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Combining microdilution with MicroResp™: Microbial substrate utilization, antimicrobial susceptibility and respiration

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ABSTRACT

Pharmacological studies focus on susceptibility of pathogenic microbes against specific drugs or combinations of them, ecological studies on substrate utilization efficiency of variable microbial communities. The MicroResp™ system was especially developed to study soil microbial communities. It was slightly modified to facilitate exploring of microbial growth efficiency in a concentration-dependent fashion (microdilutions of carbohydrate mixtures or specific toxic chemicals). After turbidimetric growth assessment, colorimetric indicator plates (cresol red agar) were mounted to the assay plates. The substrate utilisation design is illustrated by glucose and a plant carbohydrate mixture, the antimicrobial susceptibility design by the naphthoquinone juglone. Dose–response effects are explored by curve fitting of nonlinear models that especially have been developed to detect hormetic effects that are characterized by stimulation at lower followed by inhibition at higher dosages (U- and inverse U-shaped effects). Multivariate analyses are presented utilizing metavariables that were obtained in the curve fitting process of the measured parameters growth and respiration and the factor growth efficiency.

1. Introduction

The exploration of microbial substrate utilisation and susceptibility by and against various substances or substance mixtures has received a lot of attention in medical and environmental science. Depending on the specific research question, studies differ in their focus.

Clinical studies mainly target single specific pathogens, either by identifying the strain based on its specific utilization of a substrate set, or determining its susceptibility against established antimicrobial drugs; besides, the potential of specific synthetic compounds, natural products or antibiotics as "chemical weapons" against other microbes are explored in on-going screening efforts to identify potential candidates for drug development, especially when facing multidrug resistance of microbial pathogens (Coates et al., 2002). Environmental studies, by contrast, are aimed more at exploring the extent of changes caused by specific factors on the structure of microbial communities that is revealed by community level physiological profiling by assessing utilisation pattern of a standardized substrate set (Campbell et al., 2003).

Broth microdilution methods are established as standard in determining MIC values (minimal inhibitory concentration) of antimicrobial substances (Pfaller et al., 2011; Wiegand et al., 2008). Today, turbidimetric assays in 96-well microplates are prevalent in academic and clinical studies (De La Fuente et al., 2006; Engelmeier and Hadacek, 2006).

Environmental studies are aimed at determining community level physiological profiles (CLPP) by assessing microbial substrate utilization of a wide range of carbon sources, but only at a single concentration (Garland, 1997; Lehman et al., 1995). Again, the most recommended scoring method is turbidimetry. Kits such as Ecoplate™ (Biolog Inc, Hayward CA, USA) assess the growth on 32 substrates using optical density (turbidimetry) at 590 nm (Garland and Mills, 1991). In 2003, researchers from the Macaulay Land Use Research Institute in Aberdeen (Scotland, UK), introduced the MicroResp™ kit, in which substrate induced respiration (SIR) was measured colorimetrically on a second indicator microplate that is clamped to a deep well plate containing the soil samples (Campbell et al., 2003). Both plates are connected by an air-tight seal that facilitates the movement of the evolving carbon dioxide to the indicator plate that contains agar augmented with cresol red and $NaHCO_3$. The following chemical reaction occurs:

$$CO_2 + H_2O + HCO_3^- \rightarrow 2CO_3^- + 3H^+$$

The cresol red changes from pink to yellow colour with decreasing pH. The advantage of the MicroResp™ method is that it relies on a

Abbreviations: CFU, colony forming units; G, growth; GE, growth efficiency; MS, Murashige–Skoog; GC–MS, gas chromatography–mass spectroscopy; LB, Luria-Bertani; MDS, multidimensional scaling; MEA, malt extract agar; R, respiration; TSA, tryptic soy agar.

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colour change of a pH sensitive dye that is not in direct contact with the medium and tested microbes. In solutions, water soluble tetrazolium salts are often used as redox-sensitive indicators of microbial growth. The risk of a potential reduction of the dye by other unattended strong chemical reducers that also may be present in the medium does not apply to MicroResp™ (Bhupathiraju et al., 1999).

Here we want to introduce a modified MicroResp™ method that explores substrate utilization and susceptibility of specific compounds or compound mixtures that are added to liquid culture media in dilution series. This assay provides both growth and respiration as quantitative data, allowing to calculate a growth efficiency factor (Del Giorgio et al., 1997) that interrelates changes in growth and respiration rates of treatments compared to untreated controls (cultures on non-supplemented media). It represents a highly sensitive assay not only to study substrate utilisation but also susceptibility against toxic carbohydrate compounds, such as natural products, drugs or xenobiotics.

Media for carbon utilization and susceptibility differed in their initial carbon content; the former had a low carbon content to force microbes utilizing the offered carbohydrate. Conversely, higher carbon content facilitates more vigorous growth that helps detecting inhibitory effects. To illustrate the applicability of this assay, we chose the carbohydrate glucose, a substrate that is used to study microbial carbon-use-efficiency (Keiblinger et al., 2010), a complex carbohydrate mixture as it occurs in plant tissues, and a secondary metabolite, the naphthoquinone juglone that is well known for its antimicrobial activity (Hadacek and Greger, 2000). To explore dose–response relations we used several non-linear growth models that were especially developed to study hormetic effects, stimulation at lower dosages that is followed by inhibition in higher. This effect is not limited to pharmacological but also occurs in biological models (Calabrese and Blain, 2009).

The fact that the pepper bark tree, *Warburgia ugandensis*, of the family Canellaceae, is used as source of plant carbohydrates and bacterial and fungal isolates (endophytes), is due to a current research project, from which data have been chosen in attempts to illustrate specific aspects of the introduced assay.

2. Material and methods

2.1. General

Tryptic soy broth (TSB), juglone, KH_2PO_4, KCl, $NaHCO_3$ and Amberlite XAD-1180 were purchased from Fluka (Buchs, Switzerland); methanol (p.a.), MS medium, and cresol red (indicator grade) were obtained from Sigma Aldrich (Schnelldorf, Germany), D-(+)-Glucose, peptone and ethanol absolute from Merck (Darmstadt, Germany), LB broth and sucrose from Roth (Karlsruhe, Germany). NaOH (50% aqueous solution) was purchased from J.T. Baker (Deventer, Netherlands).

DNA polymerase was obtained from Solis BioDyne, (Tartu, Estonia), endonucleases *Hae*III and *Alu*I and Go Taq® Green Master kit from Promega GmbH (Mannheim, Germany). Water had Milli-Q quality.

2.2. Plant carbohydrates

The plant carbohydrates were obtained by extraction of 3 g dried leaves of *W. ugandensis* Sprague (Canellaceae) at ambient temperature (80 ml methanol for 24 h). The extract was filtered (MN 615; Macherey-Nagel, Düren, Germany) and concentrated to 10 ml under vacuum. Two hundred milligrams of the crude extract were fractioned over Amberlite XAD-1180. Glass columns (15 mm diameter) were filled with 20 g resin and prepared according to manufacturer's guidelines. Two 50 ml fractions were eluted, one with water, one with absolute ethanol. The concentrated eluates were stored at −20 °C until further use.

For GC–MS measurements, 100 µg of the dried aqueous eluate were dissolved in 100 µl *N*-methyl-*N*-TMS-trifluoroacetamide (MSTFA, Thermo Scientific, Waltham, MS) for derivatisation into trimethylsilyl ethers and esters. One microliter of this solution was injected into an AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MS) in splitless mode, the injector temperature was 250 °C. The column was a Zebron 5 ms column (18 m × 0.18 mm, 0.18 µm film thickness; Phenomenex, Torrance, CA), the helium follow rate 0.8 ml/min. The temperature gradient started at 70 °C and, after 3 min, rose to 300 °C at a rate of 3 °C/min. The gas chromatograph was linked to a TurboMass™ quadrupole mass analyzer (Perkin Elmer, Waltham, MS); the transfer line temperature was set to 280 °C, the ion source to 200 °C, the filament current to 70 eV. The mass spectrometer was run in the TIC mode from 40 to 620 amu. The obtained chromatograms were integrated with TurboMass 4.1.1 (Perkin Elmer, Waltham, MS) and the peak areas were expressed as relative amounts of the total peak area (100%). Mass spectra were identified tentatively with the Golm Metabolome Database (Potsdam, Germany; http://gmd.mpimp-golm.mpg.de; Kopka et al., 2005).

2.3. Microorganisms

2.3.1. Strain isolation and identification from Warburgia ugandensis

2.3.1.1. Bacteria. For isolation of endophytic bacteria, leaves and roots embedded in MS agar were carefully pulled out of the agar and remaining agar was removed with a sterile scalpel. The plant material was washed in sterile, distilled water, rinsed with ethanol, and finally flamed. Roots and leaves were tested for sterility on tryptic soy agar plates incubated for 8 days at room temperature. No growth was observed. All plant material was cut into small pieces, macerated by grinding in sterile mortars, and suspended in 5 ml of 0.9% NaCl solution. Portions of 50 µl of the supernatant and of a 10^{-1} dilution were spread on 10% TSA and R2A plates, respectively. Plates were incubated for 2–8 days and room temperature. Ninety-six colonies in total were randomly picked and further analyzed. For isolation of genomic DNA, bacteria were grown overnight in 5 ml of tryptic soy broth in a rotatory shaker at 27 °C. DNA was isolated as described (Reiter and Sessitsch, 2006). 16S rDNA PCR was carried out by using primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and 1520r (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al., 1989) and primers pHr (5′-TGCGGCTGGATCACCTCCTT-3′) (Massol-Deya et al., 1995) and P23SR01 (5′-GGCTGCTTCTAAGCCAAC-3′) (Massol-Deya et al., 1995) were used for amplification of the 16S–23S rRNA IGS. PCRs were performed in a total volume of 50 µl and contained 1 µl of extracted DNA, 1× PCR reaction buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.15 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 2.5 U FIREPOL DNA polymerase. Cycler conditions were as following: 5 min denaturation at 95 °C, 30 cycles consisting of denaturation for 30 s at 95 °C, primer annealing for 1 min at 53 °C, polymerization for 2 min at 72 °C, and final extension for 10 min at 72 °C. Aliquots of the PCR products containing 200 ng of amplified DNA were digested with 5 U of endonucleases *Hae*III and *Alu*I individually for 3 h at 37 °C. The resulting DNA fragments were analyzed by gel electrophoresis in 2.5% agarose gels. One isolate of each ribotype was identified by 16S rDNA sequencing with the primer 8f making use of the sequencing service of the company AGOWA (Berlin, Germany). Retrieved sequences were visualized with sequence alignment editor package of BioEdit (Ibis Biosciences, Carlsbad, CA) and identified by BLAST analysis.

2.3.1.2. Fungi. For isolation of fungi MS agar-embedded plant tissue was incubated at room temperature for two weeks. Growing fungal hyphae were carefully examined with the naked eye and with an Olympus SZH10 research stereo microscope (×140) to distinguish endophytic fungi that grew directly out of plant material from

eventually contaminations on the agar surface. Parts of fungal hyphae with different habitus were then transferred aseptically to malt extract agar. Cultures were grown in the dark at room temperature. The isolation procedure was repeated in case of mixed habitus types growing on one Petri-dish until a pure culture was obtained. For storage cubes with actively growing mycelia were cut out of solid media and transferred aseptically to 2 ml vials containing a 14% sucrose-1% peptone solution and kept at −20 °C until further use.

For DNA isolation fungi were grown on MEA at room temperature. Fungi material was washed from the plates with 1–2 ml of 0.1% Triton X100 and collected by centrifugation. DNA was isolated with the same procedure described for bacteria. PCR was carried out by using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and TW13 (5′-GGTCCGTGTTTCAAGACG-3′) (White et al., 1990) to amplify a 1.5 kb PCR product spanning the ITS and part of LSU sequence. PCRs were performed in a total volume of 15 µl using Go Taq® Green Mastermix, 1 µM of each primer, 0.67 mg/l BSA and 1 µl of undiluted DNA as template. Cycler conditions were as following: 2.5 min denaturation at 95 °C, 35 cycles consisting of denaturation for 30 s at 94 °C, primer annealing for 30 s at 54 °C, polymerization for 2 min at 72 °C, and final extension for 5 min at 72 °C. Sequencing was done with the primers ITS1 and TW13, respectively making use of the sequencing service of the company AGOWA (Berlin, Germany). Retrieved sequences were visualized with sequence alignment editor package of BioEdit (Ibis Biosciences, Carlsbad, CA) and identified by BLAST analysis.

2.3.2. Strains from culture collections

An airborne isolate of *Penicillium expansum* Link (MA 2811) was obtained from the culture collection of the Austrian Centre of Biological Resources and Applied Mycology (ACBR), Department of Biotechnology, Agricultural University of Vienna, Austria.

2.3.3. Inoculants and stock cultures

Bacteria were grown on LB agar plates and fungi on MEA (15 g/l malt extract, 1.5 g/l peptone) at room temperature in dark. Bacterial growth curves were determined as described by Cappuchino and Sherman (2005). Colony forming units of fungal and bacterial stock solutions were determined by plate dilution. For the assay, the stock inoculum solution was adjusted to 10^5 CFU/ml. Fungal conidia were harvested as described elsewhere (Engelmeier and Hadacek, 2006). Bacterial and fungal stock cultures as well as fungal conidia suspensions were stored in 14% sucrose-1% peptone solution at −20 °C.

2.4. Assays

2.4.1. Substrate utilization

Substrate utilization of glucose and plant carbohydrates was assessed by broth microdilution (Wiegand et al., 2008). Twelve concentrations were prepared by 1:1 serial dilution (0.005–10 mg/ml). The test was performed in sterile 96-well U-shaped microplates (Greiner BioOne, Kremsmünster, Austria). The growth medium for bacteria was diluted LB broth (0.19 g/l), for fungi diluted MS broth (0.1 g/l). Both still supported detectable growth of the respective strains. The media were sterilized with a table top autoclave (Certo-Clav Sterilizer GmbH, Traun, Austria) at 121 °C for 15 min. Substrate stock solutions (20 mg/ml) were prepared in the respective medium. One hundred microliters of the stock solution were pipetted in the first well and serially diluted (1:1). To the 50 µl of substrate solution, 50 µl of inoculate was added. At the starting point, turbidity was measured at 600 nm with a microplate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). For the assay duration, the microplate lids were sealed with Parafilm M (Brand GmbH, Wertheim, Germany) and incubated at room temperature on a horizontal shaker (120 rpm). End point turbidity measurements were performed after 24 h (bacteria) and 48 h (fungi). To overcome detection problems due to heterogeneous fungal growth, turbidity measurements were performed using the 4×4 scan per well option. The values were corrected by deducting respective blanks of identical solutions without inoculate to exclude turbidity effects that were caused by chemical reactions and solute precipitation.

Immediately after end point measurements were finished, respiration was assessed with a modified MicroResp™ technique (Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen, UK; http://www.microresp.com). For each detection plate (wells filled with cresol red agar; 1 l: 18.75 mg cresol red, 16.77 g KCl, 0.315 g $NaHCO_3$, 30 g agar), absorbance measurements were performed at 570 nm before fixing them upside down on the microplates that were used for turbidity measurements before. The original MicroResp™ system was slightly modified by using flat instead of deep well microplates. The MicroResp™ metal clamp was replaced by a plastic clamp fixing detection and culture microplate with the aid of two glass plates or, better, fibre boards; the recommended seal facilitated a tight connection of the two microplates (Fig. 1). After 6 h incubation, the detection plates were again scored at 570 nm, a decrease in absorbance indicated CO_2 development that was used to quantify microbial respiration. Data were normalized prior to statistical analysis (percentage of growth/respiration of the respective strain on non-supplemented medium).

2.4.2. Antimicrobial susceptibility

Procedures were similar as mentioned above, but media composition differed: LB broth (10 g/l) for bacteria, malt extract broth (15 g/l malt extract, 1.5 g /l peptone) for fungi. The media composition was optimized to obtain optimal growth conditions for detecting inhibitory effects. All media were prepared with a 0.025 M NaOH/KH_2PO_4 buffer (pH 7.4) instead of pure water. Sixteen juglone concentrations were prepared by 1:1 serial dilution (0.01–348 µg/ml).

2.4.3. Controls

Controls were performed similarly as treatments. The control data were required to transform the experimental values into quantitative

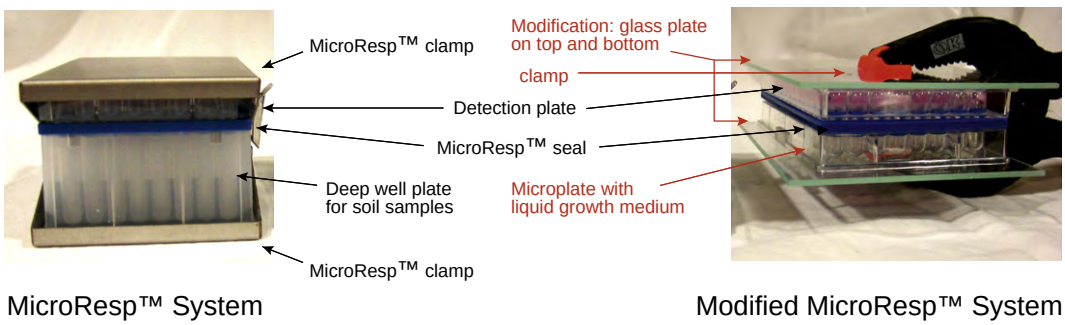


Fig. 1. Comparison of the original versus the modified MicroResp™ setup: the deep well plates for soil samples were replaced by microplates with liquid growth media containing a substrate or toxic chemical compound in microdilutions.

data. The substrate solution was replaced by medium in the respective control. In the substrate utilization version, the composition of the medium was chosen to facilitate some minimal growth of which the rate increased if the substrate was utilized by the tested microorganism. In the antimicrobial susceptibility version, the nutrient concentration was higher to facilitate vigorous growth to detect inhibitory effects.

In the tested concentration range, juglone, the secondary metabolite, was soluble in the medium. For more lipophilic compounds, however, it may be necessary to employ organic solvents as emulsifiers. In this case, the concentration of the organic solvent should not exceed 5% (v/v). Moreover, if added the whole effect of the organic solvent in the dilution series has to be assessed in addition (Engelmeier and Hadacek, 2006).

2.4.4. Replicates

Dilutions were performed in triplicates at least. All replicates were used for curve fitting and their quality was determined by a lack-of-fit test (simple one-way ANOVA, R package drc, R Development Core Team, 2011). This statistical test also was applied to assess result replicability of independently performed assays. The results are not presented as dose–response curve of the values that were obtained from various concentrations in the dilution series directly in the assay but as model-fitted curve (see Section 2.5) calculated from these data.

2.5. Curve fitting and multivariate analysis

The assay was designed as quantitative biological assay (Roberts and Boyce, 1972); 100% was the mean of control growth and respiration. Based on the measured parameters growth (G) and respiration (R), growth efficiency (GE) was calculated as proposed by Del Giorgio et al. (1997) for bacteria.

$$GE = \frac{G}{G+R}$$

The sum of growth and respiration reflects the amount of assimilated carbon (A) and, in studying the production of planktonic bacteria (BP), the factor BP/A more or less aims to characterize the organic carbon flow through the studied aquatic system. By contrast, the main goal of calculating GE is to compare the substrate use efficiency of selected fungal and bacterial isolates. Instead of comparing the carbon utilisation within a given time period, as for example in batch cultures, dose–response effects assessment of specific carbohydrates or mixtures represents the major focus of this assay design. Curve fitting of dose–response models for G, R, and GE, was performed in the R environment for statistical computing and graphics (R Development Core Team, 2011) and, specifically, the package drc was used (Ritz and Streibig, 2005). To account for U-shaped and inverse U-shaped hormesis effects, the following extended log-logistic models were selected:

(1) Brain–Cousens model (Brain and Cousens, 1989), inverse U-shaped dose–response relationship:

$$f(x) = c + \frac{d - c + fx}{1 + \exp(b(\log(x) - \log(e)))}$$

(2) Cedergreen–Ritz–Streibig model (Cedergreen et al., 2005), inverse U-shaped dose–response relationships with variable α:

$$f(x) = c + \frac{d - c + f\exp(-\frac{1}{x^a})}{1 + \exp(b(\log(x) - \log(e)))}$$

(3) Cedergreen–Ritz–Streibig model (Cedergreen et al., 2005), U-shaped dose–response relationships with fixed α (0.0, 0.5, 1.0):

$$f(x) = c + d - \frac{d - c + f\exp(-\frac{1}{x^a})}{1 + \exp(b(\log(x) - \log(e)))}$$

The variables that were obtained in the curve fitting procedure represented metavariables that characterized the response to a dose range. A Manhattan distance matrix was calculated from the normalized meta-variables for various model and parameter combinations. The resemblance matrix was analysed by non-metric multidimensional scaling and illustrated by a 2D scatterplot that shows ellipses denoting the 10, 25, 50, 75 and 90 distance boundaries (determined in a cluster analysis, group linkage mode, of the Manhattan resemblance matrix). All these analyses were performed with Primer 6 (Primer-E Ltd., Plymouth, UK).

3. Results

3.1. Strain BLAST analyses

The closest relative of the bacterial strains WB5 Pamy was an isolate of *Paenibacillus amylolyticus* (Nakamura) Ash et al. emend. Shida et al., of WB70 Cfla an isolate of *Curtobacterium flaccumfaciens* (Hedge) Collins and Jones, and of the fungal strain WF1 Famb an isolate of *Fusarium ambrosium* (Gadd & Loos) Agnihothr. & Nirenberg (Table 1). To avoid the excessive use of culture codes, the closest related anamorph taxa are used in the on-going text as working names.

3.2. Substrate utilisation and susceptibility

3.2.1. Plant carbohydrates

The water-soluble fraction of the *W. ugandensis* leaf extract contained amino acids, mono- and dicarbonic acids, pentoses, hexoses, disaccharides, and sugar alcohols. The most prominent compounds were the sugar alcohols mannitol and xylitol, Table 2 shows the complete list of analytes that were detected by GC and identified on basis of their EI mass spectra.

The plant carbohydrates stimulated the development of the bacterial isolates, *C. flaccumfaciens* and *P. amylolyticus*; growth (G), respiration (R) and growth efficiency (GE) increased with higher dosages. At the highest concentrations tested (3000 μg/ml), an inhibition started to show. Lower concentrations, interestingly, inhibited respiration compared to control, allowing curve fitting of a U-shaped model in case of both tested strains (Fig. 2a). GE values of 0.7 indicated growth stimulation. Of the two tested fungal isolates, *P. expansum* utilized the plant carbohydrates similarly to the bacteria, but *F. ambrosium* was less efficient in metabolizing them in higher concentrations. R increased faster than G in higher dosages (Fig. 3a) that resulted in an inverse U-shaped growth curved with decreased GE in higher concentrations.

3.2.2. Glucose

When tested in similar concentration range as the plant carbohydrates, glucose turned out to be a less suitable substrate for the two bacterial strains. Whilst lower concentrations stimulated growth, higher concentrations inhibited. A similar inverse U-shaped effect was visible in the respiration and also in the dose–response ratio of the GE factor (Fig. 2b). By contrast, the fungi utilized glucose differently. While *P. expansum* growth was stimulated by higher glucose concentrations, *F. ambrosium* growth was inhibited. The effect on respiration was opposite; *P. expansum* respiration decreased with higher dosages and *F. ambrosium* respiration increased. The respiration of *P. expansum* was stimulated by more than the factor 2 (Fig. 3b).

Table 1
BLAST analysis-retrieved closest relative to 16S rRNA (bacteria) and ITS region (fungi) of *Warbugia ugandensis* isolates.

Strain	GenBank ID	origin	BLAST analysis		
Bacteria			Closest identified relative (16S rRNA)	Accession no./identity [%]	Phylogenetic group
WB5 Pamy	JF836824	*W. ugandensis*	*Paenibacillus amylolyticus*	AM237378/98	*Paenibacillaceae*
WB70 Cfla	JF836838	*W. ugandensis*	*Curtobacterium flaccumfaciens*	EU236753/99	*Microbacteriaceae*
Fungi			Closest identified relative (ITS region)	Accession no./identity [%]	Phylogenetic group
WF1 Famb	HQ130667	*W. ugandensis*	*Fusarium ambrosium*	AF178397/95	*Sordariomycetes*

Increasing glucose concentrations increased GE of *P. expansum* but decreased that of *F. ambrosium*.

3.2.3. Juglone

The naphthoquinone juglone, a natural product, proved as antimicrobial by decreasing G, R, and GE of all tested strains with higher dosages. The inhibition appeared within few dilution steps before its onset (Figs. 2c and 3c).

3.3. Growth and susceptibility, a multivariate comparison

The variables that were obtained in the curve fitting of various U-shaped and inverse U-shaped models served as metavariables for multivariate analyses of the assay results. Resemblance matrices (Bray–Curtis) of Manhattan distances yielded specific MDS plots for each model (Fig. 4a–e). A combination of all models, however, considerably improved case point distances from 25 to 90% (Fig. 4f). Similarly, multivariate analyses were performed for G, R, and GE alone (Fig. 5a–c). Again, distances between case points only were improved (from 50 to 90%) by a combination of all three parameters (Fig. 5d), but not by GE alone (Fig. 5c).

Table 2
GC–MS analyses of plant carbohydrate mixture that was used in the bioassay (% rel. peak area, TIC signal); for GC–MS parameters see Material and methods; tentative identification on basis of MS spectra and elution sequence comparison with the Golm Metabolome database (http://gmd.mpimp-golm.mpg.de). Major analytes are printed in bold.

Identified analyte	Compound class	%
	Amino acids	
Pyroglutamic acid		0.1
Threonine		0.2
	Organic acids	
Fumaric acid		0.7
Malic acid		**3.6**
Quinic acid		0.2
Succinic acid		0.5
	Fatty acids	
Palmitic acid		< 0.1
	Monosaccharides	
Fructose		**6.0**
Fucose		1.1
Glucose		**5.7**
Xylose		0.5
	Disaccharides	
Maltose		**1.1**
Melibiose		0.3
Sucrose		**2.4**
Trehalose		**1.7**
	Sugar alcohols	
Erythritol		**3.5**
Mannitol		**27.6**
myo-Inositol		0.8
Ribitol		**3.4**
Xylitol		**23.4**
Mannitol dimer		0.4
Ribitol dimer		0.1
Ribitol-xylitol dimer		0.2
	Triterpenes	
β-Sitosterol		0.2
	Others	
Glycerol		**15.8**
Glyceric acid		0.1

4. Discussion

In ecological studies, especially in soil ecology, respiration rates are used traditionally to quantify microbial biomass (Anderson and Domsch, 1978), but rarely microbial biomass and respiration are both assessed within the same study (Del Giorgio et al., 1997). The herein introduced modification of the MicroResp™ method represents an *in vitro* method. Selected examples illustrate the sensitivity of the method. Traditionally, quantitative biological assays such as those employed in this study are evaluated by calculating the EC_{50}, the estimated dose of the test compound that is required to cause an effect differing from the control by 50%; in a quantal assay, LD_{50} denotes the concentration causing the death of 50% of the test organisms (Hadacek and Greger, 2000; Roberts and Boyce, 1972). The quantal assays merit especially mentioning because EC_{50} estimates are widely performed using the probit model that was introduced by Bliss (1935) to explore dosage–mortality relations in quantal assays. The probit model also is used in quantitative assays (Engelmeier and Hadacek, 2006; Hadacek and Greger, 2000). In this case, the fact that the number of surviving test organisms limits the response scale to 100% hampers detection of hormetic U-shaped and inverse U-shaped effects that are characterized by stimulatory effects at lower—as a rule beyond 100% control—and inhibition at higher dosages (Stebbing, 1982; Calabrese, 2005). Figs. 2b, c, and 3c illustrate that hormetic effects (orange lines) are not restricted to toxic compounds but can also be caused by ostensibly non-toxic carbohydrate substrates such as glucose. Today hormesis is recognized as phenomenon that deserves attention, not only in pharmacology (Calabrese and Baldwin, 2001), but also in ecology (Costantini et al., 2010). Models that describe inverse U-shaped hormetic dose–response effects have been developed originally to explore herbicide effects on weeds. They represent logistic models that have been extended by an additional linear function to suit the U- or inverse U-shaped hormetic dose–response curve (Brain and Cousens, 1989; Cedergreen et al., 2005).

Substrate-induced or -inhibited respiration is assessed after growth has been determined by turbidity that is used widely to quantify microbial growth in *in vitro* assays of bacteria (Lindqvist, 2006) and fungi (Engelmeier and Hadacek, 2006). The basic respiration is that of the control and, depending on the concentration and quality of the added substrate, a quantitative value is obtained that is smaller or larger than that of the control. Basically, substrate utilization results in higher growth and respiration. Growth efficiency that compares growth to the carbon assimilation rate (sum of growth and respiration rate) may, however, reveal additional aspects. For example, the growth of the fungus *F. ambrosium* increases with higher

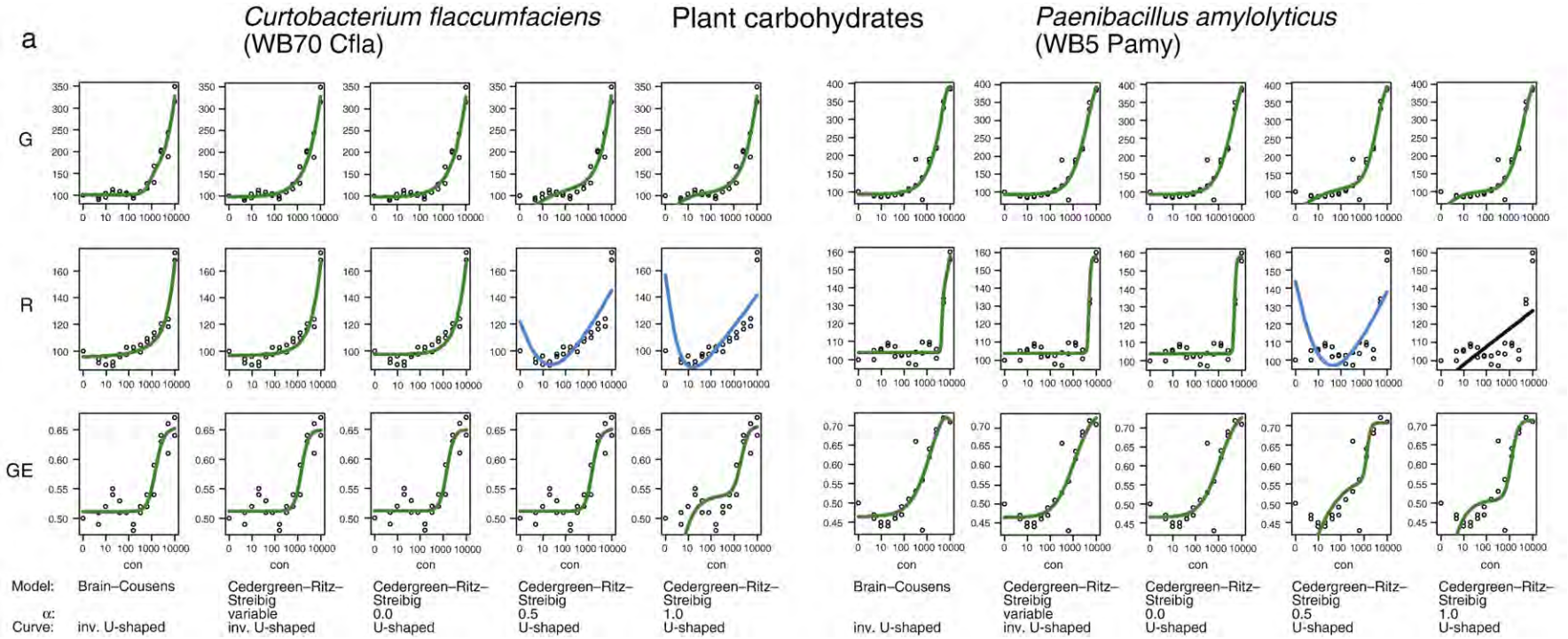


Fig. 2. Growth (G), respiration (R) and growth efficiency (GE) of exemplified bacteria; $n = 3$; Substrate utilization and antimicrobial susceptibility of a) plant carbohydrates, b) glucose, and c) juglone. Microdilutions (scales are log-transformed) in diluted (0.19 g/l) (a, b) and full LB broth (c); Scoring: G by turbidity at 600 nm after 24 h, R after additional 6 h in detection plates at 570 nm. GE = (G/G + R); for additional details, especially for models used in curve fitting, see Material and methods; colors of fitted curves: green, growth stimulation; red, inhibition; orange, inverse U-shaped hormesis; blue; U-shaped.

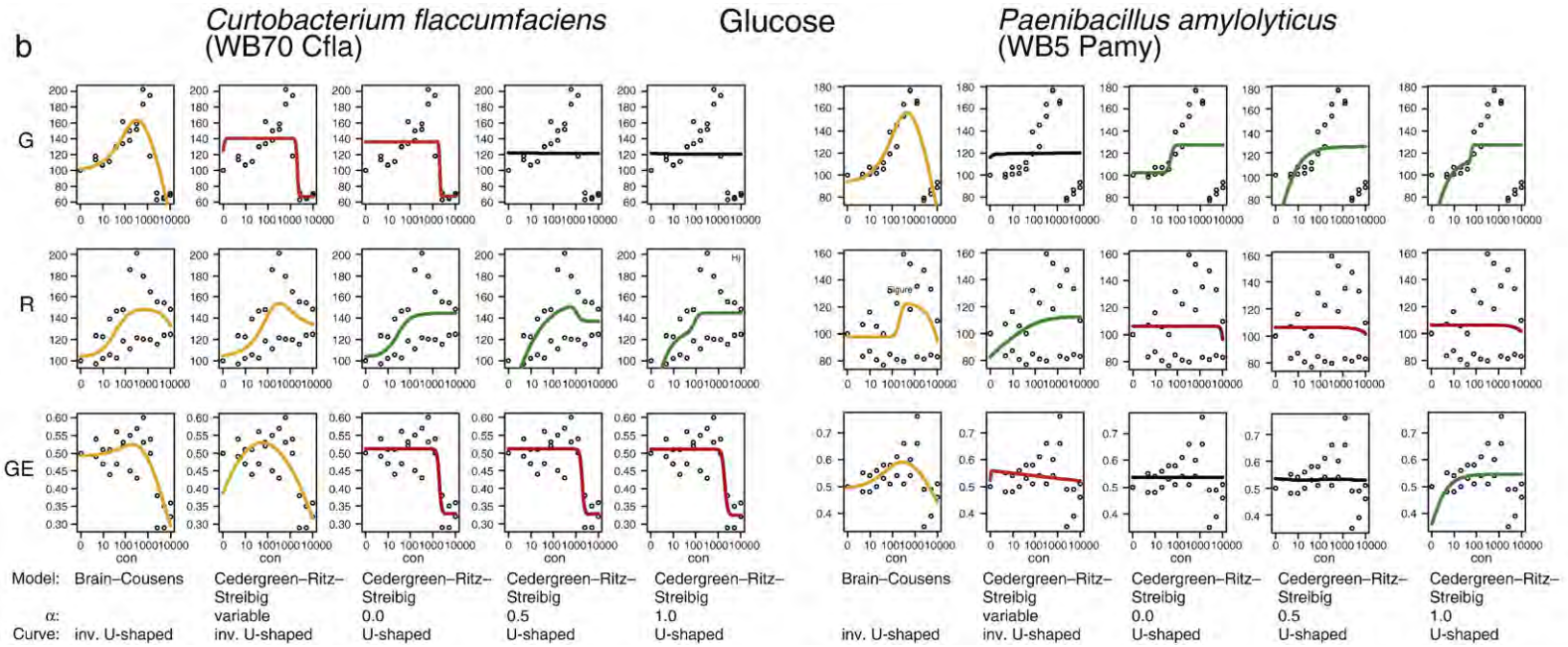
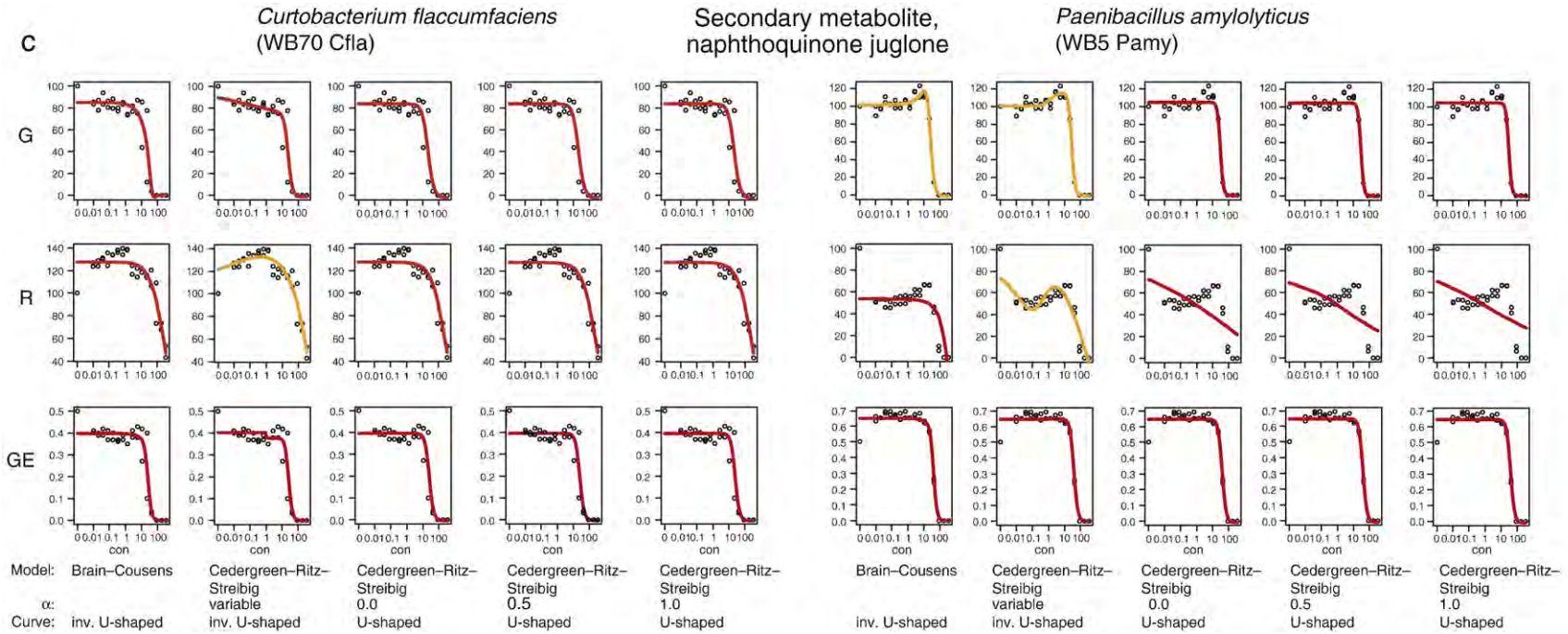


Fig. 2 (*continued*).



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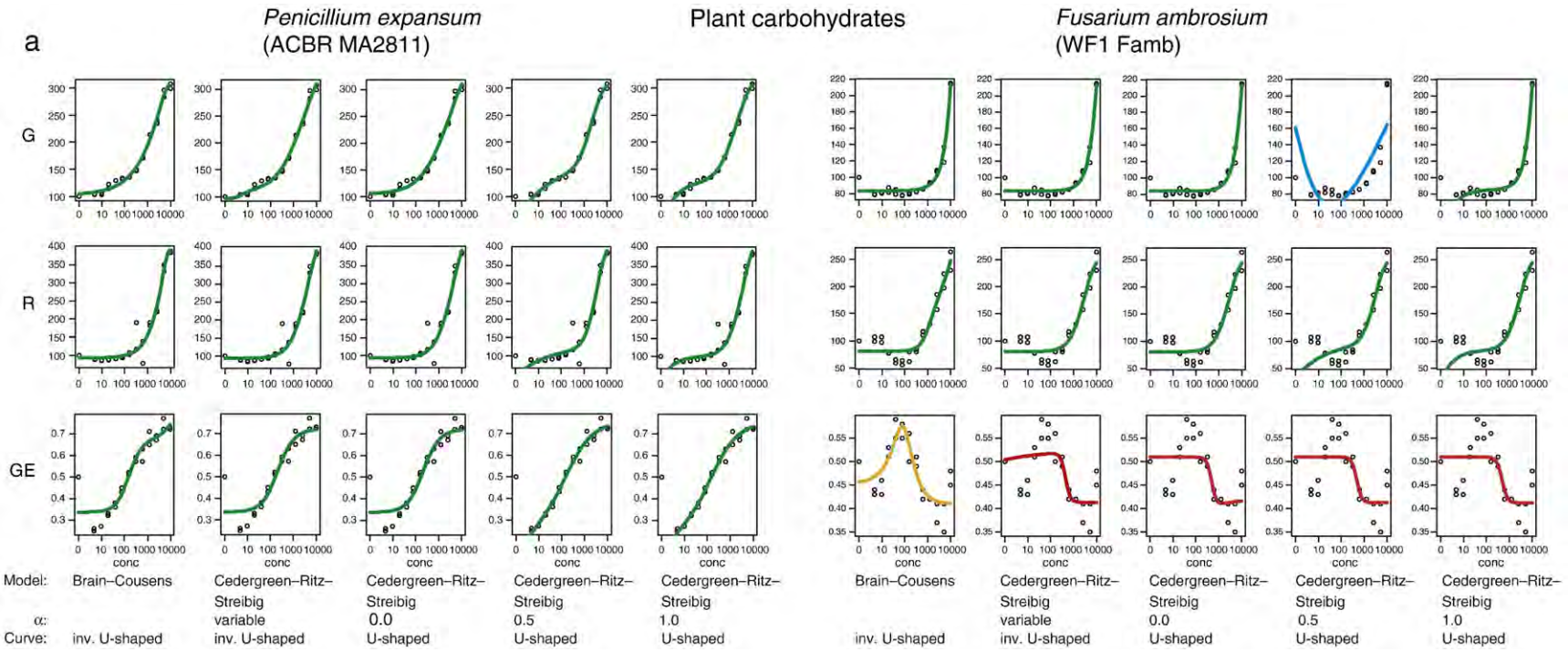


Fig. 3. Growth (G), respiration (R) and growth efficiency (GE) of exemplified fungi; $n = 3$; Substrate utilization and antimicrobial susceptibility of a) plant carbohydrates, b) glucose, and c) juglone. Microdilutions (scales are log-transformed) in MS broth (a, b) and malt extract broth (c); Scoring: G by turbidity at 600 nm after 48 h, R after additional 6 h in detection plates at 570 nm. $GE = (G/G + R)$; for additional details, especially for models used in curve fitting, see Materials and methods; colors of fitted curves: green, growth stimulation; red, inhibition; orange, inverse U-shaped hormesis; blue; U-shaped.

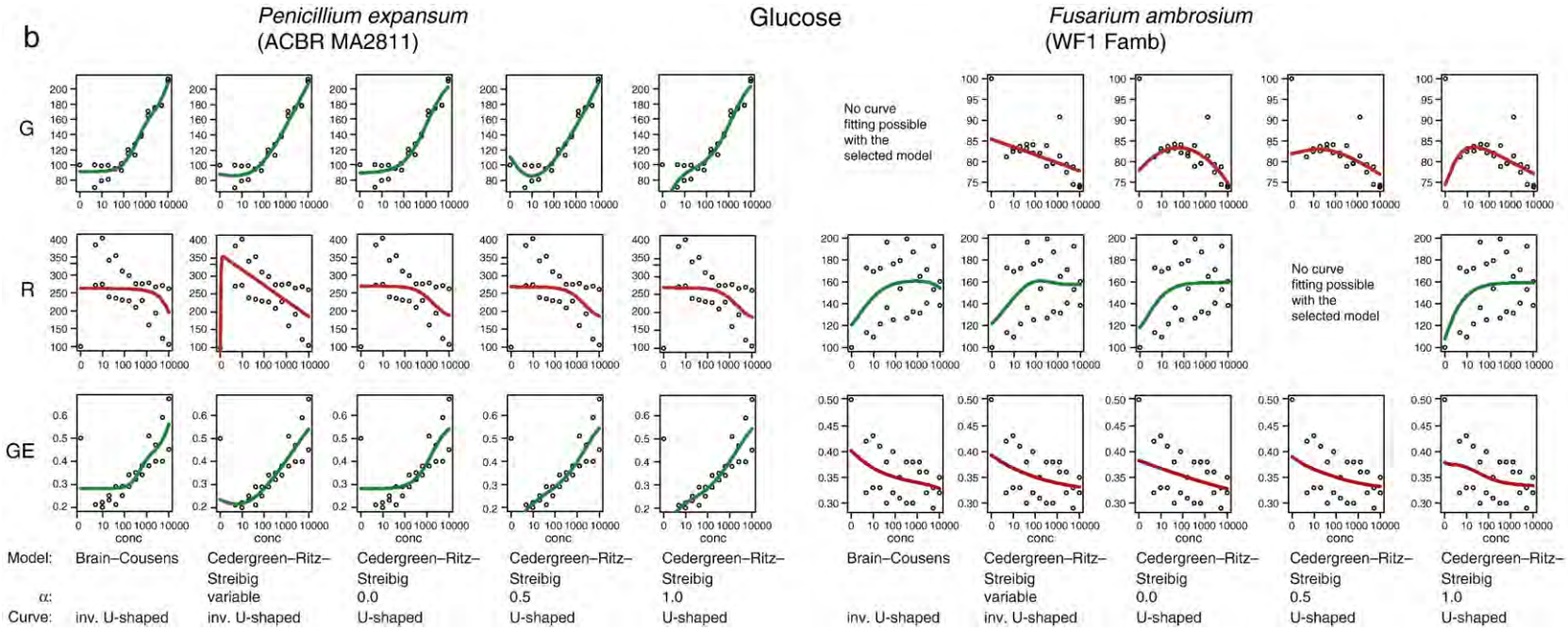
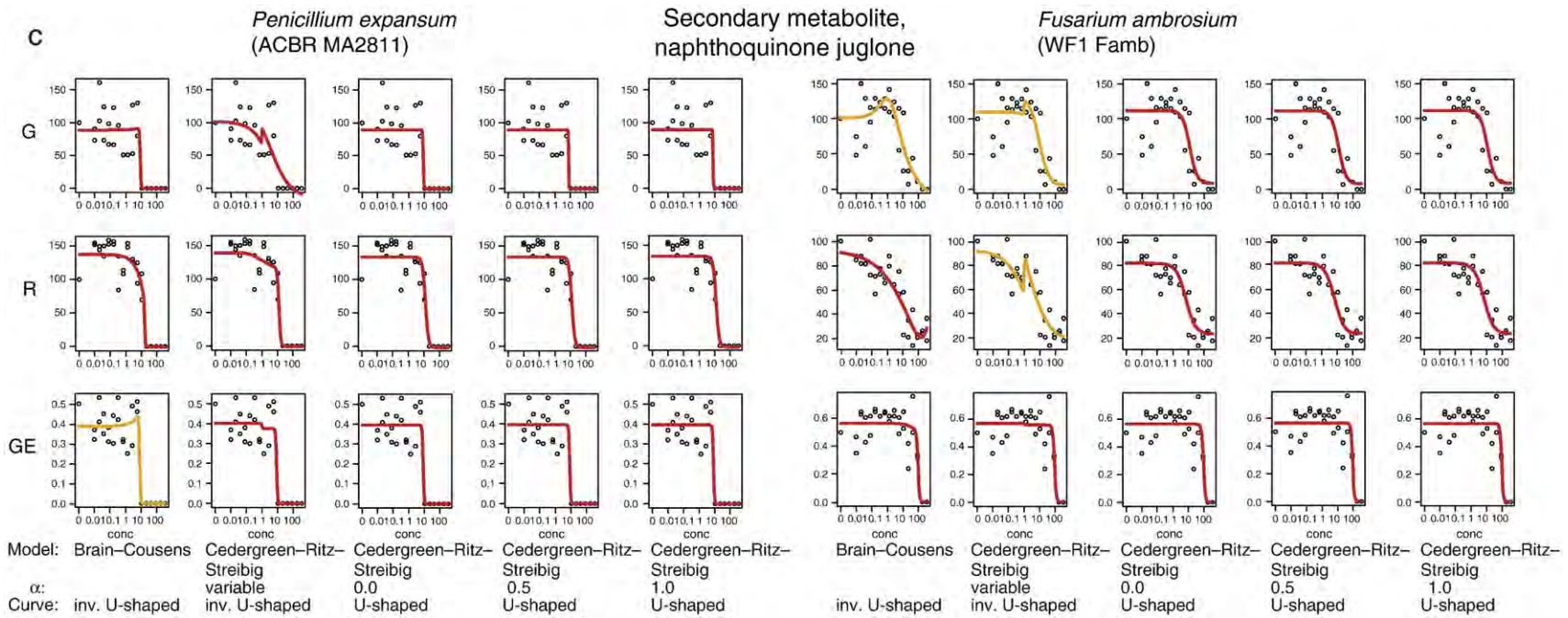


Fig. 3 (*continued*).



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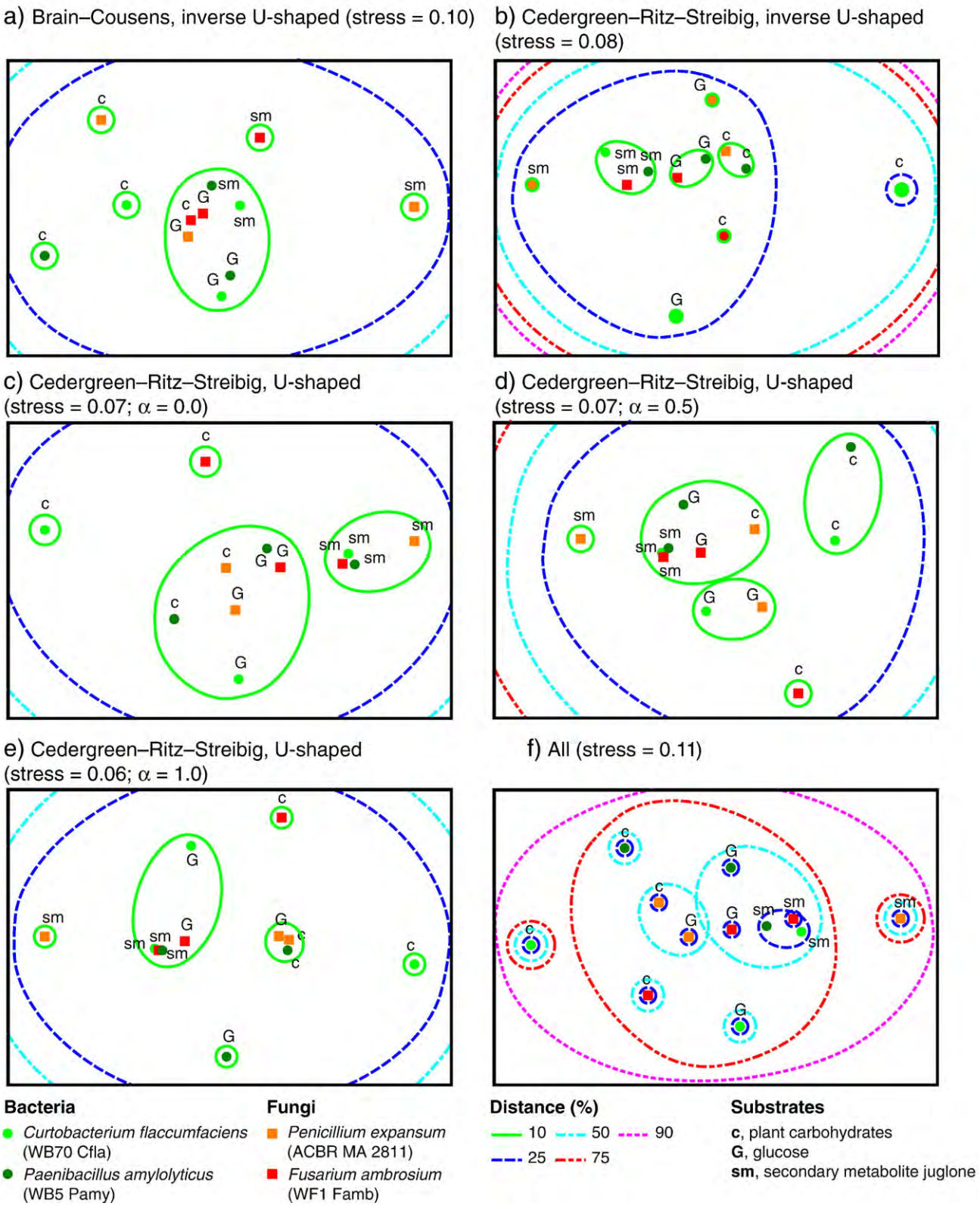

Fig. 4. Multidimensional scaling of Manhattan distance matrix of effects of plant carbohydrates, glucose, and the secondary metabolite juglone on growth of two bacterial and two fungal isolates, parameters growth (G), respiration (R), and factor growth efficiency (GE). Responses to serial dilutions were fitted to various hormetic curve models: a) Brain and Cousens (1989), b–e) Cedergreen et al. (2005); b) hormetic inverse u-shaped with variable α, hormetic u-shaped with α = 0.0 (c), α = 0.5 (d), and α = 1.0 (e); variables of fitted models were used as metavariables for distance matrix: a–e) single models; f) all combined.

dosages of plant carbohydrates and increased growth is accompanied by increased respiration (Fig. 3a). Respiration, however, increases faster than growth. This is only indicated by GE.

In plants and animals, respiration usually increases with oxidative stress (Barré et al., 1986; Tiwari et al., 2002; Bernabucci et al., 2010; Lafta and Fugate, 2011). Antibiotics can accelerate bacterial

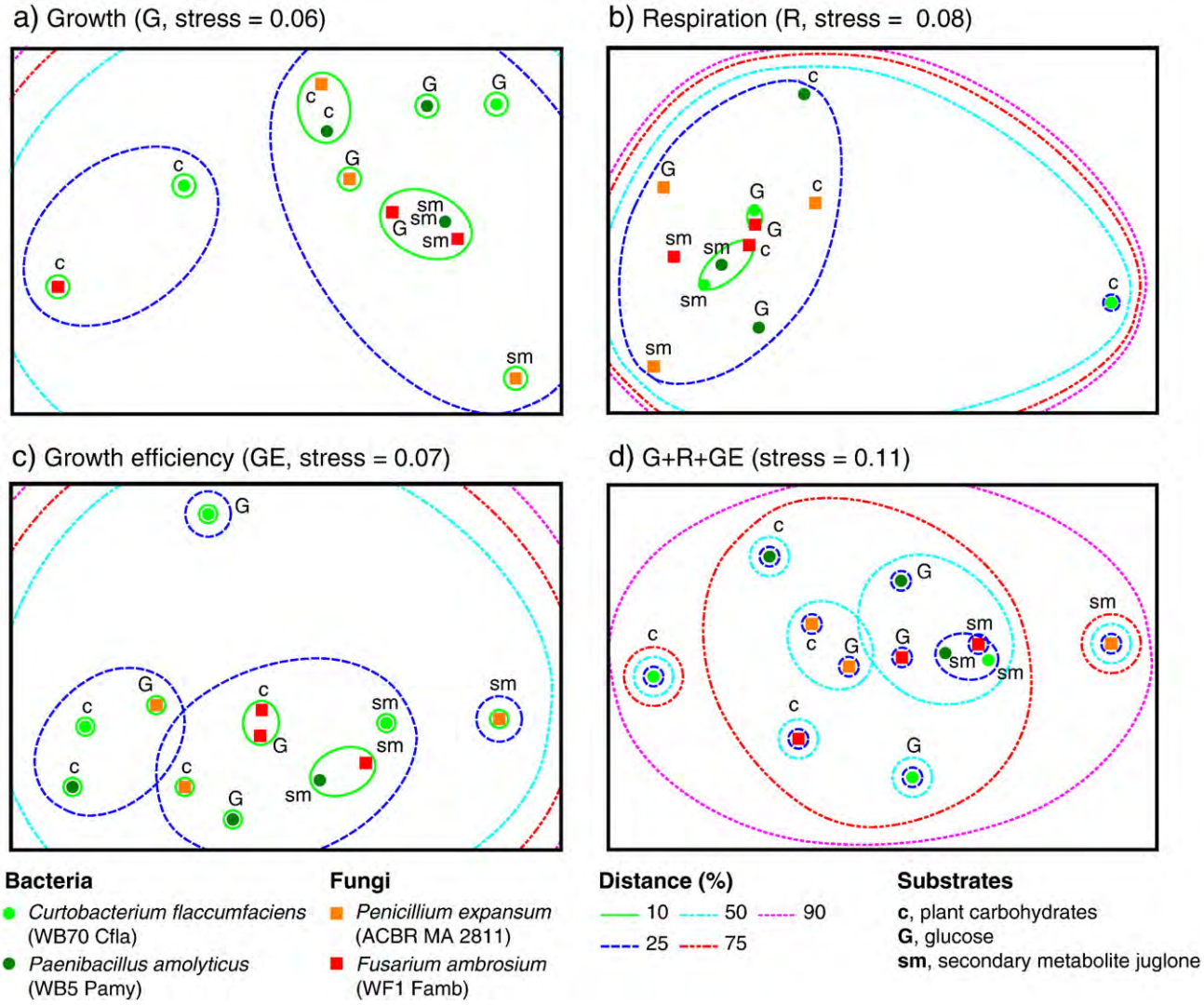

Fig. 5. Multidimensional scaling of Manhattan distance matrix of effects of plant carbohydrates, glucose, and the secondary metabolite juglone on growth of two bacterial and two fungal isolates, Responses to serial dilutions were fitted to various hormetic curve models introduced by Brain and Cousens (1989) and Cedergreen et al. (2005), for effects of the respective models on the ordination see Fig. 4; variables of fitted models were used as metavariables for distance matrix: a) growth (G), b) respiration (R), c) growth efficiency (GE), d) all three combined.

respiration by various modes of action, also including oxidative stress, that ultimately result in the hyper-activation of electron transport chains leading to the formation of superoxide anion radicals that are reduced further to the strongly oxidative hydroxyl radicals by the transition metal-catalysed Fenton reaction (Kohanski et al., 2010). Matching with this context are hormetic effects caused by toxic compounds, such as the naphthoquinone juglone, a secondary metabolite that is a well-known redox cycler similarly as the herbicide paraquat (Cocheme and Murphy, 2009). Hormetic dose–response effects are characterized by inhibitory effects at higher concentrations that are preceded by a stimulation at lower dosages (Stebbing, 1982). If reactive oxygen species, such as superoxide anion radical or hydroxyl radical, are present, juglone or its higher reduced derivatives, such as trihydroxynaphthalene, can act as scavengers by reducing them to water (Chobot and Hadacek, 2009). The juglone effects on the bacterium *P. amylolyticus* and the fungus *F. ambrosium* illustrate such hormetic effects that can be caused by the previously described mechanism (Figs. 2c and 3c), but which, however, do not appear as a rule when juglone and other redox cyclers are assayed.

The illustrated effects of various carbohydrates and juglone on the growth and respiration of the two bacterial and fungal isolates are diverse, which credits the assay but also raises questions about mechanisms causing this variability. For example, the fungus *P. expansum* is utilizing glucose as substrate as efficiently as a complex mixture of plant carbohydrates; for *F. ambrosium* and the two bacterial isolates, higher glucose concentrations reduce GE. Importing too much substrate may cause deleterious effects to the cell; some microbes, however, seem to have developed mechanisms with a feedback control that limits the uptake (Koch, 1997). Acetate, a metabolic oxidation production of glucose, was pointed out to represent a component of such a feedback control (Luli and Strohl, 1990), another might be the limitation of the respiration rate by concomitant ATP synthesis (Andersen and von Meyenburg, 1980). In this context, systems biology approaches might prove as useful tool to obtain more conclusive insights (Goelzer and Fromion, 2011).

This modified MicroResp™ method is a miniaturized assay and thus allows a certain extent of high throughput screening that demands standardized statistical procedures, best multivariate, for their evaluation. We tested various existing models fur curve fitting of the dose–response values and identified models that were developed for detecting hormetic effects in attempts to find the most suitable. The first model that was explicitly introduced for this purpose was that of Brain and Cousens (1989), followed by another one by Cedergreen et al. (2005). We used both and found them to be alternatively efficient to detect hormetic effects in growth (G), respiration (R), and even the factor growth efficiency (GE). All these models

describe inverse U-shaped dose–response effects that, sometimes, also are denoted as J-shaped. In addition, we use a model described by Cedergreen et al. (2005) that was optimized for curve fitting of U-shaped effects and includes a factor α that describes the increase rate of growth stimulation; the drawback of this model is that α is fixed and we use the conventional values 0.00, 0.50 and 1.00. In most of the cases, we obtained fitted curves that reflected the dose–response effect. The variables that were obtained from the curve fitting then were incorporated as metavariables in a multivariate analysis. A MDS plot of the Manhattan distance matrix showed that the combination of the five curve fitting models for the measured variables growth (G) and respiration (R) as well as the calculated factor GE provides a set of variables that yielded an ordination that clearly illustrated the similarities and dissimilarities of the effects of the three substrates on the two fungal and bacterial isolates (Figs. 4f and 5d), showing that the fungus *P. expansum* utilized glucose similarly efficiently as the plant carbohydrates, but was the most sensitive isolate to the secondary metabolite juglone.

The obtained results recommend further exploration of the applicability of this assay. Compared to existing antimicrobial and substrate utilization assays in the microtiter plate format, the presented MicroResp™ modification provides a hitherto unavailable combination of growth and respiration data. It not only allows the characterisation of fungal and bacterial isolates on basis of their substrate utilisation efficiency and susceptibility against toxins, but also a multivariate comparison of different chemicals or substrates in terms of effects on various microbes.

Acknowledgments

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