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Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities

C.D. Campbell[a,*], S.J. Grayston[a], D.J. Hirst[b]

[a]*Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB15 8QH , UK*
[b]*Biomathematics and Statistics Scotland, Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB15 8QH, UK*

Abstract

Sole carbon source tests (Biolog™), designed to identify microbial isolates can be used to metabolically fingerprint soil microbial communities, although the carbon source profiles were not selected for this purpose. This paper reports on the use of alternative carbon sources not available in the Biolog™ GN plates to characterize soil microbial communities. The carbon sources used are compounds commonly found in plant root exudates and are, therefore, ecologically more relevant and representative of the types of substrates available to microorganisms in rhizosphere soil. The additional carbon sources tested included various phenolic acids, amino acids, carboxylic acids, long chain aliphatics and carbohydrates. In total, 125 different carbon sources were used to discriminate between soil samples from 9 different sites each with three types of grassland vegetation. The growth curves for different groups of carbon sources were all sigmoidal, but the maximum rate of utilization was faster for carbohydrates, amino acids and carboxylic acids than for amides, phenolic and long chain aliphatic acids. Significant discrimination of soil microbial communities between sites, but not grassland types, was shown and was more distinct using the exudate carbon sources than those in the Biolog GN plate. The use of fewer carbon sources which are more ecologically meaningful constitutes a more efficient and economical technique. © 1997 Elsevier Science B.V.

Keywords: Biolog; Grassland; Microbial diversity; Sole-carbon-source; Rhizosphere; Root exudates

1. Introduction

The requirement to place biodiversity within the context of the sustainability of agricultural and natural ecosystems has increased interest in methods of measuring soil microbial diversity since soil functionality depends so much on the activity of micro-organisms [1]. There are several techniques which can be used to study microbial communities and quantify diversity in genetic, taxonomic or functional terms [1,2]. The use of sole carbon source tests is one way to measure functional diversity [2] and has been applied to a wide range of soil habitats [3–6] undergoing changes in land use or disturbance due to pollution [7,8]. Sole carbon source tests have been used in a variety of ways to classify or characterise both single species isolated from soil [9,10] and mixed populations, either extracted from the environment [9] or in model systems [11]. The method first described by Garland and Mills (1991) uses a commercially available microtitre plate (Biolog™) which can be used to simultaneously test the utilisation of 95 different substrates as sole carbon sources [9]. Carbon source utilisation is indicated by colour development of a redox indicator

*Corresponding author. Address for correspondence: Soil Science Group, Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB15 8QH UK. Tel.:+44 1224 318611; fax: +44 1224 311556; e-mail: c.campbell@mluri.sari.ac.uk

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dye and changes in the overall patterns of carbon source utilisation rates indicate differences in community composition. The technique has become popular because it is simple, uses automated measuring apparatus and yields a great deal of information about an important functional attribute of microbial communities, although the analysis and interpretation of such data is often more complicated [11,12].

In previous studies [8,13,14] it was noted that there was significant redundancy in the Biolog GN plate carbon sources, in that many of the carbon sources were highly correlated and contributed little to the discrimination of microbial populations. In studies using Biolog plates, 95 carbon sources have been used and when both GN and GP type plates are combined 138 carbon sources can be tested [2]. However, there is no reason why fewer carbon sources might not be able to discriminate adequately between populations. In studies characterising isolates, as few as 20 [15] or even 9 carbon [10] sources were enough to determine shifts in carbon source utilisation profiles.

This paper reports on the use of a wider range of carbon sources than is currently available in the GN type of Biolog plate. The additional carbon sources were chosen to represent compounds reported in the literature as plant root exudates to make the tests ecologically more relevant for testing soil microbial communities and rhizosphere isolates. By selecting only relevant carbon sources this might also allow the use of fewer tests, allowing greater replication and more economical use of microtitre plates. In order to test such ideas statistically, a large sample set was required. Data from soil samples collected from a range of upland grasslands in the United Kingdom (UK) were used to test these rhizosphere carbon sources. The soil samples had been collected as part of a UK co-ordinated project, called 'Micronet'. The aim of this project is to develop and apply different molecular, taxonomic and physiological techniques to assess the biodiversity of soil microbial populations and their relationship to plant community structure. Data from 9 of the 10 'Micronet' sites were used here to allow a comparison of the widest range of carbon sources. The full findings of this study, and the relationship between carbon source utilisation and soil/plant/climate interactions, will be published elsewhere and discussion herein is limited to the comparison of the carbon sources tested.

2. Materials and methods

The 9 sites were sampled during the period February to June 1995 (Table 1) by taking core samples (bulked from 50 cores) from a $25m^2$ quadrat in each of three replicate plots of improved, semi-improved and unimproved grassland dominated by *Lolium*, *Holcus* and *Agrostis*/*Festuca* vegetation respectively, to a depth of 5 cm using a stainless steel corer (3.5cm diameter). Samples were stored at 4°C no longer than 24 h before they were sieved (6mm) and sorted to remove organic debris, roots and soil animals.

2.1. Soil extraction

Fresh soil (10 g) was added to 100 ml 0.25 strength Ringers solution (Oxoid) and shaken on a wrist action shaker at full speed for 10 min. Ten fold serial dilutions were made and the 10^{-4} dilution, which was the lowest dilution with minimal background colour, was used to inoculate the Biolog plates. The 10^{-4} dilution was centrifuged at 750 g for 10 min to separate the soil and 150 μl of supernatant were inoculated into each well of a Biolog GN type plate and an MT plate prepared with additional carbon sources (Table 2a and b).

Table 1
Names, geographical location and date sampled for 9 grassland sites in the United Kingdom 'MicroNet' project

Site	Latitude/Longitude	Date sampled
Kirkton	56°35′N/4°45′W	02/02/95
Glensaugh	56°58′N/2°30′W	03/02/96
Borrowdale	54°30′N/3°10′W	23/02/95
Aber	53°10′N/3°55′W	07/04/95
Bronydd Mawr	51°50′N/3°25′W	07/04/95
Kinder	53°10′N/1°50′W	19/05/95
Garrigill	54°50′N/2°50′W	05/05/95
Torridon	57°35′N/53°5′W	27/06/95
Castle Douglas	54°55′N/4°0′W	27/06/95

Table 2
A: List of carbon sources reported as constituents of root exudates found in the Biolog GN and MT (*) plates; B: List of carbon sources not reported as constituents of root exudates found in Biolog GN and MT (*) plate

Carbohydrates	Carboxylic acids	Amino acids	Phenolic acids	Long chain aliphatic acids
A				
arabinose	acetic acid	L-alanine	hydroxy benzoic acid*	oleic acid*
D-fructose	citric acid	L-alanyl-glycine	ferulic acid*	palmitic acid*
D-galactose	alpha-hydroxy butyric acid	L-asparagine	coumaric acid*	stearic acid*
alpha-D-glucose	alpha-keto valeric acid	L-aspartic acid	sinapic acid*	linolenic acid*
maltose	malonic acid	hydroxy L-proline	caffeic acid*	
D-raffinose	propionic acid	L-leucine	chlorogenic acid*	
L-rhamnose	succinic acid	L-ornithine	protocatechuic acid*	
sucrose	fumaric acid*	L-phenylalanine		
ribose*	oxalic acid*	L-serine		
xylose*	glycolic acid*	L-threonine		
	tartaric acid*	gamma-amino butyric acid		
	malic acid*	tryptophan*		
	oxaloacetic acid *	methionine*		
		lysine*		
		arginine*		
		glycine*		
		valine*		

B

Carbohydrates	Carboxylic acids	Amino acids	Amides
N-acetyl-D-galactosamine	methyl pyruvate	L-glutamic acid	succinamic acid
N-acetyl-D-glucosamine	mono-methyl succinate	glycyl-L-aspartic acid	glucoronamide
adonitol	cis-aconitic acid	glycyl-L-glutamic cid	phenyl ethylamine
D-arabitol	formic acid	L-histidine	putrescine
cellobiose	D-galactonic acid lactone	L-proline	alaninamide
i-erythritol	D-galacturonic acid	L-pyroglutamic acid	2-amino ethanol
L-fucose	D-gluconic acid	D-serine	
gentiobiose	D-glucosamine acid	D,L-carnitine	
m-inositol	D-glucoronic acid		
alpha-D-lactose	beta-hydroxy butyric acid		
lactulose	gamma-hydroxy butyric acid		
D-mannitol	p-hydroxy phenylacetic acid		
D-mannose	itaconic acid		
D-melibiose	alpha-keto butyric acid		
beta-methyl D-glucoside	alpha keto glutaric acid		
D-psicose	D,L-lactic acid		
D-sorbitol	propionic acid		
D-trehalose	D-saccharic acid		
turanose	sebacic acid		
xylitol	bromo succinic acid		

Polymeric	Phenolic acid	Miscellaneous
alpha cyclodextrin	vanillic acid*	urocanic acid
dextrin	syringic acid*	inosine
glycogen		uridine
tween 40		glucose-1-phosphate
tween 80		glucose-6-phosphate
		2,3-butanediol

2.2. Additional carbon source MT plate

Additional carbon sources were tested using Biolog MT plates in which the wells have no carbon source but have the buffered nutrient medium and the tetrazolium indicator dye. The additional carbon sources were chosen to augment the carbon sources available in the Biolog GN plate by reviewing the literature [16–18] on which carbon sources might be expected to be found in soil, specifically as plant root exudates from herbaceous species (Table 2a), but also tree root exudates and other compounds important as substrates in soil, such as lignin monomers (Table 2b). The MT plates were prepared using the manufacturer's instructions [19], in that 0.3 mg of each carbon source was dispensed into each well. All stock solutions were, however, prepared in ethanol to enhance substrate solubility, to sterilize the substrate and to assist in the drying down procedure. After dispensing, the solutions in the wells were dried down, at room temperature in a laminar sterile air flow cabinet, prior to use. For all additional sugars and amino acids individual carbon source stock solutions were prepared aseptically and each well was filled by dispensing 15 μl of a 2% solution. Similarly, the carboxylic acids were prepared from 2% stock solutions except, for fumaric, maleic, malic and oxaloacetic acids which were made as 1% stock solutions and 30 μl was dispensed into each well. The long chain fatty acids were made as 0.5% stock solutions and 60 μl were dispensed into each well. For the phenolic acids, more dilute solutions were prepared at 10^{-2} M and the final concentration of 0.3mg per well was achieved by adding up to 100 μl at a time with drying between successive additions. Stock solutions were prepared fresh using analytical grade chemicals. Sterility was checked by inoculating the prepared plates with sterile de-ionized water and incubating at the same time as the test samples, and by plating out carbon source stock solutions on TSA agar (Oxoid). One well was left blank, as a control and one well contained glucose as a 'positive' control, to check that its utilization was equivalent to the glucose utilization in the GN plate. Each set of additional carbon sources, including the blank and glucose controls (32 in all), were replicated three times in a single 96 well MT plate. In preliminary tests, the amino acid cysteine was also tested but consistently gave false positive color reactions due to an abiotic chemical reaction and consequently was not tested.

Plates were incubated at 15°C for between 4 and 7 days on an orbital shaker at 100 rpm. Colour development was measured as optical density (OD) using an automated plate reader (EMAX, Molecular Devices, Crawley, UK) at 590 nm every 24 h and the data collected using 'Softmax' (Molecular Devices) software. Data from 1, 2 and 4 days are used in this paper because only these times were measured for all sites. Some of the additional carbon sources (phenolic acids) were visibly coloured and so the initial ODs were measured immediately after inoculation and were subtracted from subsequent daily readings. The average well colour development (AWCD) for all carbon sources [9] and for different groups of substrates, as defined by Zak et al. [2], were calculated as indicative of total activity. After transformation, by dividing by the AWCD [9,12], the optical density data were analysed by canonical variate analysis (CVA), after first reducing the dimensionality by principal components analysis (PCA). This reduction in dimensionality was necessary to avoid bias in the CVA which would arise from having a greater number of variates than samples. The mean Mahalanobis distance between all groups was used to measure the separation of the groups. The first 15 principal components were used in the CVA because they explained at least 85% of the variance in each case. This ensured a large amount of the between sample variation was included in the CVA, but kept the number of variables sufficiently small for the variance of the mean distance to be acceptable. The significance of the mean distance was tested by simulation by taking 15 normally distributed variables of length 81 (the number of samples) generated with no group differences. CVA was performed and the mean distance noted. This was repeated 1000 times and the percentiles of the distribution recorded. For 9 groups (sites) of 9 samples (replicates), the 95 and 99% percentiles were 2.31 and 2.44, respectively, and for 3 groups (vegetation type) of 27 samples (replicates) the 95 and 99% percentiles were 1.40 and 1.55, respectively. The hypothesis that there was no separation between any of the groups was tested by comparing the observed mean distance with these percentiles.

In order to compare the discriminant ability of different sets of variables, the standard error of the mean distance was estimated by simulation by creating 15 fixed variables with genuine differences between the groups. This simulation consisted of generating 15 normal random variables, adding them to the fixed variables, performing a CVA and recording the mean distance. This was repeated 1000 times and the mean and standard deviation of the mean distances noted. This was repeated for a range of fixed variables which gave different expected mean distances. The standard error of the actual observed mean distances was then interpolated from the simulated values. A one way analysis of variance was used to compute treatment means and least significant differences (LSD) and all computations were performed using Genstat Rel 5.3 (NAG Ltd., Oxford, UK).

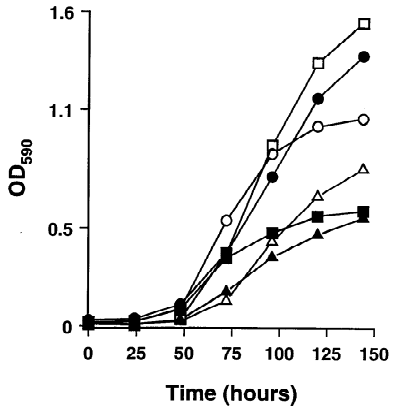

Fig. 1. Mean rate of colour development for samples from Borrowdale over 144 h for different groups of carbon sources: carbohydrates (●), carboxylic acids (○), amino acids (□), amides (■), phenolic acids (△) and long chain aliphatic acids (▲)

3. Results and discussion

There were no significant differences in OD between either the blank control wells or glucose containing wells in the GN and MT plates, suggesting the preparation methods used for the MT plate carbon sources were satisfactory. The rate of substrate utilisation (colour development) at each site, was typically most rapid for the carbohydrates, amino acids and carboxylic acids and is illustrated using data from the Borrowdale site (Fig. 1). Although the utilisation of amides was initially as fast as the carbohydrates, carboxylic and amino acids, the overall colour development was lower. The long chain aliphatic acids and phenolic acids were the most slowly utilised and had much lower AWCD after 144 h with an indication of longer lag times also (Fig. 1). The colour development over time was generally sigmoidal for all groups, but for the more slowly utilised carbon sources longer incubation times would be required to examine the full extent of carbon source utilisation. The AWCD for root exudates (Fig. 2a), which included the phenolic and aliphatic acids, was therefore, generally lower than the non-exudate sources (Fig. 2b) and was significantly different for the different sites. The sites at Torridon, Castle Douglas and Bronydd Mawr had the fastest rates of utilisation over 96 h. This may have

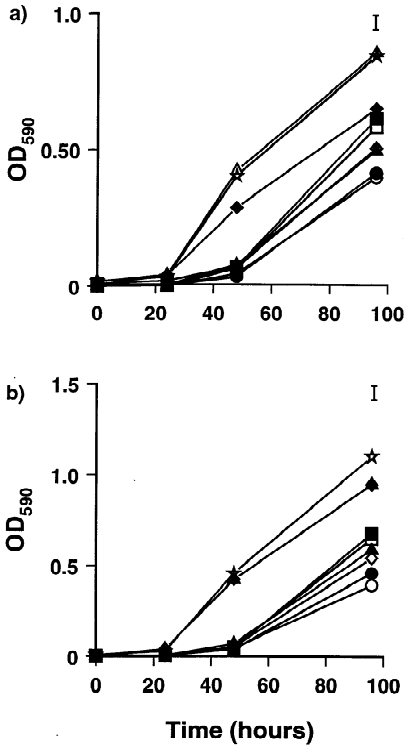

Fig. 2. Rate of AWCD for a) exudate carbon sources and b) non-exudates over 4 days for 9 different upland sites: Kirkton (●), Borrowdale(○), Glensaugh (■), Kinder (□) Aber (◇), Bronydd Mawr (☆), Garrigill (▲), Torridon (△), Castle Douglas (♦). Error bars equal to least significant difference at 5%.

been partly due to the temporal differences in sampling combined with their geographical location because these sites were amongst the last and most westerly of the sites sampled (Table 1) and plant growth would have been more advanced. The other sites were largely similar in their AWCD.

Canonical variate analysis, using the different subsets of 125 carbon sources, showed that use of the exudate carbon sources produced the highest mean distances (differences) between the sites (Table 4). These were followed by the whole 125 carbon sources, with the GN plate and non-exudates having similar mean distances, although they varied with time (Table 4). CVA of all 125 carbon sources showed significant discrimination on CV 1 and CV 2 of the Kinder and Aber sites, as one cluster and also the Torridon and Bronydd sites, as two distinct clusters, with the other sites all clustered together (Fig. 3a). Further separation on CV 3 was evident for the Bronydd and Torridon sites, but the separation was not significant (Fig. 3b). The loadings computed for each carbon source suggested a high proportion of the most influential carbon sources were in the exudate carbon sources from the MT plate (Table 3). Analysis using only the root exudate carbon sources (Table 2a) discriminated between the sites in a similar manner (Fig. 4) to the use of all 125 carbon sources (Fig. 3) but the separations were more distinct (Table 4). This was most evident on CV 3 and 4 (Fig. 4b) in which discrimination of the Borrowdale and Kirkton sites was more apparent. In contrast, the discrimination using the non-exudate carbon sources was not significant (Fig. 5a and b), with the exception of some separation of the Torridon and Bronydd Mawr sites on CV1 (Fig. 5a).

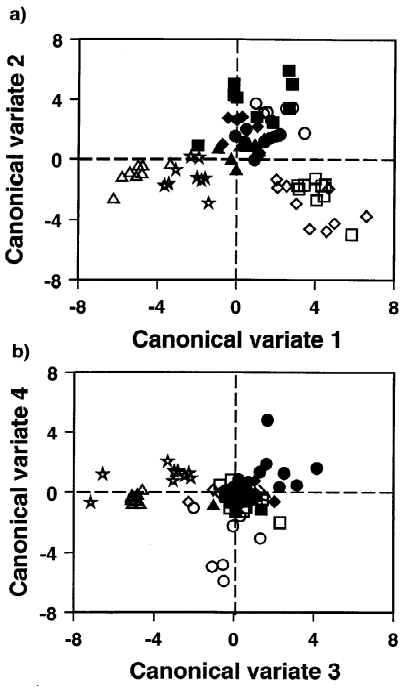

Fig. 3. Plot of ordination of canonical variates (CV) a) CVs 1 against CVs 2 and b) CV3 against CV 4 generated by canonical variate analysis of sole carbon source tests after 48 h for 125 carbon sources in Biolog GN and MT plates showing discrimination between 9 grassland sites: Kirkton (●), Borrowdale(○), Glensaugh (■), Kinder (□) Aber (◇), Bronydd Mawr (☆), Garrigill (▲), Torridon (△), Castle Douglas (♦).

There was no significant discrimination between vegetation types (data not shown). Although these results suggest that site differences were much larger than vegetation differences, plant growth was quiescent at most sites. Consequently, the extended time period over which samples were taken and the effect of soil-plant-climate interactions may confound site and plant effects on the composition of the soil microbial communities.

Of the 125 carbon sources tested, the major compounds responsible for the separation of the sites were root exudate constituents and were predominantly aliphatic acids (the long chain fatty acids and short chain carboxylic acids). This probably accounts for the improved discrimination using the exudate carbon sources alone. Significantly, nearly all these

Table 3
List of top ten carbon sources influencing separation of sites and their sum of squared (SSQ) loadings from CVA of 125 carbon sources showing predominance of exudate carbon sources and their AWCD after 96 h (* = carbon source in MT plate)

Carbon source	SSQ Loadings	AWCD 96 h
Linolenic acid*	0.0560	0.29
Malic acid*	0.0501	0.22
Oleic acid*	0.0352	0.17
Arginine*	0.0323	1.12
Serine	0.0219	0.72
Lactic acid	0.0183	0.61
Oxalic acid*	0.0126	0.16
Trehalose	0.0103	1.56
Stearic acid*	0.0086	0.18
Beta methyl D-glucoside	0.0086	0.18

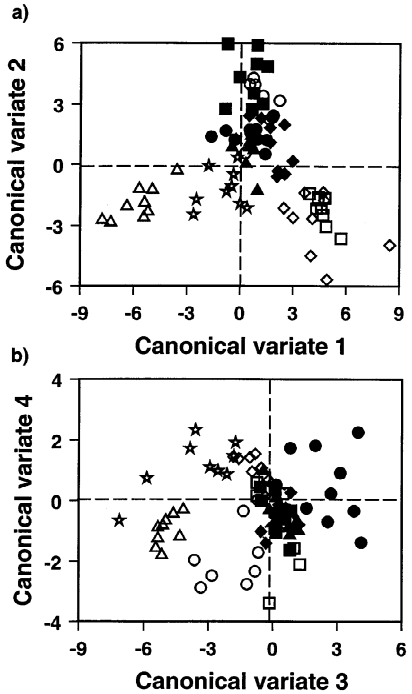

Fig. 4. Plot of ordination of canonical variates (CV) a) CVs 1 against CVs 2 and b) CV3 against CV 4 generated by canonical variate analysis of sole carbon source tests after 48 h for exudate carbon sources in Biolog GN and MT plates showing discrimination between 9 grassland sites: Kirkton (●), Borrowdale(○), Glensaugh (■), Kinder (□) Aber (◇), Bronydd Mawr (☆), Garrigill (▲), Torridon (△), Castle Douglas (♦).

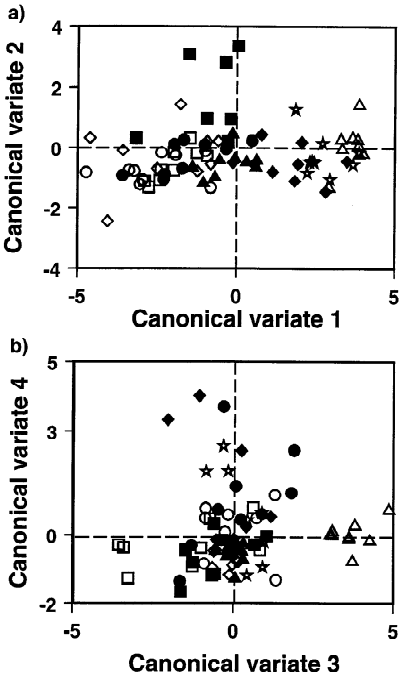

Fig. 5. Plot of ordination of canonical variates (CV) a) CVs 1 against CVs 2 and b) CV3 against CV 4 generated by canonical variate analysis of sole carbon source tests after 48 h for non-exudate carbon sources in Biolog GN and MT plates showing discrimination between 9 grassland sites: Kirkton (●), Borrowdale(○), Glensaugh (■), Kinder (□) Aber (◇), Bronydd Mawr (☆), Garrigill (▲), Torridon (△), Castle Douglas (♦).

compounds had low utilization rates (Table 3) suggesting that they are used by microorganisms present in low numbers. In fact, some of these compounds were not used at certain sites e.g. oleic acid at Torridon, Castle Douglas, Garrigill or Glensaugh; stearic acid at Kirkton, Glensaugh, Borrowdale, Torridon or Garrigill; oxalic acid at Aber, Bronydd Mawr, Castle Douglas, Torridon or Garrigill; linolenic acid was not utilized at Glensaugh, Kirkton, Torridon and Castle Douglas; and malic acid was not utilized at Castle Douglas, Borrowdale or Kirkton. This would have a significant influence on the utilization pattern.

Another important consideration with respect to substrate utilization is that of substrate concentration. Preliminary work on the use of the phenolic acids described in this paper [20] showed that high concentrations were necessary to get detectable colour development in the Biolog MT plates. Such high concentrations, especially for phenolic and certain organic acids are likely to be inhibitory or even toxic

Table 4
Mean distances (standard errors in parenthesis) between group (site) clusters from CVA of sole carbon source tests using different classes and numbers of carbon sources over 3 times of incubation

Time (h)	All carbon sources	GN plate carbon sources	Exudate carbon sources	Non-exudate carbon sources
24	6.72 (0.38)	4.89 (0.32)	7.02 (0.42)	4.85 (0.32)
48	6.30 (0.38)	4.12 (0.29)	6.62 (0.41)	4.06 (0.29)
96	4.69 (0.32)	3.25 (0.25)	5.17 (0.33)	3.16 (0.24)

to some microorganisms [21]. This may partly explain the extended lag phases in colour development for these compounds, since extended adaptation and selection may be required, within the population before significant growth and colour development occurs (Fig. 1).

The reasons why certain carbon sources increase the discrimination of this technique may be complex. This is because the discriminatory power of multivariate techniques lies not in the use of many different carbon sources, but in the use of combinations of carbon sources. The exudate carbon sources were a more diverse set of compounds which might, therefore, be expected to differentiate to a greater extent between microbial populations. In contrast, many of the carbon sources in the GN plate are similar and may, therefore, add little to the discriminatory power of this technique. It is hypothesized that the exudate substrates select for a greater diversity of organisms, which include relatively slower growing organisms, present in lower numbers. This greater diversity would then be more likely to catabolise more unusual carbon sources, either as individual species or as consortia catabolising a sequence of reactions. If true this approach would then be more representative of microbial mineralisation functions in the soil.

The benefit of using carbon sources which are ecologically more relevant, is that more convincing conclusions may be made if the function of those carbon sources is known or understood. However, this would only be useful if it resulted in equal or better discrimination. As we have demonstrated, the use of the rhizosphere carbon sources has provided greater discrimination, illustrating that these carbon sources have significant advantages over the existing carbon sources in the GN plate. An additional benefit of using fewer tests is that greater efficiency and economy is possible, which facilitates more replication and increases the separation power when using discriminant types of statistical analysis.

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