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Laboratory analysis of soil respiration using oxygen-sensitive microplates

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ABSTRACT

Soil respiration is usually monitored by measuring CO_2 efflux. Most techniques available for such kind of analyses are inconvenient and difficult to adapt to micro-method format. The present study evaluates a new microplate-based method for studying soil respiration in the laboratory. Second-generation oxygen-sensitive microplates (OSM - containing a fluorescent probe attached to the bottom of the well which provides time-resolved fluorescence data) were used to measure soil respiration either in microcosm assays or in soil water extracts. The latter procedure (water extracts) was the least cumbersome, hence it was selected for further experiments. Soil respiration curves generally showed no lag phase, starting with an exponential oxygen consumption phase, followed by a period where respiration became stable after 8–10 h of incubation at 25 °C. Once the procedure for measurement of oxygen consumption in soil was established, the acute toxic effect of diverse chemicals on soil was analysed with OSM. Streptomycin and penicillin failed to reduce soil respiration. Kanamycin plus neomycin, trimethoprim and 5-fluorocytosine exhibited limited inhibitory effects. In contrast, some chemicals (copper sulphate and amphotericin B) and fungicides (such as dodine and fosetyl) noticeably reduced fluorescence readings, showing concentrations to give half-maximal inhibition of respiration (ICR_{50}) ranging from 0.0064 to 0.082 g/L. Finally, some insecticides and soil amendments assayed were either neutral or increased respiration.

It is concluded that OSM are reliable, convenient, and yield quantitative results. Moreover, the system is relatively inexpensive and amenable to automation. However, results obtained using soil water extracts may be different from those derived from undisturbed soil aggregates, clods or slurries studied under field conditions.

1. Introduction

Inadequate use of pesticides and fertilizers causes worldwide concern. Monitoring environmental changes in terrestrial ecosystems caused by chemical pollution requires a better understanding of soil respiration. The latter is defined as the production of carbon dioxide by heterotrophic soil microorganisms (mainly bacteria and fungi - Sandor, 2010). However, the CO_2 efflux is not always an accurate measurement of the respiration rate, since under field conditions carbon dioxide may migrate with soil water, be part in a carbonate dissolution reaction or in plant root uptake (Angert et al., 2015). In addition, CO_2 efflux is often measured with equipment obtaining data in situ, which show great differences in accuracy, spatial and temporal resolution and applicability (Janssens et al., 2000). On the other hand, in a closed environmental system, CO_2 measurement is hampered in alkaline (calcareous) soils for artefacts due to $CaCO_3$–CO_2–H_2O equilibria (Oren and Steinberg, 2008). One of the best options available for laboratory studies on soil CO_2 efflux are Microresp® microplates, an indirect system, amenable to automation, which measures respiration as a halochromic indicator changes colour when CO_2 reacts with bicarbonate (Campbell et al., 2003).

The CO_2 production/O_2 consumption ratio is another parameter that can be used in respiratory studies, but it varies with the kind of substrate consumed and may be biased when [O_2] drops below 0.5% due to partial anaerobiosis. Therefore, the best alternative to CO_2 determination under aerobic conditions is the analysis of oxygen consumption. There are diverse methods for measuring dissolved oxygen. Jorge et al. (2004) described an approach based on a device using fluorophores and optical fibres to determine [O_2] in water. In edaphology, a number of studies based on the use of electrodes and microelectrodes for oxygen detection have been published (Reddy et al., 1980; Sexstone et al., 1985; Pang et al., 2007; Fan et al., 2014; Angert et al., 2015). Unfortunately, the methods used by these authors showed low sensitivity or proved uneconomic or cumbersome. In contrast, Garland et al. (2003) performed their soil respiration analyses choosing a different approach, based on the BD oxygen microsensor

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system®. The method consisted of an oxygen-sensitive fluorophore (tris 4,7-diphenyl-1,10-phenanthroline ruthenium (II) chloride) included in a silicone rubber matrix in the bottom of each well of a standard microplate. These plates may be read with most commercial fluorescence plate readers available on the market, with optimal results obtained when an excitation filter at 485 nm was used in conjunction with an emission filter at 590–630 nm (Rolo et al., 2009). However, this first-generation system relying on direct fluorescence readings became obsolete with the arrival of microplates based on time-resolved fluorescence, much more sensitive to [O_2] variations, hence being the BD microplates supply discontinued a few years ago. Second-generation microplates (namely Oxoprobics® OSM) are now available and capable of performing a highly-sensitive measurement of oxygen content. These new plates have functionalized Pt(II) complexes, acting as long-decay oxygen-sensitive photoluminescent probes (excitation at 340 nm/emission at 642 nm). Furthermore, the fluorophore is covalently bound to the bottom of their wells to prevent probe leakage and, hence, cytotoxicity problems, allowing the assessment of time-resolved fluorescence instead of direct fluorescence. These microplates have been successfully used in our laboratory in previous respiration and viability studies in *Acanthamoeba* cultures (Heredero-Bermejo et al., 2015). Here, we report the application of this new approach as a novel method for monitoring growth of diverse microorganisms in pure cultures. Once this approach proved successful, microplates were explored as a tool for measuring soil respiration. In addition, diverse chemical products (drugs, pesticides, fertilizers and a pollutant) were tested for their acute toxic effect on soil samples in order to confirm that the OSM model system provides similar or better results than other methods currently being used in soil biology studies.

2. Materials and methods

2.1. Soil samples

Soil samples were obtained from an on-campus *Quercus coccifera* stand near our laboratory (40° 30′ 50.951 N; 3° 20′ 27.344 W) in the period of March – June 2016. According to the WRB system, the site's brown soil belongs to the Reference Soil Group rhodic-chromic luvisols (Table 1). Samples were obtained at three different places in a digging depth of 5–10 cm. After collection, samples were pooled and kept in an incubator for one day at either 105 °C (to determine soil composition and structure) or 30 °C (for biological purposes). In the latter case, final soil sample humidity was always below 5%. Soil was passed through a 1 mm mesh sieve and kept in the dark at 4 °C (for < 45 days) until used, in agreement with the OECD guidelines for the preservation of soil biota integrity (OECD, 2000). Controls of sterile soil were prepared by incubating sifted soil at 180 °C for 18 h.

2.2. Microorganisms and culture conditions

Control microorganisms used in this study and their respective culture conditions are shown in Table 2. All strains were purchased from the Spanish Type Culture Collection (CECT-Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain).

Table 1
Characteristics of the soil used in the present study.

Composition/parameter	Percentage/pH units
Sand	35%
Silt	50.24%
Clay	14.76%
pH (1:3 in water)	7.72 ± 0.23
Carbon (%)	3.19 ± 0.33
Hydrogen (%)	1.06 ± 0.13
Nitrogen (%)	1.11 ± 0.18
Sulphur (%)	0.34 ± 0.25

Table 2
Microorganisms and culture conditions employed in the present study.

Species	Origin/ reference	Temperature of growth	Growth media used
Bacillus atrophaeus	CECT 38	32 °C	Luria-Bertoni (USB corp – ref. US 75852)
Pseudomonas aeruginosa	CECT 108	32 °C	Luria-Bertoni (USB corp – ref. US 75852)
Candida albicans	CECT 1002	32 °C	Sabouraud (Scharlau, ref. 02–165-500)
Aspergillus niger	CECT 2090	25 °C	Malt extract (Scharlau, ref. 07–080-500)

2.3. Measurement of microbial growth based on optical density analysis

This procedure was used as a standard method to assess microbial growth and its correlation with the new OCR assay. Basically, microorganisms were grown in microplates at their optimal temperature for 20–24 h and optical density was measured at 630 nm every hour on an ELX 808 IU microplate reader (BioTek, VT, USA).

2.4. Measurement of oxygen consumption in microbial pure cultures and soil

Oxygen consumption was measured on OSM (LT-96 plates from Oxoprobics Biosciences, Madrid, Spain). Positive (oxygen-saturated) and negative (oxygen-free) control reagents, as well as mineral oil (necessary to avoid any oxygen exchange between medium and air), were supplied with the commercial plates. Attempts to measure oxygen content either without adding mineral oil or using an adhesive plastic top (Titer-tops®, Diversified Biotech, Dedham, MA, USA) were made as well. Further details on microplate-related features, reagents and procedures may be found elsewhere (Heredero-Bermejo et al., 2015). The oxygen content of the medium (OC) or the oxygen consumption rate (OCR) were both calculated by converting differences in fluorescence intensity into changes in dissolved oxygen concentration using the Stern-Volmer equation as described by Olry et al. (2007). Most respiration data in this study are presented as $1/[O_2]$ (expressed as nM^{-1}) for the fact that normalized relative fluorescence is difficult to correlate with results based on [CO_2], the most common parameter used in soil respiration studies. Alternatively, data are also expressed as OCR in nmol h^{-1} mL^{-1}.

For respiration assays, microbial overnight cultures were diluted 100-fold into fresh medium, except for *Aspergillus niger*, where a concentrated spore suspension prepared from solid cultures was used following the same procedure as for bacteria. Assays were started by inoculating each well with 100 μL of diluted microbial suspension containing approximately 0.1–1 × 10^8 colony forming units depending on the microorganism. Control wells with 100 μL of either sterile culture medium or the positive or negative controls supplied by the manufacturer were always included in each experiment. Sterile mineral oil was added (100 μL per well) to seal all the wells, as recommended by the manufacturer. The microplate was covered with its lid to avoid contamination and then placed in a plate reader (VICTOR® - Perkin Elmer, Waltham, MA, USA), which was programmed to obtain two readings per well and hour (for up to 24 h). Time-resolved fluorescence (excitation at 340 nm/emission at 642 nm) was measured at delay times of 30 and 70 microseconds. Experiments were carried out at 25 °C or 32 °C depending on the microorganism (Table 2).