation in stubble retained cereal crop systems (Gupta et al., 2006). Our purpose is thus to provide a summary of available information on measurements of non-symbiotic N_2 fixation in Australian agriculture.

2. Methods for field measurement of asymbiotic N_2 fixation

2.1. $^{15}N_2$

Field measurement of free-living and associative N_2 fixation is problematic as rates of N_2 fixation are typically very low (Kennedy and Islam, 2001). The most direct measure is that using ^{15}N labelled nitrogen gas, N_2, which is exposed to the N_2 fixing system in a closed or flow through chamber. Recovery of ^{15}N in the N_2 fixing system is then a direct measure of the amount of N_2 fixed over the period of exposure (usually 24 h), with assays needing to be conducted on many different occasions to obtain an approximate time-integrated measure (kg N ha^{-1} y^{-1}). There are few examples of this in the literature, and indeed we can find no published examples for Australian soils, although there have been measurements made under laboratory conditions (e.g. Rogers et al., 1966). A system similar to that of Ross et al. (1964) and De-Polli et al. (1977) for measuring $^{15}N_2$ fixation in intact soil–plant cores could be used for either associative or free-living N_2 fixation. Practical details of such assays are given in Dobereiner (1980) and Knowles (1980). There has been a tendency with such studies to select systems for $^{15}N_2$ assay on the basis of high acetylene reduction rates, however this may bias the results toward higher N_2 fixation estimates. While there are considerable logistic difficulties in using $^{15}N_2$ in field studies (Wood and McNeill, 1993; Giller and Merckx, 2003), which have rarely been overcome, new technologies in plastics, and gas handling and control systems (see e.g. Hunt et al., 1989) may provide opportunities for greatly improved applications of the $^{15}N_2$ technologies.

Measurement of N_2 fixation using $^{15}N_2$ requires that soil $^{14}N_2$ is displaced with $^{15}N_2$. This is difficult to do in intact soil cores, as the resistance to N_2 diffusion in soil pores is great. Disaggregation of the soil and flushing with an inert gas such as argon and replacement of the atmosphere with an appropriate mix of $^{15}N_2$ and O_2 would be most efficacious. However, an ideal system for assessing non-symbiotic $^{15}N_2$ fixation would use intact soil cores of at least 10 cm diameter and 10 cm depth, and preferably larger for associative N_2 fixing systems. Disturbance of such cores must be kept to a minimum so as not to destroy anaerobic microsites and so ideally the cores should not be transported far. In the absence of plants it may be possible to slowly and constantly recycle $^{15}N_2$ through such soil cores and estimate N_2 fixation. Achieving this at the high soil water contents typically used for free-living N_2 fixation studies is an added challenge and may require recourse to a disaggregated soil at a range of O_2 concentrations. Alternatively intact cores could be air-dried, $^{14}N_2$ displaced with $^{15}N_2$, followed by a rewetting and incubation of the cores at a desired or range of water contents. This should help to ensure an even distribution of $^{15}N_2$ through the soil, but preliminary analyses would be required to define the incubation duration needed to allow the activity of asymbiotic N_2 fixing organisms to re-establish. It is acknowledged that air-drying will perturb the microbial community; however, under natural conditions in Australia, soils are frequently exposed to such a drying regime. Despite the methodological complications, it is worth persevering with $^{15}N_2$ studies because, as is shown in Fig. 1, the method is highly sensitive and should enable rates of N_2 fixation equivalent to *ca* 0.3 mg N m^{-2} day^{-1} (1 kg N ha^{-1} y^{-1}) to be quantified.

2.2. ^{15}N isotope dilution

An alternative approach in using $^{15}N_2$ is to label soil with ^{15}N in the form of salts (e.g. $(^{15}NH_4)_2SO_4$ or urea) and observe dilution of this ^{15}N with atmospheric ^{14}N from biological N_2 fixation. However, detecting low rates of ^{14}N addition (N_2 fixation) against the high total N background in the soil remains problematic (Fried et al., 1983), although some successes have been achieved with associative N_2 fixing systems (Boddey et al., 1995a; James, 2000). In the case of associative N_2 fixing systems with plants, establishing suitable non-N_2 fixing controls has proved very difficult. A similar approach exploiting natural variations in ^{15}N abundance ($\delta^{15}N$) has also been applied to studies of associative N_2 fixation (Yoneyama et al., 1997) but these may be less reliable than ^{15}N enriched methodologies with the low N_2 fixation rates anticipated (Unkovich and Pate, 2000), and because most natural variations in ^{15}N of plants are not due solely to differences in N sources, $\delta^{15}N$ should not be used for proof of N_2 fixation, but may be useful for quantification where N_2 fixation is known to occur and other processes responsible for altering $\delta^{15}N$ are absent or minimal (see e.g. Boddey et al., 2001).