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The effect of elevated atmospheric CO_2 concentrations on the abundance and community composition of heterotrophic and methylotrophic bacteria in the phyllosphere of abundant plant species of the permanent grassland ecosystem

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submitted by

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Abstract

The consequences of global climate change are currently a major problem. Elevated CO_2 concentration in the atmosphere can affect plants, bacterial communities in the phyllosphere and plant-bacteria interactions. Phyllosphere bacteria play an important role in plant health and growth.

In this study, it was hypothesized that long-term elevated CO_2 concentration (17 years) affects the abundance and composition of oligo/heterotrophic and methylotrophic bacterial communities of the phyllosphere of abundant plant species (*A. elatius*, *G. album*) of the permanent grassland ecosystem of the Giessen free-air carbon dioxide enrichment (GiFACE) system:

The abundance of *Sphingomonas*, *Pseudomonas* and *Methylobacterium* spp. will increase due to the increase in plant substrate (sugar, methanol).

Increased CO_2 concentration will lead to an adaptation of oligo/heterotrophic and methylotrophic bacteria, especially to a functional adaptation.

To prove these hypotheses, oligo/heterotrophic and methylotrophic bacteria of the phyllosphere of both plant species (*A. elatius*, *G. album*) were cultivated for the first time using the dilution-to-extinction method in 96-well microtiter plates. The concentration of cultured bacteria was determined using the most probable number (MPN) method. The shift in the composition of the most enriched bacteria assemblages were presented using denaturing gradient gel electrophoreses (DGGE) and non-metric multidimensional scaling (NMDS) approaches. For analysis of a bacterial adaptation, the most abundant enriched bacteria were first isolated and then phylogenetically identified (using partial 16S rRNA gene sequence analysis). Isolates were assigned to phylotypes, and their occurrence was correlated with environmental factors (eCO_2, plants) was presented using canonical correspondence analysis (CCA). Pink-pigmented, facultative methylotrophic (PPFM) phylotypes, as abundant inhabitants of the phyllosphere, were analyzed at functional adaptation using changes of partial nucleotide sequences and especially amino acid sequences of *mxaF*-gene to find specifically adapted ecotypes.

Another aim of this study was to establish a highly efficient DNA extraction method for the phyllosphere-associated bacteria of the two plant species (*A. elatius*, *G. album*) without plant co-extracts (chloroplast and mitochondrial DNA) to enable the analysis of the phyllosphere microbiota of the two plant species using cultivation-independent methods (e.g. DGGE, next-generation-sequencing (NGS)) without co-amplification. For this

establishment, direct DNA extraction (such as cetyltrimethylammonium bromide (CTAB)-methods, various commercial kits) and alternative DNA extraction (bacterial cells harvesting before DNA extraction) methods on total fresh and frozen, frozen-homogenized, as well as freeze-dried leaves were tested. For alternative DNA extraction methods, bacterial cells were first homogenized or detached from the leaves and then collected by a centrifugation density gradient medium (Percoll, Nycodenz), centrifugation or Sterivex filtration with/without prefilter, and then DNA was extracted using kit or CTAB method. The efficiency of the DNA methods was checked on co-extracts by polymerase chain reaction (PCR) with the universal bacterial primer systems.

The results showed that long-term elevated CO_2 concentration affected the composition and abundance of the cultured oligo/heterotrophic and methylotrophic bacterial communities of the two plant species (*A. elatius*, *G. album*). The abundance of oligo/heterotrophic and methylotrophic bacteria of the two plant species was different and plant genotype specific. *Sphingomonas* and *Pseudomonas* were the most abundant genera only on *G. album* leaves and their abundance increased under elevated CO_2 concentrations. *Methylobacterium* spp. were the most abundant methylotrophs on the two plants and their abundance was not affected by elevated CO_2 concentrations. Among the PPFM phylotypes, single plant-specific isolates were found, indicating a potential functional plant-specific adaptation and potential genetic adaptation to elevated CO_2 concentrations. No highly efficient DNA extraction method without co-extracts could be established from *A. elatius* and *G. album* leaves. DNA extraction using the NucleoSpin® for Soil Kit (Macherey-Nagel) was the most efficient method and showed the best detection of extracted bacterial DNA from the *G. album* leaves in the presence of co-extracts and proved to be reliable. The kit was one of the few kits that allowed bacterial community analysis of the two plants (*A. elatus*, *G. album*) after the integrated co-extract removal step. This study was the first to demonstrate a correlation between the long-term elevated CO_2 effect and the oligo/heterotrophic and methylotrophic bacterial communities of *A. elatius* and *G. album* phyllosphere and provided the first indication for the potential functional adaptation of PPFMs to elevated CO_2 concentrations.

Zusammenfassung

Die Folgen der globalen Klimaveränderungen stellen derzeit ein großes Problem dar. Die erhöhte atmosphärische CO_2 Konzentration kann die Pflanzen, bakterielle Gemeinschaften der Phyllosphäre und die Wechselwirkungen Pflanze-Bakterien beeinflussen. Die Bakterien der Phyllosphäre spielen eine große Rolle in der Gesundheit und im Wachstum der Pflanzen.

In dieser Studie wurden folgende Hypothesen aufgestellt:

Langzeit erhöhte CO_2 Konzentrationen (17 Jahre) beeinflussen die Abundanz und Zusammensetzung der oligo/heterotrophen und methylotrophen Bakteriengemeinschaften in der Phyllosphäre der abundanten Pflanzenarten *(A. elatius*, *G. album*) des Dauergrünland Ökosystems des Giessen free-air carbon dioxide enrichment (GiFACE) Systems.

Die Abundanz von *Sphingomonas*, *Pseudomonas* und *Methylobacterium* spp. nimmt aufgrund der Zunahme von Pflanzenproduzierten Substraten (Zucker, Methanol) zu.

Erhöhte CO_2 Konzentrationen werden zur spezifischen Adaption von oligo/heterotrophen und methylotrophen Bakterien führen, insbesondere zu funktioneller Adaption.

Um diese Hypothesen zu prüfen, wurden die oligo/heterotrophen und methylotrophen Bakterien beider Pflanzenarten (*A. elatius*, *G. album*) zum ersten Mal mittels der „dilution-to-extinction“ Methode in 96-Wells-Mikrotiterplatten angereichert. Die Konzentrationen der oligo/heterotrophen Bakterien und der pink-pigmentierten fakultativen methylotrophen Bakterien (PPFM) wurden mittels most probable number (MPN) bestimmt. Die Veränderungen in der Zusammensetzung der am häufigsten auftretenden Bakterien wurde mit Hilfe der Denaturierenden Gradienten-Gelelektrophorese (DGGE)- und nicht-metrischen multidimensionalen Skalierung (NMDS)-Methoden analysiert. Zur Analyse der Anpassung der oligo/heterotrophen Bakterien und Methylotrophen wurden die am häufigsten angereicherten Bakterien isoliert und dann phylogenetisch (mit Hilfe partieller 16S rRNA-Gensequenzanalyse) identifiziert. Die Isolate wurden in Phylotypen eingeteilt und eine Korrelation mit den Umweltfaktoren (eCO_2, Pflanzen) mittels kanonischen Korrespondenzanalyse (CCA) dargestellt. PPFM-Phylotypen als häufige Bewohner der Phyllosphäre, wurden auf eine mögliche funktionelle Anpassung analysiert, indem Änderungen der partiellen Nukleotidsequenzen und insbesondere der

Aminosäuresequenzen des Methanoldehydrogenase Gens (*mxaF*-Gens) verwendet wurden, um spezifisch angepasste Ökotypen zu finden.

Ein weiteres Ziel dieser Studie war es, eine hoch effiziente DNA-Extraktionsmethode für die Phyllosphäre-assoziierten Bakterien der beiden Pflanzenarten (*A. elatius*, *G. album*) ohne pflanzlichen Co-Extrakte (Chloroplasten und Mitochondrien DNA) zu etablieren, um die Analyse der Phyllosphäre- Mikrobiota der beiden Pflanzenarten mit kultivierungsunabhängigen Methoden (z.B. DGGE, Sequenzierung der nächsten Generation (NGS)) ohne Co-Amplifikation zu ermöglichen. Dafür wurden die direkten DNA-Extraktionen (wie Cetyltrimethylammoniumbromid (CTAB)-Methoden, verschiedene kommerzielle Kits) und alternative DNA-Extraktionsmethoden (mit Ernte bakterieller Zellen vor DNA-Extraktion) mit verschiedenen Blattmaterialien (frischen, gefroren, gefrorenen-homogenisierten sowie gefriergetrockneten Blättern) getestet. Für den alternativen Methoden wurden die bakteriellen Zellen vor der DNA-Extraktion zuerst von Blättern abgelöst oder homogenisiert, dann entweder mittels Zentrifugationsmedien (Percoll, Nycodenz) oder Zentrifugation, Sterivex-Filtration mit/ohne Vorfilter gesammelt und anschließend wurde die DNA mittels Kit- oder CTAB-Methode extrahiert. Die Effizienz der DNA-Methoden wurde auf Co-Extrakte mittels der Polymerase-Kettenreaktion (PCR) mit den universellen bakteriellen Primersystemen überprüft.

Die Ergebnisse zeigten, dass die Langzeit-erhöhten CO_2 Konzentrationen die Zusammensetzung und Abundanz der kultivierten Oligo/Heterotrophen und der methylotrophen Bakteriengemeinschaften der beiden Pflanzenarten (*A. elatius*, *G. album*) beeinflussten. Die Abundanz der oligo/heterotrophen und methylotrophen Bakterien der beiden Pflanzenarten war unterschiedlich und Pflanzengenotyp-spezifisch. *Sphingomonas* und *Pseudomonas* waren die abundantesten Gattungen auf den Blättern *von G. album* und ihre Abundanz nahm dort unter erhöhter CO_2 Konzentrationen zu. *Methylobacterium* spp. waren die abundantesten Methylotrophen auf den beiden Pflanzen und ihre Abundanz wurde von erhöhten CO_2 Konzentrationen nicht beeinflusst. Dafür wurden einzelne pflanzen-spezifische Isolate unter PPFM-Phylotypen gefunden, die auf eine potenzielle funktionelle pflanzen-spezifische Anpassung und eine potenzielle genetische Anpassung an die erhöhten CO_2 Konzentrationen hindeuten.

Es konnte keine hocheffiziente DNA-Extraktionsmethode ohne Co-Extrakten aus *A. elatius* und *G. album* Blättern etabliert werden. Die DNA-Extraktion mit dem NucleoSpin® for Soil Kit (Macherey-Nagel) war die effizienteste Methode und zeigte den besten Nachweis

extrahierter bakteriellen DNAs aus den *G. album* Blättern in der Gegenwart von Co-Extrakten und erwies sich als zuverlässig. Dieser Kit war einer der wenigsten Kits, mit dem die Bakteriengemeinschaftsanalyse der beiden Pflanzen (*A. elatius*, *G. album*) nach dem integrierten Schritt zur Entfernung von Co-Extrakte möglich war. Diese Studie ist die erste, die eine Korrelation zwischen dem langfristigen eCO_2-Effekt von 17 Jahren und den oligo/heterotrophen und methylotrophen Bakteriengemeinschaften der *A. elatius* und *G. album* Phyllosphäre demonstrierte und liefert einen ersten Hinweis auf die potenzielle funktionelle Anpassung von PPFMs.

Content

Abbreviations

% (v/v)	volume in percent
% (w/v)	weight/volume in percent
&	and
µl	microliter
µm	micrometer
µM	micromol
16S rRNA gene	16 subunit ribosomal ribonucleic acid gene
1^{st}	first
2D	bidimensional
2^{nd}	second
3D	tridimensional
8f, 1003f	forward primer
907r, 1942r	reverse primer
A	*Arrhenatherum elatius*
a.m.	ante meridiem
AC	*Arrhenatherum elatius* from elevated carbon dioxide ring
ACC	1-aminocyclopropane-1-carboxylic acid
aCO_2	ambient atmospheric carbon dioxide
eCO_2	elevated carbon dioxide
ACT	*Arrhenatherum elatius* from control block and from elevated carbon dioxide ring
A.elatius	*Arrhenatherum elatius*
AK	*Arrhenatherum elatius* from control block
ALF968	*Alphaproteobacteria* 968
ARB	arbor (the Latin word for tree) software
ARISA	automated ribosomal intergenic spacer analysis
aT	ambient surface temperature
AU	absorbance units
bacteria/cm^2	bacteria per square centimeter
BET42a	*Betaproteobacteria 42a*
BLAST	basic local alignment search tool
BLASTN	basic local alignment search tool for nucleotide
BOX-PCR	repetitive extragenic palindromic-PCR based on BOXA1R
bp	base pair
BSA	bovine serum albumin
BSFL	black soldier fly (*Hermetia illucens*) larvae gut bacteria
C_3	three-carbon compounds
C_4	four-carbon compounds
CARD-FISH	catalyzed reported deposition-fluorescence *in-situ* hybridization

CCA	canonical correspondence analysis
CF319a	*Cytophaga-Flavobacterium 319a*
CFU	colony-forming unit
CLSM	confocal laser scanning microscopy
ClustalW	clustering with alignments and weighting
cm^2	square centimeter
CO_2	carbon dioxide
CTAB	cetyltrimethylammonium bromide
Cy3 or Cy5	3´ or 5`-end labeled indocarbocyanine
DAPI	4´,6-diamidino-2-phenylindol
ddGTPs	didesoxyguanintriphosphat
ddNTP	didesoxyNTPs, stop nucleotide
DES	DNase/Pyrogen-Free water
DGGE	denaturing gradient gel electrophoreses
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
dsDNA	double-stranded deoxyribonucleic acid
DSM 29344^T	type strain of German collection of microorganisms and cell cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen)
Prof. Dr.	Professor, Doktor der Naturwissenschaften (engl. Professor, Doctor of natural sciences)
Prof. Dr. Dr.-Ing.	Professor, Doktor der Naturwissenschaften (engl. Professor, Doctor of natural sciences) und Doktor der Ingenieurswissenschaften (engl. Doctor of engineering)
E786f	Eubacteria 786 forward primer
e.g.	in example
eCO_2	elevated carbon dioxide
EDTA	ethylenedinitrilotetraacetic acid disodium salt dihydrate
EPS	extracellular polysaccharides
eT	elevated surface temperature +2°C
et al.	"et alia" (Eng. and others)
EUB338 I, II, III	Eubacteria 338 I, II, III
FACE	Free-Air Carbon dioxide Enrichment system
FACE2FACE	FACE to FACE
FAO	food and agriculture organization of the United Nations
Fig	figure
FISH	fluorescence in-situ hybridization
FISH-CLSM	fluorescence in-situ hybridization with confocal laser scanning microscopy
G	*Galium album*
G. album	*Galium album*

GAM42a	*Gammaproteobacteria 42a*
GC	*Galium album* from elevated carbon dioxide ring
GC-968/1378R	forward and reverse primer with GC-clamp (guanine and cytosine bases)
GCT	*Galium album* from control block and from elevated carbon dioxide ring
GES buffer	guanidium thiocyanate buffer
GFP	green fluorescent protein
gfp-gene	gene for green fluorescent protein
GiFACE	Giessen free-air carbon dioxide enrichment
GK	*Galium album* from control block
H	height
h	hour
HGC69a	high G+C Gram-positive bacteria (specific for *Actinobacteria*)
IAA	indoe-3-acetic acid
IFZ	Interdisziplinäre Forschungszentrum (engl. interdisciplinary research center)
IPCC	Intergovernmental Panel on Climate Change
IR	infrared
IRT	inhibitor removal technology
JTT matrix-based method	Jones-Thornton matrix-based method
kg N ha^{-1} year	kilogram nitrogen per hectare year
Km/h	kilometers per hour
km^2	square kilometers
leaf FW	leaf fresh weight
LGC354MIX	low G+C Gram-positive bacteria (specific for *Firmicutes*)
LTP	"all species living tree " project
m	meter
M	mol
M125	mineral salt medium (M125) supplemented with 0.5% methanol (v/v)
MEGA7	Molecular Evolutionary Genetics Analysis version 7
mg	milligram
mg/l	milligram per milliliters
min	minute
ml	milliliters
MN	Macherey-Nagel
mod	modification
MPN	most probable number
mxaF-Gen	Methanoldehydrogenase funktionales Gen (deu.)
mxaF-gene	methanol dehydrogenase functional gene (eng.)

mybm-1388	*Methylobacterium* -1388
N_2	nitrogen gas
NaCl	sodium chloride
NBCS	newborn calf serum
ng	nano gram or 10^{-9} gram
ng/µl	nano gram per microliter
NGS	next-generation-sequencing
nm	nanometer
NMDS	non-metric multidimensional scaling
NMR	nuclear magnetic resonance
nt	nucleotide
OH group	hydroxyl group (oxygen and hydrogen atom)
One-way ANOSIM	one-way analysis of similarities
p.m.	post meridiem
PAST software	paleontological statistics software
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG 6000	polyethylene glycol
pH	the potential of hydrogen
PPFM	pink-pigmented, facultative *Methylobacterium*
PSE227	*Pseudomonas* 227
p-value	probability value
R2A	Reasoner's 2A agar
rcf	relative centrifugal force
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RT	room temperature
s	seconds
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SPB	sodium phosphate buffer
spp.	species (plural)
SSCP	single-strand conformation polymorphism
Pseudomonas syringae pv. *syringae* B728a	*Pseudomonas syringae* pathovar *syringae* B728a
Quercus ilex L. leaves	*Quercus ilex* Linnaeus (described by Carl Linnaeus) leaves
Tab	table
TAE buffer	tris base, acetic acid and EDTA buffer

TAPE-FISH	combination of adhesive tape used for sample collection from surface with fluorescence in-situ hybridization
TBE buffer	tris -borate-EDTA buffer
TGGE	temperature gradient gel electrophoresis
TGS	third generation sequencing
t-RFLP	terminal restriction fragment length polymorphism
Tris-Cl	tris-hydrochloride
TS medium	tryptic soy medium
UPGMA	unweighted pair group method arithmetic average
U.S. or USA	United States of America
UV light	ultraviolet light
V	volume
V/cm	volt per centimeter
V3-V4	hypervariable regions of 16S rRNA gene
VBNC-bacteria	viable but not cultivable bacteria
W	width
W/m^2	watts per square meter
w/o	with/without
ZR	Zymo Research

1 Introduction

Global climate changes caused by increased atmospheric CO_2 concentration (IPCC, 2007) can affect plants, bacterial communities colonizing the total aboveground surface of plants (phyllosphere) (Vorholt, 2012) and the interactions of plants and phyllosphere bacteria. The CO_2 concentration has increased significantly, and it is believed that it will continue to rise faster than expected (Dyson, 2005, Canadell et al., 2007, Hanson et al., 2008). There are many free-air carbon dioxide enrichment (FACE) studies that have already shown the response of elevated CO_2 to plant growth (Jongen et al., 1995, Ainsworth&Rogers, 2007, Jones et al., 2014, Ainsworth& Long, 2021), on the microbiome of the rhizosphere (Wang et al., 2017, Yin et al., 2018, Bei et al., 2019) and even less on the microbiome of the phyllosphere (Ren et al., 2014).

Currently, there are no conducted studies regarding the long-term (17 years) effects of elevated CO_2 on the oligo/heterotrophic and methylotrophic bacteria on the leaves of *Arrhenatherum elatius* and *Galium album* using culture-dependent and culture-independent methods. In addition, there is no highly efficient DNA extraction method for phyllosphere-associated bacteria without co-extracts (chloroplast and mitochondrial DNA), which are especially problematic during the 16S rRNA gene amplicon sequencing based analysis of microbial community composition. The microorganisms of the phyllosphere are of great importance for the growth and health of plants (Lindow& Leveau, 2002, Lindow&Brandl, 2003, Vorholt, 2012, Rastogi et al., 2013, Koskella, 2020).

1.1 Free air carbon-dioxide enrichment (FACE) systems

The FACE system is a technically unique method to study the effect of increased CO_2 on the plant´s physiology and its ecosystem ecology under natural environmental conditions (Mcleod&Lang, 1999, Ainsworth&Long, 2005). FACE systems have been used worldwide (the U.S., Italy, Japan, Germany, China, India, Australia and Brazil) to study different crops (cotton, wheat, winter wheat, sorghum, potato, grape, rice, sugar beet, corn, soybean, cassava, rapeseed, mung bean, mustard, peanut, chickpea, field pea, faba bean, lentil and coffee) (Kimball, 1983, Long et al., 2004, Dong et al., 2018, Ainsworth&Long, 2020, Wang&Liu, 2021, Mollah&Fitzgerald, 2022, Gamez et al., 2023) as well as different grasslands (e.g. Giessen-FACE system in Germany (Jäger et al., 2003), in the U.S. (Dukes et al., 2005), in New Zealand (Newton et al., 2014)). The plant microbial communities in different plant species have been studied in the soil (Hayden et al., 2012, Qiu et al., 2023), in the rhizosphere (Runion et al., 1994, Schortemeyer et al., 1996, Montealegre et al., 2002, Xie et al., 2005), in the endosphere

(Christian et al., 2021), as well as in the phyllosphere (Runion et al., 1994, Müller et al., 2006, Ren et al., 2014), because the microbial communities are vital for the plants, either as plant growth promoters or as pathogen or antagonists (Antoun&Prevost, 2006; Spaepen et al., 2007, Glick et al., 2007, Dawar et al., 2010). For the first time, the response to eCO_2 was tested in a field with cotton (*Gossypium hirsutum*) in the FACE system with a ring diameter of 2 m, which has been developed in the year 1990 by Brookhaven National Laboratory (USA, New York) (Hendrey et al., 1999). The ring diameters of FACE systems varied between 0.2 and 50 m (McLeod&Long, 1999; Miglietta et al., 1996, 1997, 2001, Hendrey, 1999). FACE technology has improved over time (Okada et al., 2001) and is now at a sophisticated standard, but there is still room for improvement (Jäger et al., 2003). FACE systems have also been used in combination with other factors such as temperature (Lukac et al., 2010, Ren et al., 2015, Weigel, 2005, Vu et al., 1997), drought (Yuhui et al 2017, Yang et al., 2023, Abdelhakim et al., 2022), nitrogen (Ren et al., 2014) to better study global climate change conditions. One of the oldest running long-term FACE in Europe (Jäger et al., 2003) is the Giessen-FACE (GiFACE) system in the „Environmental Monitoring and Climate Impact Research Station Linden" in Linden, near Giessen (Germany) which has been in operation since 1998. In the GiFACE system, a new technique (PlumeX) which is based on the classical FACE technique was used to fumigate the permanent grassland, which is dominated by the grass species *Arrhenatherum elatius*, forb *Galium album* (Jäger et al., 2003). The permanent grassland makes up about 13% of the agricultural area in Germany and is an important habitat for animals and plants.

1.2 Effects of elevated CO_2 on plants and phyllosphere-associated bacterial communities

Elevated CO_2 leads to an increased rate of photosynthesis through plant-leaves (Ainsworth&Rogers 2007, Xu et al., 2013). Thereby, elevated CO_2 can affect photosynthesis differently, depending on the species of plant, C_3 (directly) and C_4 (non-directly) (Drake et al., 1997, Seneweera&Conroy, 2005, Ainsworth&Rogers 2007). In addition, elevated CO_2 also affects stomata (Xu et al., 2016) and leads to decrease of stomatal density (Lin et al., 2001, Teng et al., 2009), stomatal conductance (Medlyn et al., 2001, Ainsworth&Rogers 2007, Gao et al., 2015) and leaf transpiration (Teng et al., 2009, Katul et al., 2010). Plant physiology can be indirectly affected by elevated CO_2. The plant growth under elevated CO_2 concentration led to increased and faster photosynthesis, resulting in more biomass production (Ainsworth&Long 2005) and therefore a higher harvest yield of crops (Long et al. 2004, Ainsworth&Long, 2021, Fitzgerald et al., 2022). The elevated CO_2 also affects the chemical

composition of plant tissue. The increased photosynthesis leads to increased nonstructural soluble carbohydrate (sugar, starch) in leaves (Ainsworth&Long 2005, Watanabe et al., 2014, Dong et al., 2018), which improves plant tolerance to environmental stress and raises defense capabilities against pathogenic bacteria (Ehness et al., 1997, Roitsch et al., 2003, Bolton, 2009, Drake et al., 2011, Huang&Xu, 2015). Moreover, carbohydrates, amino acids and passively leaked C-sources like organic acids by plants can use leaf-associated oligotrophic bacteria (Mercier&Lindow, 2000, Trouvelot et a., 2014), which live in habitats with a low level of nutrients (Leveau&Lindow, 2001, Delmotte, 2009, Lindow&Brandl, 2003). The effect of increased CO_2 concentration on bacterial communities in the phyllosphere of rice plant species have already been reported in the study by Ren et al. (2014) and Ikeda et al. (2015). Ren et al. (2014) showed shifts in the structure, composition, diversity and abundance of rice-associated bacteria by increased CO_2 concentration. Ikeda et al. (2015) showed that the blade-associated bacteria of rice were more sensitive to the eCO_2 effect than sheath-associated bacteria and that the diversity of symbiotic bacteria depends more on the sugar content of the plant rather than on other environmental factors. A bacterial adaptation to long-term changes of environmental conditions could be linked to the establishment of specifically adapted plant ecotypes (Cohan, 2002).

1.3 Phyllosphere and their oligotrophic bacterial abundance

The phyllosphere is the whole above-ground area of the plant that is dominated by leaves (Vorholt, 2012) and Bacteria is the highest inhabitant of leaves (Lindow&Brandl, 2003). The leaf surface from below and above is twice as large as the land surface and is approximately 1 017 260 200 km^2 (Woodward et al. 2004). This concludes an estimated average, of 10^6 to 10^7 bacteria/cm^2 of leaf area (Lindow&Brandl, 2003). This accounts for up to 10^{26} bacterial cells on the global phyllosphere (Vorholt, 2012). The phyllosphere is a habitat for epiphytic bacteria which colonizes the plants surface and they have adapted to extreme environmental conditions such as UV light, the diurnal cycle, rain, temperature, drought, geographical location, as well as seasons (Whipps et al., 2008, Knief et al., 2011, Wellner et al., 2011, Rastogi et al., 2012,Copeland et al., 2015, Ding&Melchner, 2016). In addition, the hydrophobic wax-coated cuticle (Vorholt 2012) is a habitat for oligotrophic bacteria that is adapted to an environment with a low concentration of nutrients (e.g., carbon and nitrogen) (Bringel&Couee 2015). Nutrients are not evenly distributed on the leaf´s surface, so microorganisms colonize where organic carbon sources are present (Leveau, 2006, 2009, Vorholt, 2012;

Beattie&Lindow, 1999, Lindow&Brandl, 2003). Most of these microorganisms produce extracellular polysaccharides (EPS), which provide protection against desiccation and from osmotic stress, through the intracellular accumulation of proline, choline and soluble sugars (Chang et al., 2007, Lindow&Brandl, 2003, Monier&Lindow, 2003). The most abundant phyllosphere-associated bacteria on various plants that use sugars are oligo/heterotrophs and belong to the genera *Sphingomonas* and *Pseudomonas* (Delmotte et al., 2009, Yashiro et al., 2012, Bodenhausen et al., 2013, Glenn et al., 2015, Vorholt, 2012). Furthermore, a prominent carbon source for phyllosphere–associated bacteria is methanol, which is produced as a bi-product of pectin methylesterase in the cell walls (Fall&Benson, 1996, Vorholt, 2012, Delmotte et al., 2009). Methanol is released in diurnal cycles through stomata during transpiration (Fall&Benson, 1996; Vorholt, 2012) and serves as an ecological niche for pink-pigmented, facultative *Methylobacterium* (PPFMs) (Fall&Benson, 1996, Delmotte et al., 2009, Yashiro&McManus, 2011, Bodenhausen et al., 2013, Glenn et al., 2015; Vorholt, 2012). Leaf-associated microorganisms can affect the health of the host plant in different ways, beneficial, neutral, or unfavorable (Bais et al., 2006). Abundant phyllosphere-associated bacteria (PPFMs, *Sphingomonas* spp.) can also promote plant growth and health by producing plant hormones like indoe-3-acetic acid (IAA, also known as auxin) and cytokinin (Berg, 2009, Schauer&Kutschera, 2011, Vorholt, 2012, Ismail&Mohammed, 2023) as well as vitamin B12 (Toraya et al., 1975, Basile et al., 1985, Ivanova et al., 2006). Another way, that plants can benefit from phyllospheric bacteria (*Methylobacterium* and *Sphingomonas* spp.) to protect the plants from pathogens (Innerebner et al., 2011, Duran et al., 2021, Joel et al., 2023). In addition to the synthesis of phytohormones, there are other strategies such as nitrogen fixation, phosphate solubilization, induction of defense responses, the decrease of abiotic stress etc., which promotes the plant´s health and growth (Compant et al., 2016). However, *Pseudomonas* can exist as either pathogenic or non-pathogenic bacteria in the phyllosphere, depending on whether they are in the epiphytic (on leaves), non-pathogenic phase, or the endophytic (inside leaves), pathogenic phase (Sohrabi et al., 2023). As an example, the pathogenic *Pseudomonas syringae* pv. *syringae* B728a was observed in epiphytic microbiota of bean leaves with beneficial properties such as motility, chemotaxis and promoting defense mechanisms (Yu et al., 2013), but the endosphere revealed a higher expression of phytotoxic genes. The plant-associated *Pseudomonas* can both be pathogens with pathogen virulence gene expression (Von Bodman et al., 2003, Maignien et al., 2014) and quorum quenching

bacteria that disrupt quorum sensing signals and protect plants from pathogens (Ma et al., 2013).

1.4 Overview of methods used to study phyllosphere microbiota

The phyllosphere is the largest habitat for pathogenic and beneficial microbiota (Innerebner et al., 2011, Knief et al., 2011), which plays an important role in agriculture and the environment because they can influence plant health and growth (Lugtenberg et al., 2002, Lindow&Brandl, 2003, Rasche et al., 2006, Delmotte et al., 2009, Zhou et al., 2011, Innerebner et al., 2011, Knief et al., 2011, Ali et al., 2012, Bulgarelli et al., 2013). The bacterial communities of the phyllosphere are diverse and can vary significantly in size among different plant species and even within the same species over short periods (Hirano&Upper, 1989). The microbiota is dynamic and depends on biotic and abiotic factors (Knief et al., 2010, Redford et al., 2010, Wellner et al., 2011, Horton et al., 2014). The diversity and properties of the phyllosphere microbiota and its interactions with biotic and abiotic factors are of great interest (Krimm, 2005). Over the last 30 years, visualization methods, cultivation-dependent and cultivation-independent methods or the combination of these methods have been used to contribute to new insights into the phyllosphere microbiota.

1.4.1 Microscopy-visualization of phyllosphere-associated bacteria

Phyllosphere-associated bacteria can be visualized by using different methods directly on the host or after isolating the bacteria from the phyllosphere. Bacterial investigation with single-cell resolution on the leaves is mainly performed using fluorescence microscopy (Remus-Emsermann&Schlechter, 2018). For fluorescence microscopy, the bacteria are specifically stained with organic fluorophores using various techniques. The most popular technique for staining bacteria in a habitat is “fluorescence in situ hybridization” (FISH), which is based on fluorescent labeled oligonucleotide probes that to bind a specific complementary sequence (species to phyla level) to the 16S ribosomal RNA in bacteria. FISH enables localization, visualization, identification and characterization of phyllosphere-colonizing bacteria in environmental samples (Amann&Fuchs, 2008) with the combination of epifluorescence microscopy (Morris et al., 1997) and confocal laser scanning microscopy (Morris et al., 1997, Pawley, 2006, Piccolo et al., 2010, Hofmann, 2014, Peredo&Simmons, 2018). For identification purposes, universal probes are employed to detect *Bacteria* in general (EUB338 I, II and III, Amann et al., 1990, Daims et al., 1999), and specific probes can be used to detect taxa of

interest as *Methylobacterium* (mybm-1388, Pirttilä et al., 2000) or *Pseudomonas* (PSE227, Watt et al., 2006). There are several probes available to stain dominant bacterial classes, such as *Alphaproteobacteria* (ALF968, Neef, 1997), *Betaproteobacteria* (BET42a, Behrens et al., 2004), *Gammaproteobacteria* (GAM42a, Manz et al., 1992), *Actinobacteria* (HGC69a, Roller et al., 1994), *Bacteroidetes* (CFB560, O´Sullivan et al., 2002 and CF319a, Manz et al., 1996) and *Firmicutes* (LGC354MIX, Meier, 1999). The probes are often marked with Rhodamine Red or Alexa-488, 647, Cy3 or Cy5 (3´ or 5´-end indocarbocyanine dyes). This method fails when staining bacteria that have a poor cell membrane permeability and a low content of 16S rRNAs or an inaccessible hybridization site for oligonucleotide probes (Amann&Fuchs, 2008). Remus-Emsermann et al. (2014) reported such failure in hybridization with probes (for *Alphaproteobacteria* and most bacteria) labeled with Cy3 and Cy5 in the cells of some *Methylobacterium* strains. The investigation of leaf-associated bacteria using fluorescence microscopy is further complicated by the highly uneven surface and distribution of the leaf, their autofluorescence (e.g. members of the genus *Pseudomonas*) and the autofluorescence of leaf components (e.g. chlorophyll, chloroplast, mitochondria) (Karabourniotis, 2001, Remus-Emsermann&Schlechter, 2018). Many of these problems have been solved with methods such as CARD-FISH and TAPE-FISH. CARD-FISH (catalyzed reported deposition-fluorescence in-situ hybridization) is an improved version of FISH that produces a 50x stronger signal through an enzymatic reaction (Schönhuber et al., 1997, Pernthaler et al., 2002). This even allows cells with low ribosome numbers and low physiological activity to be visualized (Matturro et al., 2021). With TAPE-FISH method, plant autofluorescence could be circumvented by transferring epiphytic bacterial communities from leaf surfaces to adhesive tape without spatial changes (Bish&Brehm-Stecher, 2009, 2010; Remus-Emsermann et al., 2014). Peredo and Simmons (2018) developed a portable leaf FISH method that obtains high-resolution images of phyllosphere-associated bacteria with reduced background autofluorescence by processing spectral images with a linear unmixing algorithm (ZEN software, Carl Zeiss) while maintaining the unaltered distribution with the strong probe signal.

For the quantification and visualization of bacterial cells on the leaves, intercalated fluorescent dyes such as 4',6-diamidino-2-phenylindole (DAPI), acridine orange, Hoechst 33342 and 33258, Syto™ is used (Remus-Emsermann&Schlechter, 2018). The dye passes through the cell membrane and due to their chemical properties, it binds to the dsDNA and can be visualized with either reflected light microscopy (Yashiro et al., 2011) or with

epifluorescence microscopy (Reisberg et al., 2012). Using fluorescent dyes Syto™ 9 (green) and propidium iodide (red), live and dead epiphytic bacteria can be identified and quantified (Remus-Emsermann&Schlechter, 2018). Peredo and Simmons (2018) use this method (Live/Dead BacLight L7012, Live Technology, USA) to check the cells density and viability of the cultures after inoculation of sterile *Arabidopsis* leaves. Postiglione et al. (2022) used this staining method (Live/Dead BacLight Bacterial Viability Kit (Invitrogen)) for visualization and localization of bacteria on *Quercus ilex* L. leaves. Niwa et al. (2011) optimized the Live/Dead technique and used it for the enumeration of live epiphytic bacteria on rice leaf sheath and seeds after washing by using epifluorescence microscopy.

By using green fluorescent protein (GFP) as a reporter gene, gene expression and protein localization in the bacterial cell can be visualized using fluorescence microscopy. The studies (Joyner&Lindow, 2000, Leveau&Lindow, 2001) demonstrate that by fusing the *gfp*-gene with the fructose- and iron-responsive promoter, the nutrient utilization (fructose, iron) of the phyllosphere-associated bacterial cells (*Erwinia herbicola*, *Pseudomonas syringae*) and their distribution on the bean leaves could be visualized. Mullens and Jamann (2021) used GFP markers to track the spread of pathogen (*Clavibacter nebrakensis*) in the maize plant. Mahmood et al. (2022) used GFP-tagged *Pseudomonas* sp. MehA-P42 to show its localization on seeds, roots, stems and leaves of sunflower.

Fluorescence microscopy was used to show the localization of cells on the surface of different plant parts, but confocal laser scanning microscopy was able to show the specific cell localization (in inter- and intracellular) in plant parts (Mahmood et al., 2022). Confocal laser scanning microscopy (CLSM) is the most used method for spatially accurate visualization and localization of microorganisms on/in the host (Pawley, 2006). CLSM is based on the detection of fluorescent light that is triggered by a laser beam and focused through a pinhole. This signal is digitized and produces a high-resolution, high contrast bidimensional (2D) or three-dimensional (3D) image up to a depth of 40 µm without needing to cut the sample (Hofmann, 2014). To visualize bacterial colonization patterns in the phyllosphere, CLSM is often used in combination with FISH (Cardinale, 2014, Wicaksono et al., 2023). Peredo&Simmons (2018) used FISH-CLSM to visualize and identify bacterial taxa on cuticular and subcuticular leaf areas at the genus level. It can also be used to confirm the results of the cultivation-dependent and -independent methods and to demonstrate the niche localization of the targeted bacteria in the phyllosphere as in the study by Alibrandi et al. (2017). Due to deep sample scanning, CLSM

is also well suited for the visualization of microbial aggregates and biofilms (Morris et al., 1997). CLSM can be combined with molecular methods (e.g. FISH) and with other microscopic techniques (e.g. SEM). Fluorescence microscopy in combination with electron microscopy offers the possibility to first identify the target objects or regions of interest and then to visualize them at a higher resolution (nm) (Jahn et al., 2012).

Scanning electron microscopy (SEM) can visualize the leaf surface with associated bacteria at the nanometer scale. SEM uses electrons instead of light to create a high-resolution 3D image which is generated in a vacuum by an electron gun. The electrons are focused on a fine point on the sample using a magnetic lens and scanned in a grid pattern across the surface of the sample. The emitted signal is captured and converted by a special electron detector. In phyllophere research, SEM has been used to investigate the surface of various plant species, for the detailed qualitative description of bacterial localization, e.g., the type of attachment and aggregation, the colony morphology, the morphological diversity of the bacteria, as well as semiquantitative parameters, e.g., the occurrence and abundance of phylloplane-associated bacteria (Baldotto&Olivares, 2008). Aydogan et al. (2020) used SEM to show the surface structure of leaves, bacterial colonization patterns, and the quantitative (number) and qualitative (size) properties of stomata. In a study by Theodora et al. (2019), this method was used for the comparative analysis of morphological changes in the extracellular matrix (biofilm) of pathogenic bacteria to confirm the biofilm disruption of biofilm-forming pathogenic bacteria by the extracts with anti-quorum sensing activity of phyllosphere isolates.

1.4.2 Cultivation methods: advantages and disadvantages

Cultivation methods have been developed since the 1880s to understand the enumeration of cells, their shape, physiology, functions and relationships with their environment (Ritz, 2007). They are traditionally still widely used to characterize phyllosphere-associated bacteria (Ritz et al., 2007, Yadav et al., 2010), because they are inexpensive and not very technically demanding. Isolation of some organisms can be time-consuming and laborious. The cultivation of phyllosphere bacteria is highly dependent on the culture media and culture conditions (Nichols, 2007, Kobayashi&Palumbo, 2000). For most bacteria from environmental samples, suitable culture media and optimal conditions have not yet been found (Müller&Ruppel, 2014). The difficulties in cultivation of phyllosphere-associated bacteria may be due to the "viable but not culturable" or "not yet culturable" bacteria themselves (Colwell, 2000, Oliver, 2010), which have developed a slower metabolic activity under stress conditions (nutrient

limitation, desiccation, temperature fluctuations and UV radiation) (McDougald et al., 1998) (Müller&Ruppel, 2014) and cannot yet be propagated in the laboratory (Oliver, 2010). There are different techniques and methods that can be used to study the bacterial population of the phyllosphere.

One of the simplest techniques is leaf printing (Corpe, 1985), in which microorganisms are transferred from leaf surfaces to an agar plate by leaf printing and then cultured under optimal conditions. This technique allows the abundance and composition of the leaf community to be observed depending on the medium. The disadvantage of this technique is that it has a low observation resolution and limited quantitative estimation. Due to the different growth rates, the slow-growing bacteria can be overgrown by fast-growing bacteria and are more likely to be missed in the analysis. However, this leaf printing technique provides the first indication of uneven localization of microorganisms on the leaf surface (Leben, 1988, Manceau&Kasempour, 2002). Afterwards, the isolates can be purified and characterized (Jacques&Morris, 1995). Better quantification of phyllosphere-associated bacteria can be achieved using colony forming unit (CFU) and most probable number (MPN) methods.

The total number of culturable bacteria per leaf, per cm^2 or per gram of fresh leaf can be estimated using the CFU method (Kinkel et al., 2000, Yadav et al., 2010, Knief et al., 2008, 2010, Yashiro et al., 2011, Jackson et al., 2013, Aydogan et al., 2020) and MPN method (Rekosz-Burlaga et al., 2006, Pariona-Llanos et al., 2010, Ingram et al., 2011, Susilowati et al., 2018). For this purpose, the bacteria are separated from the leaf by a washing step (vortexing, sonication, shaking in saline or phosphate buffer). The methos for the separation of bacteria from the leaf can vary. For example, the bacterial aggregates could be separated from the leaf surface without vortexing and sonication (Morris et al., 1998). The CFU method is based on the counting of the colony forming units per agar plate, assuming that one CFU is formed from one cell. The difficulty of this method lies in the necessary separation of cell aggregates and cell clumps. Otherwise, the CFU formation from two or more cells leads to underestimation in the determination of the total bacterial population (Leveau, 2006). The MPN method, on the other hand, is a statistically based method that estimates the number of bacteria without counting colonies. It is based on three replicate tubes per required decimal dilution level, which are inoculated with liquid nutrient medium containing the inoculum. It is assumed that the cells in the sample are randomly distributed and that any inoculum volume that has at least one viable cell shows growth (Garthright&Blodgett, 2003). Otherwise, the true bacterial

cell density will be underestimated. The presence or absence of cell growth in the tube indicates a positive or negative result. Using the last three positive dilutions and number of tubes, the estimated MPN value is taken from the MPN table (Oblinger&Koburger, 1975, De Man, 1983) and is calculated to the final concentration. One of the advantages of the MPN method is that the MPN value can also be calculated using the free MPN calculation program by Jarvis et al. (2010). This program allows the estimation of the MPN from both standardized and non-standardized parameters (Jarvis et al., 2010). The results of the MPN method as well as CFU methods can be presented logarithmically, which allows quantitative comparison of the different samples (Hirano et al., 1982, Kinkel et al., 1995, Woody et al., 2003). The disadvantage of the MPN method is that the accuracy of the probable estimate depends on many replicates and corresponding dilutions. The higher the number of replicates, the better the accuracy of this method (Oblinger&Koburger, 1975). The MPN method is only suitable for the investigation of bacteria (Sandle, 2015) and due to its inaccuracy, it is recommended to verify this method with other methods, e.g. microscopy, direct plating methods (Herigstad et al., 2001, Chen et al., 2003, Rekosz-Burlaga et al., 2006, Yashiro et al., 2011). A major advantage of this method is that it can be minimized by using 96-well microplates, thus saving the higher material effort, processing time and incubation space (Hernandez et al., 1993). Subsequently, MPN method can be verified using the economical drop plate method, whereby a drop of serial dilutions is pipetted onto culture medium agar plates, and an MPN value can be determined with high accuracy (Chen et al., 2003). Using the drop plating method, a CFU value of leaf-associated bacteria can be determined (Knief et al., 2010) and pure cultures can be isolated from highest dilutions (Knief et al., 2010). The disadvantage of the drop plating method is that it has been used worldwide for many years, but has not yet been standardized (Herigstad et al., 2001, Chen et al., 2003), e.g. the size of the droplets (Hoben&Somasegaran, 1982, Barbosa et al., 1995), number of replications or number of dilutions (Herigstad et al., 2001).

Dilution-to-extinction is another cultivation method besides the MPN method. It is based on the growth of cells in small amounts of nutrient-poor liquid medium and serial dilutions. It was developed by Button et al. (1993) to better cultivate the typical small, abundant but fastidious, slow growing (low growth rate) bacteria that can grow under oligotrophic heterotrophic conditions in seawater and to isolate novel bacteria. For this purpose, serial dilutions of filtered, autoclaved seawater with the addition of amino acids

(casein hydrolysate) as a growth medium and an inoculum were first prepared in culture tubes with screw caps, incubated and then cell growth was checked microscopically (Button et al., 1993). This method was miniaturized by Hoefman et al. (2012) through 96-well microplates and used to enrich and isolate bacteria from samples such as wastewater treatment, wetlands, manure pits biofilter material (Hoefman et al., 2013) and the intestine of black soldier fly larva (Cifuentes et al., 2022). However, it has never been used for cultivation of phyllosphere-associated bacteria. In this work, the enrichment of abundant phyllosphere-associated bacteria especially for oligo/heterotrophic and methylotrophic groups was performed using this method for the first time. There are many advantages to this method. Dilution-to-extinction is not as complicated to handle as the conventional MPN method with test tubes and can be used for counting bacteria. By using 96 well microplates, this method is more cost-effective, simpler, more sensitive to detection and can be automated (Collado et al., 2007). This method allows for better enrichment and isolation of oligotrophic heterotrophic bacteria from environmental samples because serial dilutions mimic nutrient availability in the phyllosphere (Lindow&Brandl, 2003). Because with increasing dilution and decreasing inoculum, the number of culturable cells increased due to reduced competition between bacteria in the community, improved cell division and ideally lead to the growth of pure cultures (Collado et al., 2007, Vartoukian, 2016). Furthermore, the small, abundant cells with a low growth rate can be incubated for long periods of time (Collado et al., 2007) and are not overgrown by rapidly growing bacteria as is the case with direct culture on agar plates (Button et al., 1993). According to new findings, this method can achieve a higher diversity of cultured bacteria than a direct plating cultivation method as shown on BSFL gut bacteria in the study by Cifuentes et al. (2022). In addition, the bacteria can be further characterized directly or after isolation of pure cultures by microscopic, culture-dependent and culture-independent methods.

1.4.3 Culture-independent methods: advantages and disadvantages especially of 16S rRNA gene amplicon sequencing with NGS techniques

The plant-associated microbiota is also investigated using culture-independent methods because the spectrum of microbial communities in environmental samples is much broader using culture-independent methods than using cultivation methods (Head et al., 1998). The impact of most plant-associated microorganisms on plant biology also led to the approach of culture-independent detection methods in phyllosphere research (Wilson&Lindow, 2000).

The culture-independent methods provide valuable data from bacteria that are not culturable and are neglected with culture-dependent methods. For example, “viable but not culturable” (VBNC) bacteria that are unable to continue their cell division on/in standard laboratory media (Oliver, 2010).

The first cloning method which has been used for decades, is where near full-length 16S rRNA gene amplicons from complex PCR pools of bacterial communities were integrated into a host cell vector, enabling a comparative sequence analysis of the obtained clones with a high phylogenetic resolution (genus to species level) (Ionescu et al., 2016). However, this method was expensive and required a lot of labor (Singleton et al., 2001, Tyson et al., 2004, Venter et al., 2004, Nocker et al., 2007, Ionescu et al., 2016).

In contrast, community fingerprint methods (single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (t-RFLP)) offer faster diversity analysis and rapid comparison of the composition of bacterial communities (Nocker et al., 2007). These methods showed good separation of short PCR fragments (up to 500 bp) of the same length but with sequence differences (Ovreas, 2000, Nocker et al., 2007). A key problem with these methods is that the phylogenetic identification of interesting bands was only possible after gel excision and were limited (Duineveld et al., 1998, Sun et al., 2004). Another advantage for some molecular methods (e.g. SSCP, t-RFLP, Automated ribosomal intergenic spacer analysis (ARISA)) was the partial automation by replacing the acrylamide gel with a capillary array sequencer and achieving a higher throughput (Baba et al., 2003, Ikeda et al., 2007). Furthermore, all methods based on 16S rRNA gene amplification and amplicon sequencing have their disadvantage in PCR amplification (Müller et al., 2014). The reason for this is a strong sensitivity to template concentration (Chandler et al., 1997), primer mismatches and inhibitory compounds during PCR amplification (Nocker et al., 2007). Particularly, co-amplification with bacterial universal primers of 16S rRNA gene sequences of chloroplast and mitochondrial, which are homologous to 16S rRNA gene sequences of bacteria, proved to be very disruptive in the analysis of leaf microbial community composition (Saito et al., 2007, Berlec, 2012, Reisberg et al., 2012, Lefevre et al., 2020).

Since the development of next-generation sequencing (NGS) technologies between 2004 and 2006 (Mardis et al., 2013), diversity analysis and phylogenetic composition of microbial communities have dramatically accelerated (Dumbrell et al., 2016). With the

revolutionary improved Sanger method (Sanger et al., 1977), it was possible to sequence a large amount of DNA (Ari&Arikan, 2016), but the output was limited in comparison to second-generation NGS technologies. In addition, the Sanger method could only analyze one sequencing reaction (Hu et al., 2021), whereas the NGS methods could sequence many sequences (hundreds of thousands to millions) in parallel in a single run easily, quickly and efficiently (Mardis, 2013, Ari&Arikan, 2016). The large amount of 16S rRNA gene sequences has become a major problem in bioinformatics analysis. Although computationally intensive software has been developed for this purpose, the raw sequence data must first be processed by the user. However, the time and cost required for DNA sequencing have been significantly reduced compared to Sanger sequencing due to the very simple and complete automation with expensive equipment (Buermans&Dunnen, 2014, Ari&Arikan, 2016). Another important advantage of shot-read sequencing (NGS) technologies such as pyrosequencing, Illumina or Ion-Torrent is the detection of changes in the diversity and structure of microbial communities in different ecosystems (Hu et al., 2021, Nkongolo&Narendruka-Kotha, 2020). In addition to the abundance and diversity of the complex bacterial communities in the sample, it can also determine their phylogeny and taxonomy (to the genus level) (Klindworth et al., 2013, Delgado-Baquerizo et al., 2016, Farowski et al., 2016, Ionescu et al., 2016). The advantages and disadvantages of these technologies differ individually. Pyrosequencing technology (Margulies et al., 2005) was previously the most widely used NGS method for biodiversity assessment in the phyllosphere (Müller et al., 2014) because of its long-read length (average 400 bp) and very high sequencing accuracy (Ari&Arikan, 2016). The disadvantage of this method was that the sequencing of homopolymer regions, especially when they consisted of more than five identical base repeats, was incorrect (Ari&Arikan, 2016). Illumina NGS technology (Bentley, 2006, Bentley et al., 2008) has surpassed pyrosequencing in this respect thanks to the ability to incorporate one nucleotide per reaction (Loman et al., 2012, Jünemann et al., 2013, Mardis 2013) and additionally offers lower cost, higher accuracy, high output and ease of use (Nelson et al., 2014, Ionescu et al., 2016). The disadvantage of this method is the limited read length of 2 ×300 bp (Ari&Arikan, 2016). However, this read length and coverage depth of the V3-V4 sequencing regions of the 16S rRNA gene is beneficial for taxonomic resolution and diversity analysis of bacterial communities at the genus level due to a good balance between them (Jeong et al., 2021). The method is currently the most widely used method worldwide (Ari&Arikan, 2016). Compared to Illumina, Ion Torrent technology

(Rothberg et al., 2011) is the most cost-effective method, which enables rapid detection by pH measurement and does not require the optical signal of fluorescently labeled nucleotides (Levy&Myers, 2016, Goodwin et al., 2016). Although sequencing of read lengths between 200 and 600 bp is possible, the relative error rate of the data is very high compared to other methods, especially in homopolymer regions (Hu et al., 2021).

The latest generation (third generation sequencing, TGS), also known as long-read sequencing technology, has successfully improved over the limitations of NGS in recent years (Tedersoo et al., 2021). These technologies provide more accurate and reliable taxonomic and phylogenetic bacterial identification at the genus and species level by covering full-length 16S rRNA gene sequence without PCR amplification, with little effort, shorter execution times, and a low risk of contamination (Kraft&Kurth, 2020, Logsdon et al., 2020). But they remain expensive, still require large amounts of starting material and require specific solution steps in bioinformatics analysis (Tedersoo et al., 2021).

1.5. Hypotheses and aims of study

1.5.1 Hypotheses

In this study, the following hypotheses were formulated.

(i) Elevated CO_2 concentration will affect the diversity and abundance of oligo/heterotrophic and methylotrophic bacterial communities of the phyllosphere. The abundance of *Sphingomonas*, *Pseudomonas* spp. (oligo/heterotrophs) and *Methylobacterium* spp. (methylotrophs) will increase due to the increase in plant substrate (sugar, methanol) resulting from the elevated CO_2 concentration.

(ii) Long-term treatment with elevated CO_2 (17 years) leads to phylotypic adaptation of oligo/heterotrophic and methylotrophic bacteria, especially functional adaptation.

1.5.2 Aims

To prove these hypotheses, the effect of elevated CO_2 concentrations on the community composition and abundance of oligo/heterotrophic and methylotrophic bacterial communities on *A. elatius* (monocot, grass) and *G. album* (dicot, herb) leaves), which are the dominant plant species in the permanent grassland of the GiFACE system was investigated.

First, the most abundant oligo/heterotrophic and methylotrophic bacteria of both plant species (*G. album*, *A. elatius*) were cultured by the dilution-to-extinction approach using 96 well microplates. The concentration of cultured bacteria was determined using the most

probable number (MPN). The shifts in the diversity and composition of the most enriched bacteria assemblages were presented using DGGE and NMDS approaches. For analysis of new adapted ecotypes, the most abundant enriched bacteria were first isolated, then phylogenetically identified at genus level (using partial 16S rRNA gene sequence analysis) and differentiated at strain level (using BOX-PCR analysis). Isolates were assigned to phylotypes based on calculated pairwise sequence similarities in monophyletic clusters of phylogenetic trees. A correlation of phylotypes with eCO_2 concentration and plant species was presented using CCA. PPFM phylotypes, as abundant inhabitants of the phyllosphere, were analyzed at functional adaptation using changes of partial nucleotide sequences and amino acids of *mxaF*-gene to find specifically adapted ecotypes.

Another aim of this study was to establish a high efficiency DNA extraction method of phyllosphere-associated bacteria of the two plant species (*A. elatius, G. album*) without co-extraction of chloroplast and mitochondrial DNA to better investigate the phyllobiome of the two plant species. For this establishment, direct DNA extraction (CTAB-methods, various commercial kits) and alternative DNA extraction (bacterial cells harvesting before DNA extraction) methods on total fresh and frozen, frozen-homogenized as well as freeze-dried leaves were tested. For alternative DNA extraction methods, bacterial cells were first homogenized or detached from the leaves and then collected by a centrifugation density gradient medium (Percoll, Nycodenz), centrifugation or filtration with Sterivex filters with/without prefilter. The quantity, quality and presence of co-amplification (mitochondria and chloroplasts) of all DNA extracts were checked and compared.

2 Material und Methods

2.1 Material

2.1.1 Chemicals

Chemicals for DNA extraction by CTAB method

10% CTAB solution (10 ml): (Cetyltrimethylammonium bromide)	1 g CTAB 10 ml ultrapure water stirring, autoclaved
5 M NaCl solution (10 ml):	2,92 g NaCl (MG=58.44 g/mol) 10 ml ultrapure water stirring, autoclaved
1 M Tris-Cl pH 8 (100 ml):	12.14 g Tris (MG=121.14 g/mol) 100 ml ultrapure water pH 8 adjusted using concentrated HCl fill up to 100 ml with ultrapure water autoclaved
0.5 M EDTA pH 8 (200 ml):	37.24 g EDTA 100 ml ultrapure water 6 NaOH cookies pH 8 adjusted with 10 M NaOH S fill up to 200 ml with ultrapure water autoclaved

Chemicals for DNA extraction by Pitcher et al. (1998) modified

N-lauroylsarcosine 10% (v/v) (10 ml):	1 g N-lauroylsarcosine fill up to 10 ml with sterile water no autoclaved (explosive!), soapy, use blue gloves, work under hood!

GES buffer (100 ml):	60 g guanidium thiocyanate 20 ml 0.5 M EDTA, pH 8 20 ml sterile water heat to 65°C and stir until dissolved Let cool 5 ml N-lauroylsarcosine 10% (v/v) fill up to 100 ml with sterile water sterilized by filtration with filter (0.45 µm pore size) store at room temperature
7.5 M ammonium-acetate (50 ml):	28.91 g ammonium-acetate in 50 ml water dissolved sterilized by filtration with filter (0.22 µm pore size)
Chloroform/2-pentanol (24.1):	240 ml chloroform 10 ml 2-pentanol store at 4°C, use blue gloves, work under hood!
25% SDS (natriumdodecylsulfate) (100 ml):	25 g SDS resolved at 60-80°C in 100 ml sterile water no autoclaved, store at room temperature use blue gloves, glasses, mask, work under hood
Lysozyme (50 mg/µl) (10 ml):	0.5 g lysozyme 10 ml PCR water vortex, store at -20°C
RNase A (25 mg/ml):	0.05 g RNase A 2 ml PCR water vortex, store at -20°C

Proteinase (20 mg/ml):	0.04 g proteinase 2 ml PCR water vortex, store at -20°C

Chemicals for DNA extraction by Griffiths et al. (2000)

CTAB buffer:	10% CTAB in 1.6 M NaCl 240 mM potassium phosphate buffer pH 8.0
10% CTAB in 1.6 M NaCl:	0.5 g CTAB 5 ml 1.6 M NaCl autoclaved
240 mM Potassium phosphate buffer pH 8:	28.2 ml 1M dipotassium phosphate (K_2HPO_4) 1.8 mM 1M potassium dihydrogen phosphate (KH2PO4) pH 8 adjusted with KH_2PO_4 125 ml PCR water autoclaved both buffers combined in ratio of 1:1
12 mM PEG/NaCl:	3.6 g PEG 6000 (30%) fill up to 12 ml NaCl sterile filtration with 0.22 µm store at room temperature
Phenol/chloroform/isoamylalcohol (25:24:1)	5 ml phenol/chloroform/isoamylalcohol 2.4 ml Tris buffer mixed 5-10 min incubated at 4°C 500 µl from the bottom, turbidity phase

	use blue gloves, work under hood
10% N-lauroysarcosin:	turbid solution
	use blue gloves, glasses, mask, work under hood
0.5 M EDTA pH 8 (400 ml):	74.48 g EDTA
For DNA extraction and DGGE	200 ml ultrapure water (milky)
	pH 8 slowly adjusted with 25 ml 10 M NaOH
	fill up to 400 ml with ultrapure water
	autoclaved
1x PBS pH 7 (1l):	7.6 g NaCl (130 mM)
For cells detachment	0.36 g NaH_3PO_4 (3 mM)
	1 g Na_2HPO_4 (7 mM)
	450 ml ultrapure water dissolved
	pH 7 adjusted
	fill up to 1l
	store at room temperature
10x TBE buffer (1l):	107.8 g Tris
For DGGE	55.03 g Boric acid (use blue gloves, mask, under hood!)
	40 ml 0.5 M EDTA pH 8
	in 300 ml ultrapure water successively dissolved
	fill up to 1l
	resolved for 20 min at 60°C
	store at room temperature
Loading dye solution (for DGGE)	10 mM Tris-HCl (pH 7.6)
(Thermo Scientific):	0.03% bromophenol blue
	0.03% xylene cyanole FF
	60% glycerol
	60 mM EDTA

	store at 4°C
Ethidium bromide staining solution:	1 drop of ethidium bromide (10 ng/ml, Roth) 1l ultrapure water stored in a dark bottle at room temperature under hood use blue gloves
Chemicals for PCR:	10x Dream Taq buffer with 20 mM $MgCl_2$ (Thermo Scientific) 5 u/µl Dream Taq DNA Polymerase (Thermo Scientific) 2 mM dNTP mix (Thermo Scientific) primer (100 pmol/µl, Eurofins Genomics) BSA (Bovine serum albumin) (20 mg/ml, Thermo Scientific) PCR ultrapure DNase and RNase free water (Roth)

2.1.2 Kits

In this study, the following ready-to-use sets (kits) were used for DNA extraction from leaf material.

Kits	**Manufacturer**
NucleoSpin®Plant II kit	Macherey-Nagel
ZR Plant/Seed MiniPrep™ kit	Zymo Research
Fast DNA®Spin kit for Soil	MP Biomedicals
PowerSoil®DNA Isolation kit	MoBio
PowerPlant®Pro DNA Isolation kit	MoBio
NucleoSpin®Soil kit	Macherey-Nagel
NucleoSpin®96 Soil kit	Macherey-Nagel

2.1.3 Culture media

Liquid M125 medium for methylotrophs (1l):

Macronutrients: 1 g KNO_3

0.2 g $MgSO_4x7H_2O$

0.02 g $CaCl_2x2H_2O$

0.23 g Na_2HPO_4

0.07 g NaH_2PO_4

0.001 g $FeSO_4x7H_2O$

Micronutrients: 5 µl $CuSO_4x5H_2O$

10 µl H_3BO_3

10 µl $MnSO_4x5H_2O$

7 µl $ZnSO_4x7H_2O$

10 µl MoO_3

successively dissolved in 800 ml ultrapure water

adjusted pH 6.8

fill up to 1 liter *with ultrapure water

autoclaved

cool down

add 5 ml ethanol (4°C) sterile

mix, store at 4°C, dark, use blue gloves

*For first cultivation after sampling fill up to 960 ml, then after autoclaving add 40 ml sterile cycloheximide (conc. 200 mg/40 ml, blue gloves, dark) add to cooled solution

Solid M125 medium for methylotrophs (50 petri dishes, 1l):

Macronutrients and micronutrients see above

successively resolved in 800 ml ultrapure water

adjusted pH 6.8

add to 14 g washed agar

fill up to 1 liter** with ultrapure water

autoclaved

cool down until hand warms

add 5 ml ethanol (4°C) sterile

mix, pour into the plates, dry, store at 4°C, dark, use blue gloves

**for first cultivation after sampling fill up to 960 ml, then after autoclaving add 40 ml sterile cycloheximide (conc. 200 mg/40 ml, blue gloves, dark) add to the hand warm solution

Washed agar (50 petri dishes, 1l):

14 g agar (Roth)
fill up to 1 liter with ultrapure water
to stir for 20 min at room temperature
let stand for 20 min to deposit
to pour off careful supernatant
repeat it three times
add required liquid medium

Liquid R2A medium for heterotrophic growth (1l):

3 g R2A (Lab M Broth)
or
0.5 g Yeast extract
0.5 g Protease peptone
0.5 g Casein-hydrolysate
0.5 g Glucose
0.5 g Starch
0.3 g Pyruvate (pyruvic acid)
0.3 g K_2HPO_4
0.05 g $MgSO_4x7H_2O$
adjusted pH 7.2 with K_2HPO_4 or KH_2PO_4
fill up to 1 liter *with ultrapure water
autoclaved

*For first cultivation after sampling fill up to 960 ml, then after autoclaving add 40 ml sterile cycloheximide (conc. 200 mg/40 ml, blue gloves, dark) add to cooled solution

Solid R2A medium for heterotrophic growth (50 petri dishes, 1l):

Nutrients see above
successively resolved in 800 ml ultrapure water

adjusted pH 7.2 with K_2HPO_4 or KH_2PO_4
add to 14 g washed agar (see above)
fill up to 1 liter** with ultrapure water
autoclaved

**for first cultivation after sampling fill up to 960 ml, then after autoclaving add 40 ml sterile cycloheximide (conc. 200 mg/40 ml, blue gloves, dark) add to the hand warm solution

Solid ½ R2A (for spotting, 500 ml for 10 square petri dishes):

0.75 g R2A
resolved in 450 ml ultrapure water
add to 7 g washed agar
fill up to 500 ml**
autoclaved

**for first cultivation after sampling fill up to 960 ml, after autoclaving 40 ml sterile cycloheximide (conc. 200 mg/40 ml, blue gloves, dark) add to the hand warm solution

2.2 Methods

2.2.1 Field side and sampling

2.2.1.1 Field side of GiFACE system

In this study, samples were taken from the Giessen Free-Air Carbon dioxide Enrichment (GiFACE) experiment of the 'Environmental Monitoring and Climate Impact Research Station Linden' in Linden, near Giessen, Germany. The field side is a permanent grassland located at 50°32'N and 8°41.3'E, 172 meters above sea level. The site had not been ploughed for at least 100 years. During the last decades, it had been managed as a meadow with two cuts per year and fertilized with 50-80 kg N ha^{-1} $year^{-1}$. Since 1995, the amount of fertilizer had been reduced to 40 kg N ha^{-1} $year^{-1}$. The soil is a Fluvic Gleysol on sandy clay loamy sediments over clay (FAO classification) (Jäger et al., 2003). The mean annual temperature and precipitation is 9.4°C and 575 mm (observation period: 1996-2005), respectively. The vegetation was described as an *Arrhenatherum elatioris* Br.Bl. *Filipendula ulmaria* sub-community and is dominated by 12 grass species, 2 legumes and 15 non-leguminous herbs (Rodwell et al., 1992).

The GiFACE system contains six rings randomly placed in the field side (Fig. 1). Each ring has an inner diameter of 8 m and consists of 24 identical segments (H=35 cm, W=70 cm from galvanized steel). The grassland in three rings (control rings) is exposed to ambient atmospheric CO_2 and in the other three rings to an atmospheric CO_2 concentration of approximately 20% above ambient atmospheric CO_2 measured in a height of 40 cm above ground (Fig. 1).

Figure 1 Giessen Free Air Carbon dioxide Enrichment (GiFACE) system of the „Environmental monitoring and climate impact research station in Linden ", near Giessen, Germany. Six rings of the GiFACE: three rings of control with ambient atmospheric CO_2 (aCO_2) and three rings with exposed CO_2 approximately 20% above to ambient atmospheric CO_2.

The ring's pre-diluted CO_2 is spread by auxiliary air up the wind so that the wind further could be distributed and diluted. Concentration of CO_2 is continuously monitored in the ring centers; concentration controllers and the upwind controllers are used to keep the CO_2 concentration constant. Each segment has an individual segment controller. Since 1998, the CO_2 was enriched throughout whole year every day between 2 h after sunrise to 2 h before sunset (Jäger et al., 2003).

2.2.1.2 Sampling of plant material

2.2.1.2.1 For cultivation-dependent approaches

For investigations using cultivation-dependent approaches, two abundant plant species from the permanent grassland of the GiFACE system, *Arrhenatherum elatius* (*A. elatius*) and *Galium album* (*G. album*), were selected for this study. On August 27, 2015, leaves were collected from three defined regions a, b and c (Fig. 2), of each ring. Using sterile tweezers, leaves were cut, and the tweezer was cleaned with 70% (v/v) ethanol after collecting each sample. The middle four centimeters of the first and second leaves were collected from *A. elatius*, while the third and fourth wreaths of leaves were taken from *G. album*. Each replicate was placed in 120 ml sterile whirl-pack bags (Carl Roth GmbH, Karlsruhe, Germany) and stored immediately at 4°C in the dark, to be processed in the laboratory on the same day.

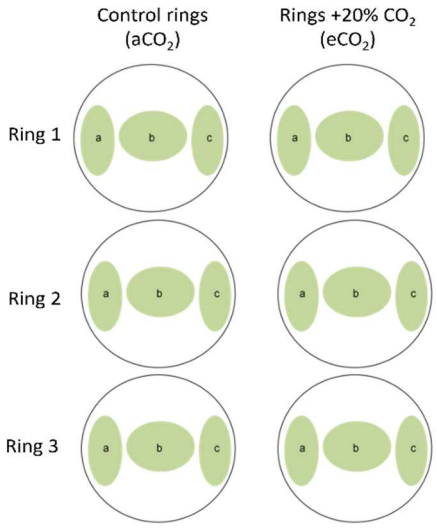

Figure 2 Sampling scheme from three control rings (aCO_2) and three rings with +20% CO_2 concentration to ambient atmospheric CO_2 (eCO_2) in the GiFACE system; a, b, c: three collection points in each ring.

The environmental conditions during the time of sampling between 8 a.m. and 3 p.m. are presented in figure 3. The air temperature was 20-22.5°C. On this day, there was no precipitation as on the two days before. During the sampling period, wind speed was 14 km/h

in the morning and decreased to 3 km/h over time. Two days earlier, the wind speed was faster during the day with max. 16-18 km/h. On that day, the global radiation reached 450 W/m^2, which was lower than the two days before with 700 W/m^2.

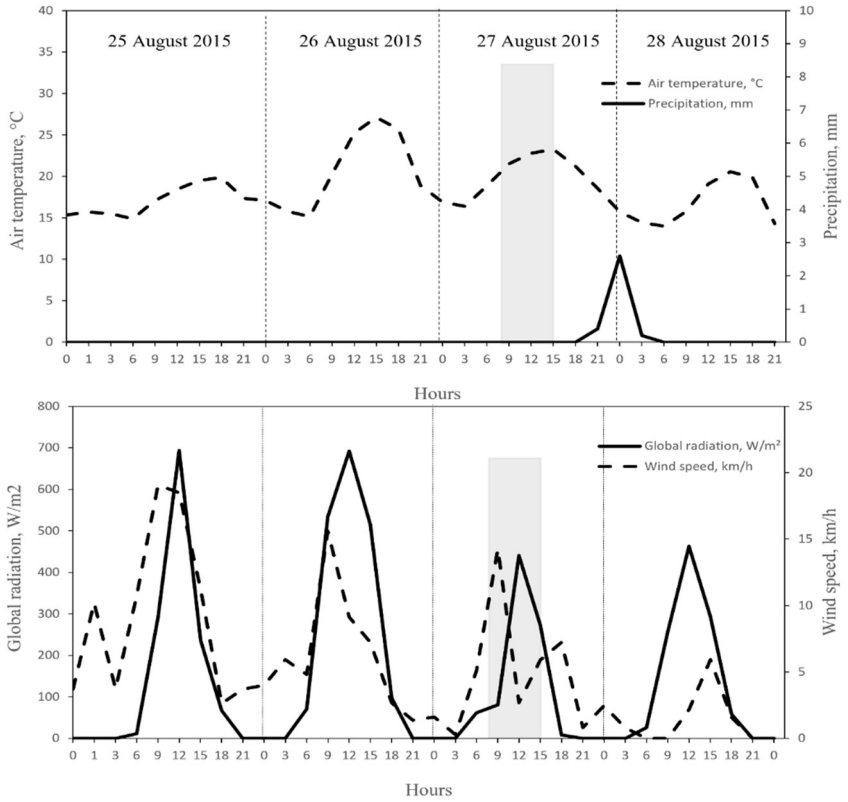

Figure 3 Meteo data of air temperature, precipitation, global radiation and wind speed on sampling day, two days before and one day after sampling. Grey marked area of sampling time in the sampling day (27 August 2015). Meteo data was provided by the Department of Plant Ecology (IFZ), Justus-Liebig-University Giessen, Germany.

2.2.1.2.2 For cultivation-independent approaches

For DNA extraction methods, part of sampling material was taken from the Giessen Free-Air carbon dioxide enrichment (GiFACE) system. There are two leaf pools (A and G) of *A. elatius* and *G. album* plant species which have been collected on 15 April 2014, before fertilizing. The *A. elatius* and *G. album* leaves were collected from one control block (AK, GK) and from three treatments (I) increased temperature of the leaves surface by +2 °C (AT, GT), (II) elevated CO_2 with +20% above ambient atmospheric CO_2 (AC, GC), III) combination of elevated CO_2 and increased temperature (ACT, GCT). Two to three leaves were taken from each of the two plant species from three different places in a block with sterile tweezers and sampled separately in 540 ml sterile whirl-pack bag (Carl Roth GmbH, Germany). For the DNA extraction of the *A. elatius* and *G. album* leaf-associated bacteria, all samples (AK, AT, AC, ACT and GK, GT, GC, GCT) were pooled and frozen at -80°C. The increased leaf surface temperature was generated

using ceramic Infrared (IR) heaters ESE of 230 V and 250 W with reflector and E27 ceramic lamp holder (Friedrich Freek GmbH, Germany). The IR-lamp was attached to three metal rods to prevent wind damage. A metal plate above the lamps provided protection against rain. The IR-lamps were placed at the height of 80 cm above the ground. In the control block of increased temperature treatment, there was no IR-lamp but only a metal plate as rain protector.

For the detachment method, the leaves of *A. elatius* and *G. album* species were collected on 17 April 2015 on a sunny, cloudy day between 11 a.m. and 2 p.m. outside on the meadow across from the Research Centre for Biosystems, Land Use and Nutrition (Giessen) (Fig.4).

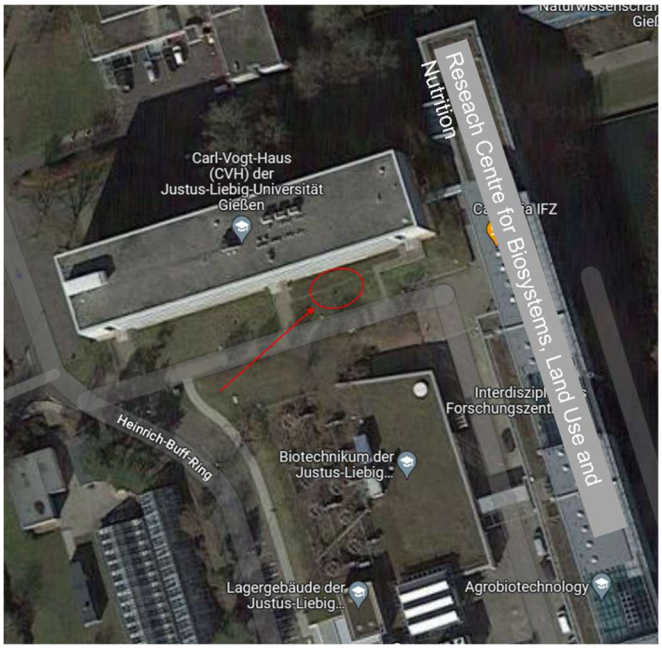

Figure 4 Sampling site of *A. elatius* and *G. album* leaves plant species across from the Research Centre for Biosystems, Land Use and Nutrition in Giessen (Germany). The screenshot of sampling site was made from google map.

The first top leaf of *G. album* was removed with a sterile tweezer and the middle leaves were collected in a sterile beaker. The beaker was cooled with ice packs and pure ice in a grey Styrofoam box and protected from the stress though ambient light. From *A. elatius*, the tip (one third of the leaf) was cut off with a sterile tweezer, then approximately four centimeters from center leaf part collected in a separate cooled beaker. The leaves of both plant species were pooled (*A. elatius* and *G. album*) and distributed into ten falcons (50 ml) according to the experiments. For the filtration experiment, 1.5 grams of pool were weighed into four falcons. For separation by Percoll, three grams of pool were weighed in two falcons. For the centrifugation experiment, one gram of pool was weighed into four falcons. All samples were processed directly. The rest of pool was transferred to a 540 ml whirl-pack bag (Carl Roth

GmbH, Germany), frozen with liquid nitrogen and stored at -80°C for further direct DNA extractions. For DNA extraction with kits PowerSoil®DNA Isolation kit (MoBio), PowerPlant®DNA Isolation kit (MoBio) and Fast DNA®Spin kit for Soil (MP Biomedicals), NucleoSpin®Soil kit (Macherey Nagel), the frozen leaves of the samplings (15 April 2014 and 17 April 2015) were stored at -80°C and then freeze-dried using the Freeze Dryer Modulyo (Edwards) for approximately 48 hours. The weight of the pool with *A. elatius* and *G. album* leaves was reduced from three gram to one gram.

2.2.2 Culture-dependent approaches

2.2.2.1 Detachment of phyllosphere bacteria by mechanical treatment

The detachment method was used to separate leaf-associated bacteria from fresh *A. elatius* and *G. album* leaves. For this purpose, 1.5 g leaf material was mechanically treated for 120 s at normal speed in Stomacher 80 (Biomaster, Seward Laboratory Systems Inc., USA). The treatment was performed in 120 ml sterile whirl-pack bags filled with 10 ml autoclaved phosphate buffered saline (PBS).

2.2.2.2 MPN analysis

The most probable number (MPN) is a statistic method to estimate viable microbial concentration in sample by means of three liquid broth replicates in ten serial dilutions. It is often used to assess the bacteria in soils, waters, and agricultural products (Oblinger&Koburger, 1975). For this method, triplicates series dilution is prepared up to 10^{-9} with 9 ml of liquid medium and 1 ml of original sample. After the incubation period, the turbidity or gas formation in the tubes is screened, which is evaluated as a positive result. The estimated density is based on (i) that microbial cells are randomly distributed in inoculum, (ii) at least one viable organism must be in inoculum to show growth in the culture medium after incubation (Jarvis et al., 2010). The evaluation was carried out using a table with the "most probable number of cells" in three final positive dilution stages and in three parallel tubes per dilution stage (de Man, 1983). With the number of stitches, the most probable number of cells per ml was read from the table and converted to initial dilution.

In this study, minimized MPN was used to estimate the concentration of oligo/heterotrophic and methylotrophic bacteria in the phyllosphere of the two plant species (*A. elatius*, *G. album*) from the rings (three aCO_2 and three eCO_2), but in smaller volume (Chen et al., 2003) in comparison to the standard method. MPN was calculated using method by

Jarvis et al. (2010). In the 96 well microplates (Thermo Fisher Scientific, Germany), 20 µl cell suspensions were serially diluted into 180 µl liquid medium of 10^{-1} to 10^{-8} (from top A to bottom H) using multi-channel micropipette (Eppendorf, Merck). For each sample, serial dilutions were generated as triplicates. A dilution in the first well (row A of 96 well plate) was mixed 10 times and then 20 µl of sample were transferred to next dilution. The pipette tips were changed for each dilution. Two different liquid media were applied; oligo/heterotrophic bacteria were enriched in ½ R2A broth and methylotrophic bacteria in mineral salt medium (M125) supplemented with 0.5% methanol (v/v). To avoid fungal growth, both media were supplemented with 200 mg/l filter-sterilized (0.2 µm Filtropur S syringe filter, Starstedt) cycloheximide (AppliChem) after autoclaving. The inoculated 96 well microplates were sealed with a sterile gas permeable adhesive seal (Thermo Schintific, UK) and put in a sterile plastic box in the presence of autoclaved pure water from Purelab Plus ultra-pure water purification system (ELGA) together with control plate. On the control plate, three columns were filled with each medium, leaving space between both media. The inoculated 96 well plates were incubated at 25°C in dark oligo/heterotrophic bacteria for six weeks and for methylotrophic bacteria eight weeks. Afterwards, bacterial growth in the 96 well plates were monitored using spots assays (see chapter 2.2.2.3) and MPN of detached cultured bacteria per g leaf material analyzed by calculation with the method described by Jarvis et al. (2010). Statistical analysis was performed in SigmaPlot (Applied Maths) using the One-Way ANOVA test to determine the significant differences among enriched leaf-associated bacteria from two plants under aCO_2 and eCO_2 conditions.

2.2.2.3 Spots assays

Spot assays or also known as drop plate method is a microbiological test to check the growth rate of bacterial or yeast cells on various media or to perform a serial dilution test of microorganisms. This method provides accuracy, sensitivity, and precision for counting bacterial concentrations and saving sample processing time, material costs, and incubator space (Chen et al., 2003).

For drop spotting, 5 µl of each serial dilution (MPN, see above) with enriched leaf-associated oligo/heterotrophic and methylotrophic bacteria were used. Spot assays were generated in square petri dishes (Greiner Bio-one, Germany) with washed agar and heterotrophic and methylotrophic media (see chapter 2.1.3). The agar was washed three times in pure water (20 min stirring/20 min sedimentation), added to the medium before

autoclave. Drops from all serial dilutions were placed using multi-channel micropipettes and incubated methylotrophic (12 days) and heterotrophic (3 days) plate at 25°C. All plates with bacterial growth were presented in Fig. 5. The results were used for calculating MPN concentration of enriched oligo/heterotrophic bacteria and PPFMs grown on *A. elatius* and *G. album* plant species.

2.2.2.4 Dilution-to-extinction and isolation of abundant oligo/heterotrophs and methylotrophs

Dilution to extinction is a widely used cultivation method based on liquid culture medium and dilution culture, which was developed by Button et al. (1993) to better cultivate from sea water, abundant but fastidious, slow growing (low growth rate) bacteria that can grow under oligotrophic heterotrophic conditions and isolate new bacteria. In this work, this method was used for the first time for cultivation of phyllosphere-associated bacteria, especially oligo/heterotrophic and methylotrophic bacteria. This is because the dilution-to-extinction method, like the minimized MPN method, is based on serial dilutions and dilution cultures, both being better suited for the analysis of bacterial communities (Shigematsu et al., 2009). The same enriched oligo/heterotrophic and methylotrophic bacteria from the 96-well microplates (described in Chapter 2.2.2.2) were used for Investigation of abundant bacterial assemblages and their isolation. After incubation, 10 µl of the three strongest positive dilutions of enriched oligo/heterotrophic, methylotrophic and PPFM bacteria were purified onto the respective agar media to isolate abundant single colonies. Of each pure bacterial culture, cell lysates were prepared using the freeze-thaw method (described below) for phylogenetic identification (chapter 2.2.2.6.2). In addition, environmental cell lysates were pooled from the triplicates of the last three highest positive dilutions to compare the bacterial assemblages between aCO_2 and eCO_2 treatment using DGGE method (see below).

For long-term preservation of the pure culture, two loops of fresh bacterial biomass were suspended in 1.4 ml u-bottom push cap tubes (Micronic, Netherlands) in 500 µl Gibco™ newborn calf serum (NBCS, ThermoFisher Scientific) and stored at -20°C. The most abundant bacterial assemblage's oligo/heterotrophic and methylotrophic leaf-associated bacteria of both plant species (*A. elatius*, *G. album*) grown under eCO_2 and aCO_2 conditions were isolated und compared using culture-dependent and -independent methods.

2.2.2.5 Preparation of bacterial samples by freeze-thaw method

The freeze-thaw is a simple thermal method to release DNA, RNA, protein or organelles from cells by repeatedly freezing and thawing a sample. When freezing, ice forms on the cell membrane, which helps to break it down. Thawing at high temperature above 90°C leads to denaturation of membrane proteins and helps to release organelles (Schütte et al., 1983, Stackebrandt&Goodfellow, 1991).

For comparison analysis using DGGE, triplicates of environmental samples (the oligo/heterotrophic and methylotrophic bacterial assemblages between aCO_2 and eCO_2 treatment) 100 µl were pooled from each dilution (three highest positive). To perform cell lysis on the pure cultures, two loops of fresh biomass of an isolate from agar plates were resuspended in 500 µl sodium phosphate buffer (SPB; 120 mM, pH 8.0). The cells were washed by using vortex and centrifugation at 4°C by 17 000 g (Micro Star 17R, VWR) for 30 min. Supernatant was discarded. The pellet was resuspended in 500 µl sterile, pyrogen free, hypotonic water (PCR-water, Roth). All samples (environmental pools, pure cultures) were frozen at -20°C, then heated at +115°C for 1 min 30 s on a heating block (HLC-Haep Labor Consult, Germany) and vortexed. This freeze-thaw cycle was repeated three times. After the procedure, cell lysates are ready to apply for PCR amplifications.

2.2.2.6 Phylogenetic identification and differentiation of oligo/heterotrophs and methylotrophs

In this study, the isolates from all three of the highest positive dilutions and PPFMs cultured using dilution-to-extinction approach were phylogenetically identified at a genera level by partial 16S rRNA gene sequencing and differentiated at the strain level by genomic fingerprinting using BOX-PCR analysis as described by Glaeser et al. (2013).

2.2.2.6.1 Genotyping using genomic fingerprinting

First, the most frequent isolates were differentiated by repetitive extragenic palindromic-PCR (Box-PCR) method using BOXA1R primer (5´-CTA CGG CAA GGC GAC GCT GAC G-3´) (Versalovic et al., 1994). Genomic fingerprint patterns of the isolates were generated by 1.4% (w/v) agarose gel electrophoresis (Glaeser et al., 2013) and agarose gel run under the same conditions (for 3.5 hours, at 140V, in 1xTAE buffer, same gel chamber). Box gels were stained in 0.1% (v/v) ethidium bromide, documented by a Quantum vilber lourmat (PeqLab, Germany) and analyzed using software BioNumerics version 8.0 (Applied Maths N.V.). Box fingerprint

patterns were selected for a similarity-matrix calculation with the Pearson correlation coefficient. Custer analysis was performed by the Unweighted Pair Group Method Arithmetic average (UPGMA) with 1% position tolerance and 0.5% optimization. Isolates with identical genomic DNA fingerprint patterns were assigned to one genotype. The 16S rRNA gene sequences of one genotype were normally identical. The different genotypes in this study were phylogenetic identified by 16S rRNA sequencing.

2.2.2.6.2 Phylogenetic identification by 16S rRNA gene sequencing using the Sanger method

For phylogenetic identification, partial 16S RNA gene amplifications were sequenced by Sanger method (Sanger et al., 1977). This method determines the order of the individual nucleotides in the DNA sequence. It is based on PCR reaction with a primer, dNTPs (deoxyNucleotide Triphosphate), fluorescence labeled ddNTPs (didesoxyNTPs, stop nucleotide) and DNA polymerase, by amplifying the PCR products of different lengths. The fragments of different lengths are created by randomly binding ddNTPs without an OH group at 3´ position to a DNA strand, which then blocks further elongation. These PCR products are separated and determined by gel electrophoresis. There are two versions of this method, manual and automated. Manual Sanger-sequencing uses four PCR reactions, each with one type ddNTPs. All PCR products are loaded onto a gel, creating a conveyor belt for each PCR, and separated by gel electrophoresis according to their sequence length (the smallest fragments at the bottom, the largest at the top). The gel is analyzed to determine the DNA sequence. First, the lowest band is determined on a gel and assigned to a PCR. This smallest band, if it is e.g. a PCR product with ddGTPs, will start the DNA sequence with guanine (G) on the 5´ end. The next longest band is also assigned to a specific PCR based on the belt and the next nucleotide is incorporated into the sequence in the 3´ end direction. In automated Sanger-sequencing, all ddNTPs with different fluorescence labels are used in a single PCR. The separation takes place in a single capillary gel electrophoresis. The PCR products migrate through the gel according to their length (the smallest fragments first). A fluorescent dye that has been incorporated at the end of the PCR product is triggered with a laser and the emitted light is detected. The result is shown directly in the computer program as a chromatogram. The DNA sequence in this chromatogram is represented as a series of colored peaks corresponding to each nucleotide.

In this study, PCR products with partial 16S rRNA gene fragments were first prepared for sequencing. Phylotypes were amplified with the primer system 8f (5´-

AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5´-ACGGCTACCTTGTTACGACTT-3´) (Lane, 1991) in a total PCR-volume of 25 µl using 1 µl cell lysate as a template and 0.2 µM of each primer with the same content of other reagents as described above. Amplification conditions were as follows: 95°C for 3 min, 30 cycles of 95°C for 30 s, 57.3°C for 30 s, and 72°C for 90 s, and finally 72°C for 10 min. After quality control of PCR product by 1% (w/v) agarose electrophoreses, partial 16S rRNA gene fragments were sequenced using primer 27f (5´-AGAGTTTGATCMTGGCTCAG-3´) (Lane, 1991) by LGC Genomics (Berlin, Germany). Chromatograms with sequences were manually trimmed at the 5´and 3´ends and internally corrected based on the electropherograms using MEGA7 (Tamura et al., 2013). Isolates were phylogenetically identified by BLASTN analysis (Altschul et al., 1997) against the EzBioCloud type strain 16S rRNA gene sequence database (Yoon et al., 2017). All phylogenetically identified isolates are represented in Fig. 10. If the sequence similarities of the 16S rRNA gene to closely related type strains are less than <98%, then nearly full-length 16S rRNA gene sequences were generated for the isolates. This was achieved by sequencing the back part of the 16S rRNA gene using the primer E786f (5´-GATTAGATACCCTGGTAG-3´ as described by Coloqhoun et al. (1997). Sequences were corrected and combined using MEGA7, respectively. Based on the sequence similarity to the next related type of strains and placement in the "All-Species Living Tree" project (LTP) database version LTPs v123 in ARB, isolates were assigned and differentiated into phylotypes containing the 16S rRNA gene sequence identical isolates. The isolates with pairwise similarity >98.65% were assigned to a phylotype and represented in Fig. 11.

2.2.2.6.3 Differentiation of PPFM based on *mxa*F-gene sequences

In this study, the different phylotypes of pink-pigmented facultative methylotrophs (PPFMs) were differentiated using a partial *mxaF*-gene sequence and translated protein sequences (MxaF), to determine the functional adaptation to elevated CO_2. Wellner et al. (2011) studied *mxaF*-gene to show the higher diversity and resolution of PPFMs. For differentiation analysis of PPFMs, a partial sequence of *mxaF*-gene of 550 bp was amplified with the primer system 1003f (5´- GCGGCACCAACTGGGGCTGGT-3´) and 1561r (5´-GGGCAGCATGAAGGGCTCCC-3´) (McDonald et al., 1995, McDonald&Murrell, 1997). PCR was performed in a total PCR-volume of 25 µl used 1 µl cell lysates of pure bacterial cultures as template. PCR master mix contained 0.16 mM of dNTPs, 0.2 µM of each primer, 0.2 mg/ml BSA and 0.02 U/µl of *Taq* Dream DNA polymerase (all chemicals from Thermo Scientific, Germany). Amplification conditions were as

follows: 95°C for 3 min, 34 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and finally 72°C for 10 min. After amplification, quality control by 1% (w/v) agarose electrophoreses, partial *mxaF*-gene fragments were sequenced with the Sanger method using primer 1003f (McDonald et al., 1995, McDonald&Murrell, 1997) by LGC Genomics (Berlin, Germany). The sequences of PPFMs were aligned with a full-length *mxaF*-gene sequence (locus tag MPOP_RS22895) of *Methylobacterium populi* BJ001^T (NC_010725.1) to manually trim at the 5´and 3´ends using MEGA7 (Tamura et al., 2013). Alignments of these sequences were generated with ClustalW (Thompson et al., 1994). DNA sequences (*mxaF*) and translated protein sequences (MxaF) were used for constructing the neighbor-joining tree with 100 bootstrap replicates. The evolutionary distances were computed using the Jukes-Cantor method (Jukes&Cantor, 1969) for *mxaF* and using the JTT matrix-based method (Jukes&Cantor, 1969) for MxaF. The *mxaF* or MxaF sequences were compared with the *Methylobacterium populi* BJ001^T (Type 1) and defined to *mxaF*-types and MxaF-types. One difference between nucleotide sequence or amino acid sequence was assigned to an allele. The typing of nucleotide and amino acid sequences of the *mxa*F-gene was used to determine functional differences of *Methylobacterium* between eCO_2 and aCO_2 conditions.

2.2.3 Culture-independent approaches

2.2.3.1 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) based on PCR-amplified 16S rRNA gene fragments were applied in this study to compare the enriched oligo/heterotrophic and methylotrophic bacterial assemblages. PCR-amplified 16S rRNA gene fragments were amplified with primer system GC-968f/1378r (Nübel et al., 1996, Heuer et al., 1997) from each pool of each of the last three highest positive serial dilutions of dilution-to-extinction cultivations or amplified with primer system GC-339f/907r (Muyzer et al., 1993) from DNA extracted using NucleoSpin®Plant II kit (Macherey-Nagel) and ZR Plant/Seed MiniPrep™ kit (Zymo Research) (Fig. 16c). Fragments of them same length were separated, based on their GC content and sequence in a 7% (v/v) polyacrylamide gel containing a 40-70% denaturation gradient of urea and deionized formamide (as described previously, Glaeser et al., 2010). A total volume of 50 µl PCR reaction including 2 µl DNA template was prepared and amplified (Tab. 10, 11). Subsequently, 25 µl of PCR product with 2 µl loading buffer DGGE per sample was loaded into the DGGE gel. DGGE was performed with an INGENYphorLI system IPU-S (Ingeny International BV, UK) with 1xTAE buffer (pH 7.4, adjusted by acidic acid) at 60°C

running for 22 hours at a constant voltage of 100V. DGGE gels were afterwards stained in 0.1% (v/v) ethidium bromide, documented by a Quantum vilber lourmat (PeqLab, Germany), and edited in Adobe photoshop CS3 extended version 10.0 (Fig. 7, 16c). For comparison of the bacterial community assemblages enriched per dilution-to-extinction method, the gels were analyzed in GelCompare II version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). DNA bands were marked manually and obtained DGGE patterns were used to calculate a similarity matrix using the Pearson correlation coefficient, which considered DNA band presence/absence and DNA band intensities. Custer analysis was performed by the Unweighted Pair Group Method Arithmetic average (UPGMA) with 1% position tolerance and 0.5% optimization. PAST version 3.11 software (Hammer et al., 2013) was used for subsequent statistical analysis. Non-metric multidimensional scaling (NMDS) was used to compare the composition of enriched oligo/heterotrophic and methylotrophic bacteria considering the atmospheric CO_2 concentrations as varying environmental parameters and groups (aCO_2, eCO_2) (Fig. 8). NMDS is based on the calculation of the Bray-Curtis similarity index of the bacterial community DGGE patterns. One-way ANOSIMs analysis was applied to test for significant differences among enriched bacterial assemblage from plant material of aCO_2 and eCO_2 treatments (Fig. 8).

2.2.3.2 Direct DNA extraction methods

2.2.3.2.1 DNA extraction by modified NucleoSpin®Plant II kit (Macherey-Nagel)

The NucleoSpin®Plant II kit (Macherey-Nagel) is for isolating total plant DNA tissue using two lysis buffer systems based on the established CTAB (PL1) and SDS (PL2) methods. DNA extraction involves a treatment step with RNAse A to remove RNA and to enable photometric quantification of pure total DNA. In addition, this kit contains an optimized Binding Buffer PC and chaotropic Wash Buffer PW1 to completely remove proteins, RNA, metabolites and other PCR inhibitors. In this study, this kit was used to extract bacterial DNA from frozen homogenized leaves of *A. elatius* and *G. album* plant species by mortars in the presence of liquid nitrogen. The leaves were collected on 15 April 2014 from the FACE2FACE system before fertilizing (see chapter 2.2.1.2.2). Two pools of each plant species (A, G) were prepared and homogenized. Two replicates of each pool were provided and weighed. The amount of leaf material was approximately 50 mg. In this study, DNA was extracted according to manufacturer's instructions via cell lysis using Buffer PL1 and eluted with 100 µl. After, the quality and quantity of extracted total DNA were determined by photometric measurements

using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). The sample was not diluted.

2.2.3.2.2 ZR Plant/Seed DNA MiniPrep™ kit

The ZR Plant/Seed MiniPrep™ Kit (Zymo Research) is for isolating plant DNA without PCR inhibitors and with PCR-quality of DNA. State-of-the-art, ultra-high-density ZR Bashingbeads are used for efficient cell lysis without the use of organic denaturants or proteases. The kit has Zymo-Spin ™ III-HRC filters to remove polysaccharides and polyphenols/tannins. In this study, this kit was used for the extraction of bacterial DNA from frozen homogenized leaves of *A. elatius* and *G. album* plant species by mortars in the presence of liquid nitrogen. The leaves were collected on 15 April 2014 from the FACE2FACE system before fertilizing (see chapter 2.2.1.2.2). Two pools of each plant species (A, G) were prepared and homogenized. Two replicates of each pool were provided and weighed 160-170 mg of leaf material). DNA extraction was performed according to manufacturer's instructions with addition of 35 µl beta-mercaptoethanol to the Genomic Lysis Buffer with a final dilution of 0.5% (v/v). After washing step, total DNA were eluted with 100 µl DNA elution buffer at 10 000xg centrifugation for 30 sec. The extracted DNA were spin down without the Zymo-Spin™ IV-HRC Spin Filter (green top) at 8 000xg for 1 min. After, the quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). The sample was not diluted.

2.2.3.2.3 DNA extraction by modified Fast DNA®Spin kit for Soil (MP Biomedicals)

DNA was extracted from total frozen, frozen and homogenized and freeze-dried leaves. For extraction, the leaves of *A. elatius* and *G. album* plant species were collected on 15 April 2014 from the GiFACE system (Linden, Germany), before fertilizing as described above. All samples (AK, AT, AC, ACT and GK, GT, GC, GCT) were pooled to two pools A and G, frozen in liquid nitrogen and stored at -80°C until further processing. The leaves were homogenized in mortar with liquid nitrogen. For extraction from freeze-dried leaves, 3 g of *A. elatius* and G. *album* frozen leaves collected on 17 April 2015 (described in chapter 2.2.1.2.2), were dried in 50 ml falcon covered with perforated aluminum foil using Freeze Dryer Modulyo (Edwards) for 48 hours at -60°C. Two replicates of each pool were prepared (Tab.1) and extracted according to manufacturer's instructions.

Table 1 Amount of leaf material used for DNA extraction by Fast DNA®Spin kit for Soil (MP Biomedicals), with modifications by Wellner et al. (2011) and with modifications for this study

Fast DNA ® Spin kit for Soil (MP Biomedicals	Leaf material	Environmental samples	Weight of total leaf, mg
Manufactures instructions	Total frozen	F2F-t0-Pool A1	184
		F2F-t0-Pool A2	168
		F2F-t0-Pool G1	400
		F2F-t0-Pool G2	202
	Frozen and homogenized	F2F-t0-Pool A1	126
		F2F-t0-Pool A2	176
		F2F-t0-Pool G1	170
		F2F-t0-Pool G2	179
Fast DNA ® Spin Kit for Soil (MP Biomedicals) mod. 1	Total frozen	F2F-t0-Pool A1	476
		F2F-t0-Pool A2	507
		F2F-t0-Pool G1	522
		F2F-t0-Pool G2	540
Fast DNA ® Spin Kit for Soil (MP Biomedicals) mod. 2		F2F-t0-Pool A1	296
		F2F-t0-Pool A2	280
		F2F-t0-Pool G1	428
		F2F-t0-Pool G2	417
Fast DNA ® Spin Kit for Soil (MP Biomedicals) mod. 3	Freeze-dried	F2F-t0-Pool A1	72
		F2F-t0-Pool A2	65
		F2F-t0-Pool G1	55
		F2F-t0-Pool G2	59

In this study, homogenization step (4) to perform cell lysis and release nucleic acids was carried out by using Retsch Mixer MM 400 at a max. speed for 40 sec. In step 5, centrifugation was performed at 14 000xg for 10 min to the pellet debris, such as insoluble cellular debris and lysing matrix. For binding DNA with the binding matrix (step 9), the solution was inverted by hand. The binding matrix (above the SPIN filter) was carefully resuspended in 100 µl DES (DNase/Pyrogen-Free water) and to increase yields incubated for 5 min at 55°C in a heat block (step 16). The quality and quantity of extracted total DNA were determined by photometric

measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

In addition, DNA-Extraction protocol was modified to increase the yield of DNA concentration. For DNA extraction, the total frozen leaves from the same pools A and G (sampling 15.04.2014, see chapter 2.2.1.2.2) with two replications were weighed (Tab. 1). The protocol was modified by following steps (mod. 1). After the first centrifugation at 14 000xg for 10 min, the supernatant was transferred to a new 2 ml microcentrifuge tube and again was centrifuged at 14 000xg for 5 min to the pellet debris, such as insoluble cellular debris and lysing matrix from supernatant. To increase the yield, the elution step was modified. After the first elution, the filter was placed in a new catch tube, added repeatedly 30 µl DES (DNase/Pyrogen-Free water), gently resuspended by snapping and incubated at 55°C for 5 min. If something had flown into the catch tube, then the filtrate was incubated on ice and in the next step, a new catch tube was used for incubation. Thereafter, the catch tube was centrifuged at 14 000xg for 2 min. The filters were discarded and the eluted DNA stored at -20°C. The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

To increase the yield of extracted DNA of total frozen *A. elatius* und *G. album* leaves, the previous modified protocol (mod. 2) has been further modified (mod. 3). After the first centrifugation, the supernatant was transferred to a new tube and then the lysis steps 3-6 were repeated. Sodium phosphate and MT buffers were added to the tube with beats and leaves, and then vortexed and homogenized at max. speed in the Retsch Mixer MM 400. Then, centrifugation was performed at 14 000xg for 10 min and the supernatant was transferred to a new tube. At the end, there are two tubes of each sample. The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to 25 ng/µl concentrations of DNA.

Protocol 1 was modified and applied to freeze-dried leaves samples from pools A and G collected on 15 April 2014 (see chapter 2.2.1.2.2). The frozen leaves were dried (see above) before DNA extraction and two replicates of pools (A, G) were prepared (Tab. 1). In the protocol (mod. 3) the following steps were modified. After repeating steps 3-6, the tubes were centrifuged again at 14 000xg for 2 min to pelletize the remaining lysing matrix E and plant

materials. The extracted DNA was resuspended with 60 µl DES gently by snapping, incubated at 55°C for 5 min and centrifuged at 14 000xg for 2 min (1. Elution). The same SPIN™ Filter was placed into a new catch tube and gently resuspend by snapping again with 60 µl DES, incubated at 55°C for 2 min, centrifuged at 14 000xg for 2 min (2. Elution). The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer after 1st and 2nd elution (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to 5 and 25 ng/µl concentrations of DNA.

2.2.3.2.4 PowerSoil ® DNA Isolation kit (MoBio)

The PowerSoil®DNA Isolation it (MoBio) is a kit established for the isolation of genomic DNA from environmental samples containing high concentrations of humic acids. This method provides a high degree of purify of DNA, which allows for more successful PCR amplification of a variety of organisms including bacteria, fungi, algae and actinomycetes. In this study, the bacterial DNA was isolated from pooled freeze-dried leaves of *A. elatius* and *G. album* plant species which were sampled on 17 April 2015 using this kit with modifications to improve handling. Two replicates were prepared and weighed (50-80 mg). The homogenization step (5) was performed using horizontal Retsch Mixer MM 400 at max. speed for 1 min. 500 µl supernatant and was transferred to a new tube (step 7). DNA was eluted with 100 µl PCR (prewarmed to 37°C) water (step 20). DNA was stored at -20°C until further application. The quality and quantity of extracted DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

2.2.3.2.5 PowerPlant®Pro DNA Isolation kit (MoBio)

The PowerPlant®Pro DNA Isolation kit (MoBio) is a method for fast and easy purification of total cellular DNA from plant cells, tissues and seeds. In this kit, Inhibitor Removal Technology ® (IRT) is used to remove PCR inhibitors from plant extracts. In this study, the bacterial DNA was isolated from pooled freeze-dried leaves of *A. elatius* and *G. album* plant species which were sampled on 17 April 2015 using this kit with modifications for better handling. Two replicates of pool were prepared and weighed (16-17 mg). The homogenization step (4) was performed using horizontal Retsch Mixer MM 400 at max. speed for 1 min. At step (7), 250 µl Solution PD3 with IRT were added to the samples. DNA was eluted with 100 µl PCR (preheated to 37°C) water, incubated for 2 min at room temperature (step 16). The eluate was transferred

to the white filtration membrane and centrifugated for 30 sec. at 10 000xg to provide maximum elution efficiency (step 17). DNA was stored at -20°C for further application. The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

2.2.3.2.6 CTAB extraction for plant

This CTAB method for DNA extraction from plant material was provided by the working group of Prof. Kogel of the department of Phytopathology in the Research Centre for Biosystems, Land Use and Nutrition in Giessen (Germany) and altered for this study. The method was used in this study to extract bacterial DNA from total frozen and homogenized leaves of *A. elatus* and *G. album* plant species. The leaves of *A. elatius* and *G. album* plant species were collected on 17 April 2014 from the FACE2FACE system (Linden, Germany), before fertilization as described in the chapter 2.2.1.2.2. All samples (AK, AT, AC, ACT and GK, GT, GC, GCT) were collected into two pools A and G, frozen in liquid nitrogen and stored at -80°C. Two replicates of each pool (A, G) were prepared (Tab. 2) and extracted by CTAB method. Before DNA extraction began, a CTAB lysis buffer was prepared (Tab. 3).

Table 2 Amount of leaf material used for DNA extraction by CTAB method

Leaves material	Environmental samples	Weight of total leaves, mg
Total frozen	F2F-t0-Pool A1	200
	F2F-t0-Pool A2	193
	F2F-t0-Pool G1	332
	F2F-t0-Pool G2	346
Frozen, homogenized	F2F-t0-Pool A1	211
	F2F-t0-Pool A2	184
	F2F-t0-Pool G1	249
	F2F-t0-Pool G2	182

Table 3 CTAB lysis buffer composition

Reagents	Amount to add (final 3 ml)	Concentration
CTAB (10%)	900 µL	3%
5 M NaCl	840 µL	28%
0.5 M EDTA pH 8	120 µL	4%
1 M Tris-Cl pH 8	300 µL	10%
PVP (MW 40 kDa)	0.09 g	3%
ß-mercaptoethanol	6 µL	0.2%
Water	744 µL	24.8%

First, to each sample in Eppendorf tube (2 ml) was added 700 µl CTAB lysis buffer, vortex and incubated at 65°C in heating block for 60 min. During this time, the solution was inverted 2 times. Then, 700 µl chloroform/isoamylalcohol (24:1) was added, mixed by inversion to form an emulsion. After centrifugation at 17 000xg for 12min, 550 µl of upper aqueous layer with DNA was transferred to a new tube. The step of DNA separation from RNA and proteins based on different solubilities of these molecules in not mixed liquids was repeated with chloroform/isoamyl alcohol (24:1) (555 µl for frozen, homogenized, 560 µl for total frozen leaves extraction). After centrifugation at 17 000xg for 12 min, about 450 µl/400 µl (homogenized/ total frozen leaves) of upper phase was transferred to a new tube. To supernatant, 2/3 volumes of ice-cold isopropanol (300 µl for homogenized/240 µl for total frozen leaves) were added, vortex and incubated at -20°C overnight. After precipitation overnight, the solution was centrifuged at 17 000xg for 30 min at 4°C. The supernatant was removed and washed with 1 ml cold 70% ethanol (4°C) at 17 000xg for 2 minutes. The supernatant was carefully removed using a pipette and the DNA pellet was dried in a SpeedVac (Bachofer). The dried DNA pellet was dissolved in 100 µl PCR water. To increase the dissolution of the dried DNA the samples were incubated in a heat block at 37°C for 5 min. DNA was stored at -20°C until further investigation. The quality and quantity of total extracted DNA was determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

2.2.3.2.7 DNA extraction by Pitcher et al. (1989), modified approach

The guanidium thiocyanate extraction method (Pitcher et al., 1989) is a convenient and cost-effective extraction method to gain high yields of high molecular weight DNA from Gram-positive and Gram-negative bacterial cells. In this study, this method was used for DNA extraction from a pure culture(I) and from environmental samples (II). For DNA extracts (I), the *Novosphingobium rhizosphaerae* strain JM-1^T (DSM 29344^T) was cultivated in liquid TS medium (200 ml) at 28°C for 24 hours. The cells were transferred to a 50 ml falcon, collected via centrifugation at 4 000xg for 30 min at RT. After removing the supernatant, cells were centrifugated again for 15 min at 4 000xg. The cells were stored at -20°C until extraction. Before DNA extraction, the thawed cells were transferred to a 2 ml Eppendorf tube, then centrifuged for 4 min by 17 000xg (Micro STAR 17R, VWR) and the DNA was extracted. The extraction protocol was the same for pure cultures and environmental samples but adapted to different reagents volumes (Tab. 4). The extracted total DNA was also used as DNA template for positive control of PCR.

Table 4 Volume of enzymes, chemicals per reaction and steps used for DNA extractions by Pitcher et al. (1989)

Enzymes, chemicals, steps of approach	Pure culture in 2 ml tube (I)	Environmental samples in 50 ml (II)
Lysozyme (50 mg/ml) (4°C)	100 µl	1 ml
GES-Puffer (RT)	50 µl	5 ml
SDS (RT)	50 µl (10% SDS)	750 µl (25% SDS)
RNase A (4°C)	15 µl	200 µl
Proteinase K (4°C)	15 µl	200 µl
7.5 M Ammonium-acetate (4°C)	100 µl	2.5 ml
Centrifugation at 4°C	25´ 17 000xg	20´ 4000xg
Chloroform/isopentanol (24:1) (4°C)	200 µl	5 ml
Centrifugation at 4°C	25´ 17 000xg	20´ 4000xg
Upper phase with DNA	150 µl	7 ml
Isopropanol, 0.54% of total volume (4°C)	81 µl	approx. 3.78 ml
Overnight -20°C	yes	no
Centrifugation at 4°C	5´ 17 000xg	20´ 4000xg
70% ethanol (4°C)	3x600 µl, 5´ 17 000xg	3x1 ml, 5´ 4000xg
Air dry	30´	30´
PCR water	75 µl	200 µl

For DNA extraction from environmental samples, leaves of *A. elatius* and *G. album* plant species were collected on 15 April 2014 from the FACE2FACE system (Linden, Germany), before fertilizing as described in chapter 2.2.1.2.2. All samples (AK, AT, AC, ACT and GK, GT, GC, GCT) were collected into two pools (A, G), frozen in liquid nitrogen and stored at -80°C. Two replicates of each pool (A, G) were prepared (Tab. 5) and extracted by a modified Pitcher et al. (1989) protocol.

Table 5 Weight of environmental samples for DNA extraction by modified Pitcher et al. (1989) method

Environmental samples	Weight of total leaves, mg
F2F-t0-Pool A1	544
F2F-t0-Pool A2	498
F2F-t0-Pool G1	562
F2F-t0-Pool G2	492

First, fresh lysozyme was added to each sample, then the sample was vortexed and incubated for 30 min at 37°C. The Incubation of the pure culture samples was performed in a 2 ml Eppendorf tube placed in a water bath and of the environmental samples were placed in a 50 ml falcon and heated in the heating block. For protein denaturation and inactivation of endogenous nucleases, GES puffer was added to the samples, vortexed and incubated for 15 min at 37°C. For further denaturation, SDS, RNase and protease were added, vortex and incubated for 45 min at 37°C. Lysates were cooled on ice for 5 min. Thereafter, proteins were salted out and nucleic acids were stabilized by adding frozen 7.5 M ammonium acetate for 10 min. Chloroform/2-pentanol was added after centrifugation to separate the DNA from the RNA and proteins. This separation is based on the different solubility of the molecules in the two-phase chloroform/2-pentanol solution. The centrifugation was performed for (I) sample using Micro Star 17R (VWR) and for (II) sample using Megafuge 1.0R Heraeus Instruments (Thermo Scientific). After centrifugation, three layers were formed, the upper liquid phase contained the soluble DNA, which was transferred to a new tube. Isopropanol was added to the supernatant (0.54% of total volume) and vortexed for 1 min. For DNA precipitation, only solution (I) was incubated overnight at -20°C. After centrifugation, the supernatant was discarded, and DNA was washed three times with 70% ethanol. A small volume of ethanol was carefully removed with a pipette. DNA pellets were dried by turning the tube over. DNA was

dissolved in PCR-water overnight at 4°C. DNA was stored at -20°C until further investigation. The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to the concentrations of 5 ng/µl and 25 ng/µl DNA.

2.2.3.2.8 CTAB extraction by Griffiths et al. (2000) modified approach

The bacterial DNA was isolated from total frozen leaf samples (see chapter 2.2.1.2.2) according to an extraction method modified by combining of two protocols (Griffiths et al., 2000, Nercessian et al., 2005) (Tab. 6). For extraction, two pools of each plant species (A, G) were prepared. Two replicates of each pool were prepared and weighed. Amount of leaf material was between 80-230 mg. Extraction was performed using 2 ml reaction tube with screwcap (ZR Plant/Seed DNA MiniPrep™ kit, Zymo Research) with 0.25g of each zirconia-silica beads 0.1 mm and 0.5 mm (Roth). Before starting, CTAB buffer was deployed from two solutions as 10% CTAB in 1.6 M NaCl (Griffiths et al., 2000, Nercessian et al., 2005) and 240 mM potassium phosphate buffer. Before, the 10% CTAB buffer was heated at 65°C to until it dissolved and was mixed with phosphate buffer in ratio (1:1). After the addition of 75 µl SDS (10%) and 75 µ N-lauroysarcosin (10%), 500 µl of phenol:chloroform:isomylalcohol (25:24:1) with pH 7 (must be pH 8) was added and homogenized for 30 sec at max. speed (Retsch Mixer MM 400). The separation of soluble DNA from protein and cell debris was performed by centrifugation at 17 000xg for 5 min at 4°C. After precipitation for 2 hours at room temperature, the DNA was obtained by elongated centrifugation to 30 min at max. 17 000xg (4°C). The DNA pellet was washed with 700 µl ethanol (70%) and then dried for 20 min using SpeedVac (Bachofer). The dried DNA was dissolved in 100 µl PCR water using a heat block heated to 37°C for 10 min. DNAs was stored at -20°C until further investigation. The quality and quantity of total extracted DNA was determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to concentrations of 5 ng/µl and 25 ng/µl DNA. The DNA extraction of the treatment (total frozen leaves without beads, leaves with 0.25g of 0.1 mm and 0.25 g of 0.5 mm beads, leaves with 0.5 g of 0.1 mm beads (Roth), was performed using a modified extraction protocol by Griffiths et al. (2000) (Tab. 6). The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). Then, each sample was diluted to ration of 1:10 and 1:100.

Table 6 Shot summary of DNA extraction protocols

Steps	Griffiths et al. (2000)	Nercessian et al. (2005)	Mod. Griffiths et al. (2000)
Zirconia -silica beads	no beats	0.5 g of 0.1 mm	0.25 g of 0.1 mm, 0.25 g of 0.5 mm
Extraction buffer: 10%CTAB NaCl Phosphate buffer	 500µl 0.7 M NaCl 240 mM	 750 µl 1.6 M NaCl 0.2 M	 500 µl 1.6 M NaCl 240 mM
SDS 10%		75 µl	75 µl
10% N-lauroysarcosin		75 µl	75 µl
Phenol:chlorophorm: isomylalcohol (25:24:1)	500 µl	750 µl	500 µl
Beat beating	30 sec at 5.5 m/s	30-60 sec 75% of power at 4°C	30 sec max. speed
Centrifugation	16 000xg 5 min 4°C	16 000xg 5 min 4°C	17 000xg 5 min 4°C
Chloroform:isomylalcohol (24:1)	equal volume	equal volume	equal volume
Centrifugation	16 000xg 5 min 4°C	16 000xg 5 min 4°C	17 000xg 5 min 4°C
30% PEG 6000-1.6 M NaCl	2 volumes	2 volumes	2 volumes
Incubation	2 h at RT	1-2 h at RT	2 h at RT
Centrifugation	18 000xg 10 min 4°C	18 000xg 10 min 4°C	17 000xg 30 min 4°C
Washed ethanol	70%	75%	700 µl of 70%
Centrifugation			17 000xg 10 min 4°C
Dry	air dried		20 min in a SpeedVac

2.2.3.2.9 DNA extraction by modified Suda et al. (2008) approach

The DNA extraction method according Suda et al. (2008) is a rapid, simple and inexpensive method for isolating microbial phyllosphere DNA from leaves without damaging leaf microstructure by using benzyl chloride, DNA purification is established by gel filtration from endogenous impurities (e.g. humic substances, polyphenol co-extracted from epicuticular wax). In this study, bacterial DNA was isolated using this method with modifications for better handling, using different amounts (approx. 0.5 g, 3 g, 5 g) of leaf material (fresh, frozen).

Table 7 DNA extraction protocol from Suda et al. 2008 and modifications

	Suda et al. (2008)	Suda et al. (2008) mod1
Extraction material	5 g non-shredded fresh mature leaf of trees	0.5 g, 3 g, 5 g total fresh and frozen leaves of *A. elatus* and *G. album*
Extraction buffer	5 ml	5 ml
10% SDS	1 ml	1 ml
Benzyl chloride	3 ml	3 ml
Incubation	At 50°C for 15 min	At 50°C for 15 min water bath
Mixing	1 min interval until 2 phases mixed	1 min interval until 2 phases mixed
Removal of leaves	yes	yes
3 M sodium acetate pH 5	3 ml	3 ml
Incubation	On ice for 10 min	On ice for 10 min
Centrifugation	6 000xg for 15 min at 4°C	**4 000 rpm (3345xg) for 15 min at 4°C**
Aqueous phase transfer		900 µl (6 ml in 2 tubes)
Isopropanol	Equal volume	Equal volume
Centrifugation	9 000xg for 15 min at 4°C	**13 300 rpm (17 000xg) for 15 min at 4°C**
Dry	Air drying	**40 min by SpeedVac at RT**
Dilution	200 µl TE buffer	**100 µl PCR water**
Incubation		10 min at 37°C

The fresh leaves of *A. elatius* and *G. album* were collected on the extraction day as presented in Fig. 4. The middle of both leaves was placed in a sterile beaker in a grey Styrofoam box with ice and then pooled. Two replicates were prepared using 50 ml Falcons (Greiner) in each pool (A, G) for each amount of fresh and frozen leaves and then extracted according to Suda et al. (2008) using the modified protocol 1 (Tab. 7). After precipitation with 3 ml 3 M sodium acetate pH 5 and incubation for 10 min on ice, samples were centrifugated for 3 345 xg at 4°C. 900 µl the aqueous phase with DNA (in total 6 ml) was transferred to a new 2 ml centrifuge tube and precipitated by equal volume of isopropanol. The total aqueous phase was completely centrifugated at 17 000xg for 15 min at 4°C and supernatant was discarded. The DNA pellet was dried for 40 min by SpeedVac (Bachofer) at room temperature and diluted with 100 µl PCR water at 37°C for 10 min. The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). For PCR reaction, replicates of each sample were merged and diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

2.2.3.2.10 NucleoSpin®Soil kit (Macherey-Nagel)

NucleoSpin®Soil kit (Macherey-Nagel) is used for isolation of total DNA from microorganisms like Gram positive and Gram-negative bacteria, archaea, fungi and algae in soil, sludge and sediment samples. This kit is based on mechanical lysis of the samples using the ceramic MN Bead tubes type A and two lysis buffer SL1 and SL2 in combination with Enhancer SX. The lysis buffer SL1 is suitable for soil samples predominantly with minerals and SL1 is for soil samples with organic carbon (e.g. humic acid). Enhancer SX guarantees high yields with excellent purity but fails when samples have a very high level of humic acid. In this case, Enhancer SX knows to reduce purity of DNA by releasing humic acid in the lysate. In this study, this kit was used to extract the bacterial DNA from different plant material (fresh, frozen or frozen dried leaves) to according to manufacturer's instructions using modifications to the process.

The DNA was extracted from freeze-dried leaves, collected in the FACE2FACE system (Linden, Germany) on 15 April 2014, see in chapter2.2.1.2.2), two replicates of each pool A and G were prepared and extracted using manufacturing protocol with the modification 1 for better handling (Tab. 8 mod 1). To all freeze-dried leaf samples (20-50mg), the SL1 buffer was added until the 1.5 ml mark on the NucleoSpin® Bead Tube Type A. Because very dry material can absorb large amounts of lysis buffer, an additional lysis buffer was added to the 1.5 ml. The cell lysis was performed in the Retsch MM 400 (Retsch) for 2 min at max speed at room

temperature. DNA was eluted with 100 µl PCR water, incubated for 1 min at room temperature, then centrifugated for 20 sec at 11 000xg. The quality and quantity of extracted total DNA was determined by photometric measurements using NanoDrop spectrophotometer (Thermo Scientific) (Tab. 13) and each sample was diluted to concentrations of 5 ng/µl and 25 ng/µl DNA. The bacterial DNA was extracted from frozen leaves and spiking fresh leaves of *A. elatius* and *G. album* plant species using the modified protocol 2 (Tab. 8). The leaves were taken (as described in chapter 2.2.1.2.2) on the extraction day across from the Research Centre for Biosystems, Land Use and Nutrition in Giessen (Germany). Some of the leaves were freshly used immediately, another part was frozen at -80°C. For spiking test I, 100 µl *Methylobacterium* cell suspension of isolate 1405 ($1.52x10^7$ not washed cells) were added to 140 mg the fresh leaves pool of *A. elatius* and *G. album* plant. Additionally, DNA was also extracted from 100 µl cell suspension of *Methylobacterium* isolate 1405 ($1.52x10^7$) to be used as a control (Tab. 8 mod 2). From 170 mg frozen leaves of *A. elatius*, of *G. album* and pool of *A. elatius* and *G. album* leaves, the bacterial DNA were obtained separately. The obtained total DNA was eluted twice with 50 µl PCR for 1 min at room temperature and centrifugated at 11 000xg for 30 s. The quality and quantity of these DNA was determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). DNA extracts from spiking samples (pool of fresh *A. elatius* and *G. album* leaves with *Methylobacterium* cells) were diluted to 5 ng/µl and extracts from frozen leaves were diluted to 5 ng/µl and 25 ng/µl DNA.

For spiking test II, to 175 mg fresh leaves pool of *A. elatius* and *G. album* plant species, 100 µl of *Methylobacterium* isolates 1405 ($7.03x10^4$ cells resuspend in 0.2% NaCl) and 100 µl *Sphingomonas* isolate 628 ($3.44x10^5$ cells resuspend in 0.2% NaCl) were added and bacterial DNA were extracted using modified protocol 4 (Tab. 8 mod 3). This protocol differs from the protocol 4 only in the elution step 10. The fresh leaves were taken (as described in chapter 2.2.1.2.2) on the extraction day across from the Research Centre for Biosystems, Land Use and Nutrition in Giessen (Germany). Additionally, DNA was also extracted separately from pool A and G (170 mg) and from 100 µl of *Methylobacterium* and *Sphingomonas* isolates. The obtained total DNA was eluted twice with 25 µl PCR for 1 min at room temperature and centrifugated at 11 000xg for 30 sec. The quality and quantity of extracted total DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13) and diluted to concentrations 5 ng/µl and 25 ng/µl DNA.

The bacterial DNA extraction from frozen leaves of *G. album* plant species grown in the GiFACE system (Linden, Germany) were performed using NucleoSpin®96 Soil kit (Macherey-Nagel) with modified manufacturing protocol 1 (Tab. 9). The 102 leaf samples were collected as described in chapter 2.2.1.2.2. For DNA extraction, approximately 175 mg of leaf material was mechanically homogenized using in Retsch MM 400 (Retsch) at 30/s sequency for 5 min. The extraction steps 1-4 were performed according to protocol NucleoSpin®Soil kit (Macherey-Nagel) with modification 4. Steps, 5-10 were performed according to protocol NucleoSpin®96 Soil kit with modification 1. The lysates with SL1 were placed on the removal plate (light gray rings), filtrated using vacuum at -0.2- 0.7 bar, collected in separate MN Square-well block, repeated with SL2 lysates (step 5). To each sample of MN Square-well blocks 1 and 2 were added 250 µl SB (step 6). All samples were placed on one Binding plate (light green rings) and using vacuum at -0.2-0.6 bar (step 7). Five samples were eluted using the protocol with modification 1, 30 µl PCR water for 1 min at vacuum at -0.2-0.4 bar, then repeated with 50 µl for 1 min. Concentration of DNA was measured and eluted again with 30 µl PCR water for 1 min (total volume 110 µl). The quality and quantity of total extracted DNA was determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13) as undiluted, as well as diluted to concentrations 5 ng/µl and to a ration of 1:10.

Table 8 Modifications to the manufacturers protocol of NucleoSpin®Soil kit (Macherey-Nagel)

NucleoSpin ® for Soil Kit (Macherey-Nagel)										
steps→	1	2	3	4	5	6	7	8	9	10
	Prepare samples	Adjust lysis conditions	Samples lysis	Precipitate contaminants	Filter lysate	Adjust binding conditions	Bind DNA	Wash silica membrane	Dry silica membrane	Elute DNA
Mod1	**SL1 buffer fill up to 1.5 mL mark**	150 µL SX	**In Retschmülle 2 min**	11 000xg 2 min, 150 µL SL3, vortex 5 s, on ice 5 min, 11 000xg 1 min	Load supernatant on column (red ring), 11 000xg, 1 min	250 µL SB vortex 5 s	Load 550 µL on column (green ring), 11 000xg 1 min, load remainder, 11 000xg 1 min	500 µL SB, 11 000xg 30 s, 550 µL SW1, 11 000xg 30 s, 700 µL SW21, vortex 2s, 1 000xg 30 s (2 times)	11 000xg, 2 min	**100 µL PCR water, RT 1 min, 11 000xg 30s**
Mod 2, spiking I	**first steps 1-5 with 700 µL SL1 then repeat with SL2**	150 µL SX	**In Retschmülle 5 min**	**11 000xg 2 min, transfer SL1 in a new tube, SL2 in old tube again, 150 µL SL3, votex 5 s, on ice 5 min, 11 000xg 1 min**	**11 000xg 1 min, transfer SL1 in a new tube, SL2 in old tube again**	**to each tube (2 lysates) add 250 µL SB**	**Both extracts on one column (green ring)**	500 µL SB, 11 000xg 30 s, 550 µL SW11,1 000xg 30 s, 700 µL SW21, vortex 2s, 1 000xg 30 s (2 times)	**11 000xg 2 min 2 times**	**2 times 50 µL PCR water**, RT 1 min, 11 000xg 30s
Mod 3 spiking II	first steps 1-5 with 700 µL SL1 then repeat with SL2	150 µL SX	In Retschmülle 5 min	11 000xg 2 min, transfer SL1 in a new tube, SL2 in old tube again, 150 µL SL3, votex 5 s, on ice 5 min, 11 000xg 1 min	11 000xg 1 min, transfer SL1 in a new tube, SL2 in old tube again	to each tube (2 lysates) add 250 µL SB	Both extracts on one column (green ring)	500 µL SB, 11 000xg 30 s, 550 µL SW11,1 000xg 30 s, 700 µL SW21, vortex 2s, 1 000xg 30 s (2 times)	11 000xg 2 min 2 times	**2 times 25 µL PCR water,** RT 1 min, 11 000xg 30s

Table 9 Modifications to manufacturers protocol of NucleoSpin®96 Soil kit (Macherey-Nagel)

NucleoSpin ® 96 Soil Kit (Macherey-Nagel)										
steps→	1	2	3	4	5	6	7	8	9	10
Extraction steps	Prepare samples	Adjust lysis conditions	Samples lysis	Precipitate contaminants	Filter lysate	Adjust binding conditions	Bind DNA	Wash silica membrane	Dry silica membrane	Elute DNA
mod 1	Steps 1-4 were performed for individual samples as in the NucleoSpin® for Soil manufactiring protocol with modification 4, mechanical homogenisation in Retsch Mixer Mil MM 400 (Germany) at 30/s sequency for 5 min				load all samples on Removal plate (light gray rings) - 0.2-0.7 bar, **MN Square-well blocks for each buffer**	add 250 µL SB to each sample of **MN Square-well block 1 and 2**	**load all samples (approx. 1 mL fit) on one Binding plate (light green rings) -0.2-0.6 bar, repeat with the rest**	500 µL SB -0.2-0.6 bar, 550 µL SW1 -0.2-0.6 bar, 700 µL SW2 -0.2-0.6 bar (2 times)	full vacuum for 15 min	**30 µL PCR water 1 min -0.2-0.4 bar, add 50 µL PCR water 1 min -0.4 bar, 30 µL PCR water 1 min -.04 bar**

2.2.3.3 Alternative DNA extraction methods

2.2.3.3.1 DNA extraction using a modified approach of Griffiths et al. (2000) after detachment and collecting cells by centrifugation, Sterivex filtration and Percoll

The detachment method in combination with cells collecting by centrifugation and filtration and separation by Percoll density gradient centrifugation (Sigma-Aldrich) was used to preserve the leaf-associated bacteria and to reduce the contamination of chloroplast and mitochondria. Subsequently, DNA was extracted from the cells using the modified protocol of Griffiths et al. (2000) (chapter 2.2.3.2.8). First, the fresh leaves of *A. elatius* and *G. album* plant species collected on 17 April 2015 as described (chapter 2.2.1.2.2) were pooled and distributed into falcons (50 ml) according to the experiment. For the centrifugation and filtration of 1-1.5 g from the pool and for separation by Percoll, 2-3 g of the pool were weighed in one 50 ml falcon. The bacteria were detached from leaf material with 15 ml autoclaved PBS buffer (4°C) by shaking it in the dark for 15 minutes at 8 levels of horizontal force (Edmund Bühler, Tübingen) at room temperature. Supernatant was transferred to a new tube. For sonification, 15 ml PBS buffer was added again to samples with leaves, sonicated for 7 min at max (Ultrasonic cleaner, Sonorex). For filtration, both supernatants of the wo samples were merged to 60 ml and filtrated by Sterivex filter (pore size 0.22 µm). Filtrate was frozen using liquid nitrogen, stored at -80°C. For bacterial cells collected by centrifugation, two 50 ml falcons with each 30 ml of supernatants were centrifuged at 16 000xg for 20 min at 4°C in the centrifuge (Sorvall with rotor SS-34 ANGLE). The supernatant was removed but each cells pellet was resuspended in 0.5 ml PBS buffer and transferred to one 2 ml centrifuge tube. After the addition of 0.5 ml PBS buffer, cells pellet was washed in 1.5 ml PBS buffer, centrifugated at 16 000xg for 20 min at 4 °C. Supernatant was removed and the collected cells were frozen and stored at -20°C until extraction. For separation of the bacterial cells from the leaves,

Percoll media was used for density gradient centrifugation (Sigma-Aldrich). Then, 30 ml cells suspension were filtered through nylon mesh filter (pore size 100 µm) and placed carefully on the top of 6 ml (80%) Percoll. After centrifugation at 800xg for 5 min at 4°C (Megafuge 1.0R Heraeus Instruments, Thermo Scientific), the separation of the phases was difficult to recognize. The cell suspension above the Percoll was transferred to a new falcon and centrifugation was repeated at 3 200xg for 15 min at 4°C. The cells pellet was washed two times with 1 ml PBS buffer in a 2 ml centrifugation tube at 3 800xg for 15 min at 4 °C and was frozen at -20°C. Before extraction, Sterivex filter, cells pellet after centrifugation and Percoll were resuspend in 500 µl CTAB buffer, transferred to a 2 ml tube with 0.25g of each zirconia-silica beads 0.1 mm and 0.5 mm and according to protocol as described above (chapter 2.2.3.2.8) was extracted with a screw. The quality and quantity of DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13) and they were diluted to a ratio of 1:10 and 1:100.

2.2.3.3.2 DNA extraction by NucleoSpin®Soil kit (Macherey-Nagel) with a modified protocol after cell separation by gradient centrifugation with Nycodenz and different detachment and filtration approaches

Nycodenz is a nonionic iodinated density gradient medium (Rickwood et al., 1982), which is suitable to separate a variety of different cell types, viruses, subcellular organelles and other membrane compartments. In this study, this technique was used to separate the bacterial cells from plant organelles. Then from the obtained cells, DNA were extracted using NucleoSpin®Soil kit (Macherey-Nagel) with modification 3 (Tab. 8) of manufacturing protocol. The fresh leaves of *A. elatius* (1 g) were collected across the Research Centre for Biosystems, Land Use and Nutrition (Giessen) (Fig. 4) as described in chapter (2.2.1.2.2). The leaves were homogenized in falcon (50 ml) with 15 ml 50 mM Tris-HCl buffer (pH 7.5) three times for 1 min using Ultra-Turrax (Janke&Kunkel, IKA Labortechnik, Germany). The supernatant was transferred to a new falcon, filled up to 40 ml with Tris-HCl buffer pH 7.5 and supplemented with 1% Triton X-100 (Roth) direct before use. Triton-100 is an effective detergent for the membrane system of chloroplasts (Deamer and Crofts, 1967) and less destructive to bacterial cells (Ikeda et al., 2009). Then 9 ml Nycodenz (80%) was added under the 40 ml sample and centrifuged for one hour at 3 345xg with a swing-out centrifuge in the slow setting. After centrifugation, three phases were observed (Fig. 21B). For bacterial cells fluorescence staining with SYBR Green I (Sigma-Aldrich), 250 µl of phases (I, II) were transferred in a 1.5 ml tube and

filled up to 1 ml with Tris-HCl buffer pH 7.5. These 1 ml of each sample were filtered on a black polycarbonate filter with 0.22 µm pore size and stained with SYBR Green I. For collecting bacterial cells via centrifugation, 1 ml from phase I and II was transferred to 2 ml tube and centrifuged at 17 000xg for 10 min. The bacterial cells in the supernatant were also stained with SYBR Green I (Fig. 21B). Because bacterial cells were still present in phase I, they were collected on a membrane filter (Sterivex, pore size 0.22 µm) and frozen at -20°C.

In this study, other methods were also tested to detach and to collect the leaf-associated bacteria from fresh leaves of *A. elatius* and *G. album* plant species. For cell detachment from approx. 10 g fresh leaves of each plant species (*A. elatius*, *G. album*), leaves were collected in Whirl-Pak® bag (540 ml) on 24 May 2016 (treatment 1) and 17 April 2016 (treatment 2) across the Research Center for Biosystems, Land Use and Nutrition (Giessen) (Fig. 4) as described in chapter 2.2.1.2.2 for detachment. Cells were washed from the leaves with cooled on ice 100 ml PBS buffer (pH 7) supplemented with 0.1% Silwet L-77 by mechanical treatment for 120 s at normal speed in a Stomacher 80 (Biomaster, Seward Laboratory Systems Inc., USA). Silwet L-77 is a surfactant with physical properties such as low interfacial tension, friction coefficient and surface tension and was added to better separate bacteria from the leaves. Additionally, cells were also detached using sonification for 3 min at max in ultrasonic cleaner (Sonorex). After, cells were separated from the leaf material by filtration through nylon mesh filter (pore size 100 µm) and cell suspension was collected in a beaker (500 ml). From the washing step (first step) to collecting the cell suspension after filtration, the procedure was repeated twice. The leaves in the Whirl-Pak® bag were rinsed with 100 ml PBS buffer (treatment 1) two times or with 50 ml (treatment 2) one time. Suspended bacterial cells of each treatment were collected in a Sterivex filter with pore size 0.22 µm (Millipore). To reduce chloroplasts in the cell suspension (treatment 1), a prefilter with a pore size of 5 µm (Sartorius, Göttingen, Germany) was used before the bacterial cells were collected on the Sterifex filter unit. After filtering 50 ml of cell suspension from *A. elatius* leaves (treatment 1), this prefilter was completely blocked, and then the cell suspension obtained from both plant species was collected in a Sterivex filter with pore size 0.22 µm. Cell suspension obtained from *A. elatius* leaves of treatment 2 was filtrated using a prefilter with a pore size 8 µm (Sartorius, Göttingen, Germany) in the vacuum filtration system (Nalgene). These prefilters were blocked after filtration of 210 ml of cell suspension. All Sterivex filters were stored at -20°C. DNA extraction from Sterivex filter (treatment 1) was performed using the NucleoSpin®Soil kit

(Macherey-Nagel) manufacturing protocol with modification 3 and from Sterivex filter (treatment 1) using the same kit but with modification 4. For DNA extraction, Sterivex filter was broken open and the filter was placed in a 2 ml tube with ceramic beads and secured with a screw. The lysis steps 1-5 of the manufacturers protocol were performed with lysis buffer SL1. After the first centrifugation in step 4, the supernatant was transferred to a new tube. Then, 150 µl of precipitation SL3 buffer was added to supernatant (tube 1). Steps 1-5 were repeated but with lysis buffer SL2. To the same NucleoSpin®Beat Tube, 700 µl SL2 were added and continued according to protocol. After lysis with both buffer and precipitation of contaminants (step 4), two tubes with supernatant were obtained. Each supernatant was filtered with extra NucleoSpin®Inhibitor Removal Column (red ring). After centrifugation, binding conditions were adjusted with 250 µl SB for each lysate (2 tubes). Both DNA extracts (550 µl of each sample) were placed on one NucleoSpin®Soil Column (green ring). After washing (step 8), silica membrane with total DNA was dried two times at 11 000xg for 2 min. For modification 3, the obtained total DNA was eluted with 25 µl PCR water for 1 min at room temperature and centrifugated at 11 000xg for 30 sec two times (end volume 50 µl) and for modification 4, DNA was eluted with 100 µl PCR water (mod 4). The quality and quantity of this DNA were determined by photometric measurements using NanoDrop spectrophotometer (see chapter 2.2.3.4, Tab. 13). DNA extracts from both treatments were adjusted to 5 ng/µl and 25 ng/µl DNA concentrations.

2.2.3.4 Total DNA quantification by spectrophotometry

Concentration and purity of the obtained total DNA after direct DNA extractions and in combination with detachment, cells collection and separation methods from environmental samples were measured using NanoDrop spectrophotometer (Thermo Scientific). The nucleic acids measurement is based on the absorption of UV-light at 260 nm and characterized with a peak. Concentration of nucleic acids were automatically calculated from measured absorbance values at 260 nm, using the Beer-Lambert law:

$$c = \frac{A}{\varepsilon L}, \text{ were}$$

C=nucleic acid concentration in molar (M)

A=UV absorbance in absorbance units (AU)

ε=wavelength dependent molar or extinction coefficient ($M^{-1}cm^{-1}$)

L=light path (cm).

At a wavelength of 230 nm, co-extracts of organic compounds such as humic acids, but also saccharides, chaotropic salts and other contaminants are measured. The ratio of the absorption A260/230 provides information about polysaccharide contamination and other contaminants. The values should be between 2.0-2.2, but values below 2.0 indicate contamination of the DNA. Values up to 1.5 are still acceptable. The absorption of the proteins is at 280 nm. The ratio of the absorption A260/A280 provides information about the protein contamination in the sample. The values should be between 1.8-2.0. If the value is below 1.8, this indicates protein contamination. Whereas higher values indicate RNA contamination. But contamination of proteins and RNA at the same time can compensate each other and lead to perfect result. To measure concentration and purity of DNA, 1.5 µl of a sample was used (Tab.13).

2.2.3.5 Agarose gel electrophoresis of DNA extracts and PCR products

Agarose gel electrophoresis is a biochemical and molecular biological method for separating extracted DNA or PCR fragments in the agarose gel according to their length in an electric field. This method was used for DNA quality and yield control, for control of the length of PCR-products and for DNA purification. Agarose powder (Sigma) is a polymer extracted from agar or agar-bearing marine algae. This linear galactan hydrocolloid consist of copolymer D-galactose and 3,6-anhadro-L-galactose. They are connected by α (1→3) and β (1→4) glycosidic bonds (Meena et al., 2014). The linear, double stranded DNA (dsDNA) sequences, which are negatively charged, migrate through the agarose gel to an anode in the chamber filled with an ionic 1xTBE buffer. Migratory speed is influenced by the length and conformation of the dsDNA, agarose gel concentration and constant voltage. Gel concentration can be between 0.5-3% and the higher the agarose is concentrated, the smaller the pores. The DNA ladder with differentiated DNA fragments is used to estimate the length of PCR-products or to detect a high-molecular weight of total DNA after the separation of the samples. After electrophoretic separation, the DNA can be stained with ethidium bromide to make them visible. Ethidium bromide is an organic dye with planar structure that can easily intercalate between nucleic bases of DNA and cause mutation. Ethidium bromide can be excited by UV radiation (254-366 nm) emitting light in the orange-red range (590 nm) (Sambrook&Russel, 2001).

In this study, 1% of agarose gel to control total DNAs as well as 1.4% agarose gel to control PCR fragments were used. For agarose gel, 1 g or 1.4 g agarose powder were boiled in

a microwave (Bosch) at max W in 100 ml 1xTBE buffer until the agarose powder has completely dissolved. The gel was cooled to 60 °C at room temperature on a magnetic stirrer and slowly poured into prepared gel slides with comb without air bubbles. After one hour, the solid gel was placed in the electrophoresis chamber (PeqLab), completely covered with 1xTBE buffer. After removing the comb, the agarose gel was loaded with 3 µl of total extracted DNA or 1.5 µl of PCR-product mixed with 2 µl loading dye solution for DGGE (Fermentas) in each slot. 1.5 µl each of GeneRuler™ 100 bp Plus DNA Ladder (Fermentas) as a control of PCR products or GeneRuler™ 1 kb Plus DNA Ladder (Fermentas)as a control of total DNAs. DNA was loaded into the first agarose gel slot as well in the last slot and asymmetrically in the middle slots. The separation of total DNAs was performed at constant voltage of 10 V/cm (130 V) and varying amperage for 45 min. After, the agarose gel with DNA was stained with ethidium bromide (one drop per liter of 10 mg/ml, Roth) for 30 min at room temperature and covered with dark lid. To remove the residuals of ethidium bromide from DNA, agarose gel was placed into ultrapure water for 15 min at room temperature. Documentation of agarose gel was performed using the documentation system (Quantum vilber lourmat (PeqLab, Germany).

2.2.3.6 Polymerase chain reactions (PCRs)

Polymerase chain reaction (Mullis et al., 1986, Saike et al., 1988) is a molecular biological method which enables exponential amplification of gene fragments using two hybridizing oligonucleotides and a thermostable DNA polymerase (*Taq*™ DNA-Polymerase, Chien et al., 1976). PCR amplification is performed in a T100™Thermal cycler (Bio-Rad, Germany) with adjusted reaction conditions. In initial denaturation, dsDNA strands were denaturalized to single strands at 94-98°C by breaking hydrogen bonds. The cycle begins with the denaturation step, to ensure that the DNA template and primers are completely separated. The primers (15-30 nucleotides), which differentiate by a beginning (forward primer) and an end (reverse primer) of amplification, bind to single stands (5´→3´ arrow direction) at primer specific annealing temperature. The next step is either extension or elongation, where DNA polymerase begins at the 3´ ends of primers and synthesizes the missing strand with free nucleotides. To obtain enough amplicon, a cycle (denaturation, annealing, extension) is repeated 30–35 times. After the last PCR cycle, final elongation is performed to fill in amplification of any single incomplete stranded DNA. For PCR amplification, PCR master mix was prepared for the number of reactions and adding one more. Master mix was distributed

into PCR tubes and then the DNA template was added. The content of master mix includes 1xDream *Tag* buffer with KCl, $(NH_4)_2SO_4$ and 20 mM $MgCl_2$ dNTPs, dNTPs for elongation, both primer (forward, reverse), BSA for stabilization of the *Taq* polymerase against inhibitors for examples as humic acids, iron residuals (Kreader, 1996). Dream *Taq*™ DNA-Polymerase (Thermo scientific) is an enhanced *Taq*™ DNA polymerase optimized for all standard PCRs, with higher sensitivity, longer PCR products with 3´-dA overhangs and higher yields. Depending on the application, PCR with different total reaction volumes were used (Tab. 10). PCR reaction volume of 10 µl was sufficient for control of PCR products, but for sequencing 25 µl of total reaction volumes was required. A higher volume of 50 µl PCR with environmental template was required for DGGE analysis. Subsequently, 1 µl of extracted DNA with concentration of 5 and 25 ng/µl as undiluted as well as diluted (1:10, 1:100) or 1 µl of undiluted DNA cell lysate as template was added to 10 and 25 µl volume master mix and 2 µl of DNA template was added to 50 µl PCR reaction volume (Tab. 10). For each PCR, no template control and positive control with bacterial DNA were used to verify amplification specificity and efficiency.

Table 10 PCR Master mix and DNA templates

Reagents	Concentration	Total vol. 10 µl	Total vol. 25 µl	Total vol. 50 µl
PCR-water		6.46	17.85	36.3
10xDream *Taq* buffer (Thermo scientific)	1x	1	2.5	5
dNTPs (Thermo scientific)	0.1 mM each	0.5	2.5	2.5
Forward primer	0.4 µM	0.4	0.5	2
Reverse primer	0.4 µM	0.4	0.5	2
BSA (Thermo scientific)	0.4 mg/ml	0.2	0.05	1
Dream *Taq* (Thermo scientific)	0.02 U/µl	0.04	0.1	0.2
Total extracted DNA	5 or 25 ng/µl	1 µl	1 µl	2 µl

All amplifications were performed in T100™ Thermal cycler (Bio-Rad, Germany) with reaction conditions presented in table 11 depending on the primer system and analysis.

Table 11 PCR program

Program №	1	2	3	4	5
Steps/primer	GC-339f/907r	GC-339f/907r, GC-968f/1378r	799f/1492r	341f/907r	799f/1492r
1.Initial denaturation	95°C 3 min	95°C 3 min	95°C 3 min	95°C 3 min	95°C 3 min
2.Denaturation	95°C 30 s	95°C 30 s	95°C 30 s	95°C 30 s	95°C 30 s
3.Annealing	55°C 30 s	55°C 30 s	55°C 30 s	55°C 30 s	55°C 30 s
4.Elongation	72°C 40 s	72°C 40 s	72°C 40 s	72°C 40 s	72°C 40 s
Cycles (go to step 2.)	x34	x34	x31	x34	x34
5.Final elongation	72°C 30 min	72°C 10 min	72°C 30 min	72°C 10 min	72°C 30 min
6.Store	10°C	10°C	10°C	10°C	10°C

For bacterial community composition based on fingerprint DGGE analysis, bacterial 16S rRNA gene fragments were amplified from extracted DNA using universal *Bacteria* targeting primers GC-339f (5`-CCTACGGGAGGCAGCAG-3` with a 40 bp-long GC-clamp 5´-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3´ at the 5´end and 907r (5`-CCGTCAATTCMTTTGAGTTT-3`) (Muyzer et al., 1993). PCRs were performed in a total volume of 50 µl (Tab. 10) with program 1 (Tab. 11). The final elongation step was extended to 30 min to avoid double band formation in DGGE gel (Jense et al. 2004). Yield and specificity/length of the amplified DNA fragments (410 bp) were controlled by 1.4% (w/v) agarose gel electrophorese and DNA staining in ethidium bromide (Roth, Germany). For control efficiency of DNA extractions, DNA extracts were checked for the presence of co-extracts such as chloroplasts, mitochondria using universal bacterial primer systems GC-339f/907r and GC-968f (5`-AACGCGAAGAACCTTAC-3` with a 40 bp-long GC-clamp 5´-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3´ at the 5´end and 1378r (5`-GGTGTGTACAAGGCCCGGGAAGG-3`) (Heuer et al. 1997) with program 2 (Tab. 11). For detection of bacterial DNA, extracted from environmental leaf samples, bacteria universal primer system 799f (5´-AACMGGATTAGATACCCKG-3´, Chelius and Triplett, 2001) and 1492r (5´-ACGGYTACCTTGTTACGACTT-3´, Lane (1991)) was used. In PCR program 3 (Tab. 11), the number of cycles was reduced to 31 cycles, because a higher PCR product yield was expected.

2.2.3.7 Identification of DGGE band sequences

Three single DNA bands of the DGGE gel (Fig. 16c) were cut out, purified using a modified protocol from Dohmann and Tebbe (2003). After purification, all single bands were amplified with a reaction volume of 10 µl with the primer system GC-339f/907r (Muyzer et al. 1993) and with PCR program 1 (Tab. 11). To control the purity of the bands, the purified bands of sufficient intensity were placed back into the DGGE gel, but parallel together with environmental samples. Two single bands with similar DNA content, extracted from different plant species leaves (A, G) were sequenced using the NucleoSpin®Plant II kit (Macherey-Nagel) and a ZR Plant/Seed MiniPrep™ kit (Zymo Research). One single band with different a content to other sequences (from *G. album* leaves and extracted using ZR Plant/Seed MiniPrep™ kit, Zymo Research) was also sequenced. The bands 1-3 (Fig. 16c) were reamplified with primer system 341f/907r (Muyzer et al. 1993) with program 4 (Tab. 11) and a reaction volume of 50 µl (Tab. 10). After quality control of PCR product by 1.4% (w/v) agarose electrophoresis, partial 16S rRNA gene fragments were sequenced with the Sanger method using primer 341f by LGC Genomics (Berlin, Germany). The band sequences were manually trimmed at the 5´and 3´ends and internally corrected, based on the electropherograms using MEGA7 (Tamura et al., 2013). Sequences were phylogenetically identified by BLASTN analysis (Altschul et al., 1997) against the EzBioCloud type strain 16S rRNA gene sequence database (Yoon et al., 2017).

3 Results

3.1 Response of eCO_2 on the abundance and the community composition of oligo/heterotrophs and methylotrophs

3.1.1 No effect of eCO_2 on concentration of oligo/heterotrophs and methylotrophs

Enrichment and cultivation of mechanically detached oligo/heterotopic and methylotrophic bacteria of *A. elatius* and *G. album* leaves grew in the three aCO_2 and eCO_2 rings using the dilution-to-extinction method and subsequent testing using the drop-plate method (spot assays) (Fig. 5) showed no visual differences between the serial dilutions from both treatment

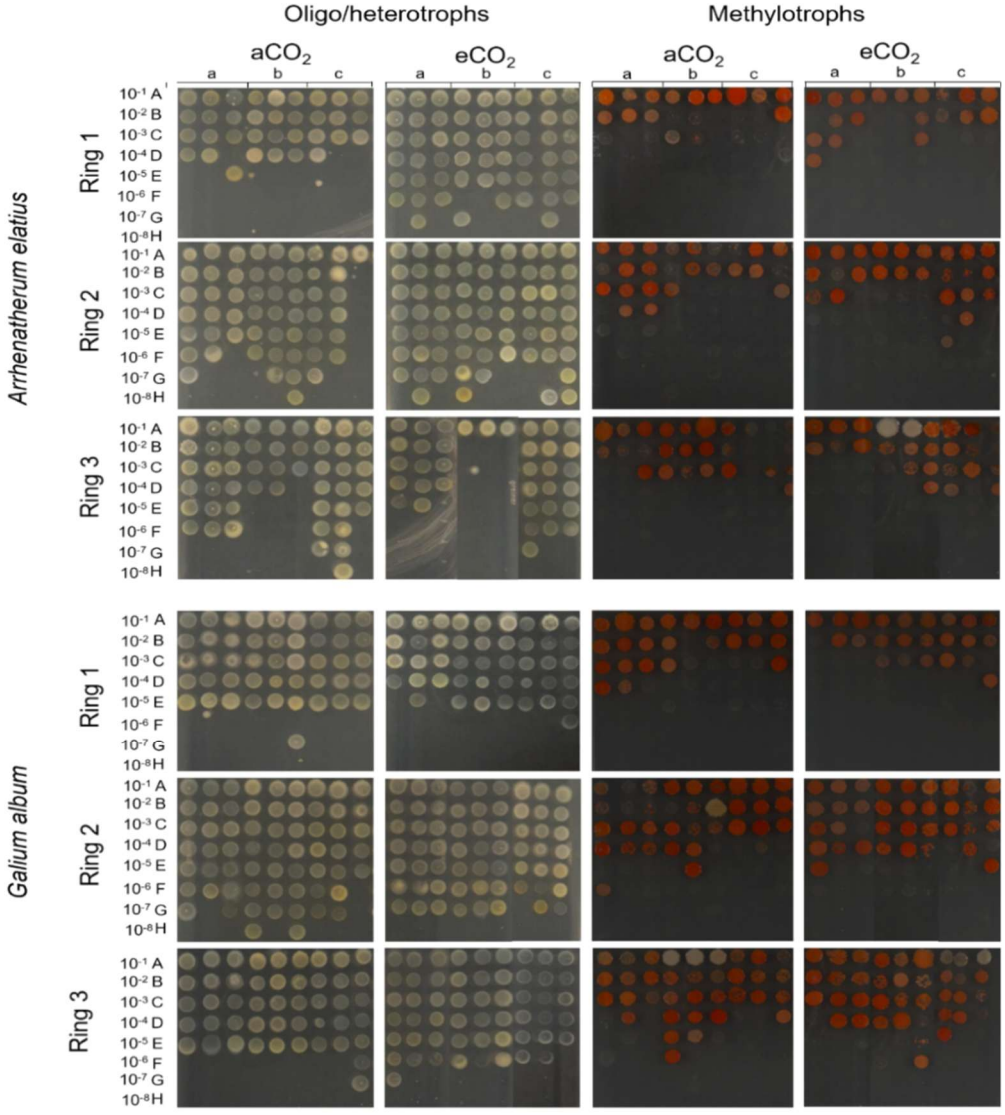

Figure 5 Spot assays of the dilution-to-extinction cultivation of abundant oligo/heterotrophs and methylotrophs enriched from leaves of *Arrhenatherum elatius* and *Galium album* grown under ambient atmospheric CO_2 (aCO_2) and elevated CO_2 (eCO_2) conditions in the GiFACE ring 1-3.Spot assays presented serial dilutions from 10^{-1} to 10^{-8} in triplicates (a, b, c) generated in 96 well plates with liquid ½ R2A medium (oligo/heterotrophs) and liquid M125 medium (methylotrophs) supplemented with 0.5% (v/v) methanol. The 5 µl of each dilution was spotted on agar plates containing the same medium and washed agar. Agar plates were inoculated for 7 days (a) / 14 days (b) at 25°C in the dark. Spot assay analysis was used for MPN calculation.

except for oligo/heterotrophic bacteria grown on *A. elatius* leaves in ring 1 under aCO_2 conditions. Furthermore, the spot assays showed that PPFMs were the most abundant among methylotrophic bacteria. MPN values were based on spot assays and estimated using the free MPN calculator by Jarvis et al. (2010) was calculated on MPN per g $_{\text{fresh leaves}}$ (Table 12). The concentration of all enriched oligo/heterotrophic bacteria was two to four orders of magnitude higher (10^8 to 10^{11} MPN per g $_{\text{fresh leaves}}$) than for PPFMs (10^6-10^8 MPN per g $_{\text{fresh leaves}}$) (Table 12, Fig. 6). There were differences in the concentration of PPFMs between plant species, but not between treatments (aCO_2, eCO_2). The concentration of PPFMs was higher on *G. album* leaves than on *A. elatius* leaves. No significant differences in the concentrations of oligo/heterotrophs were found between plant species, and PPFMs, they were also not affected by the eCO_2 concentration.

Table 12 MPN concentrations of enriched oligo/heterotrophic bacteria and PPFMs grown on *A. elatius* and *G. album* plant species.

		Experiment (aCO_2/eCO_2)	Ring	MPN means per g fresh leaves (± standard deviation)
Oligo/heterotrophs	*A. elatius*	aCO_2	1	1.6 (±1.2) ×10^8
			2	1.3 (±1.1) ×10^{11}
			3	2.3 (±3.2) ×10^{10}
		eCO_2	1	6.7 (±3.3) ×10^{10}
			2	4.0 (±3.1) ×10^{11}
			3	4.0 (±5.3) ×10^{10}
	G. album	aCO_2	1	8.6×10^9
			2	6.9 (±9.8) ×10^{11}
			3	1.2×10^{10}
		eCO_2	1	6.4 (±7.5) ×10^9
			2	1.8×10^{11}
			3	5.6 (±5.4) ×10^{10}
PPFMs	*A. elatius*	aCO_2	1	1.3×10^6 (±8.0 ×10^5)
			2	6.4×10^7 (±1.0 ×10^8)
			3	4.7 (±4.0) ×10^6
		eCO_2	1	3.1 (±2.4) ×10^6
			2	4.9 (±6.3) ×10^7
			3	1.0 (±1.1) ×10^8
	G. album	aCO_2	1	7.3×10^7(±1.2 ×10^8)
			2	3.5 (±3.4) ×10^8
			3	8.8×10^8 (±1.3 ×10^9)
		eCO_2	1	2.0 (±2.5) ×10^7
			2	9.9 (±3.5) ×10^7
			3	7.3 (±5.0) ×10^8

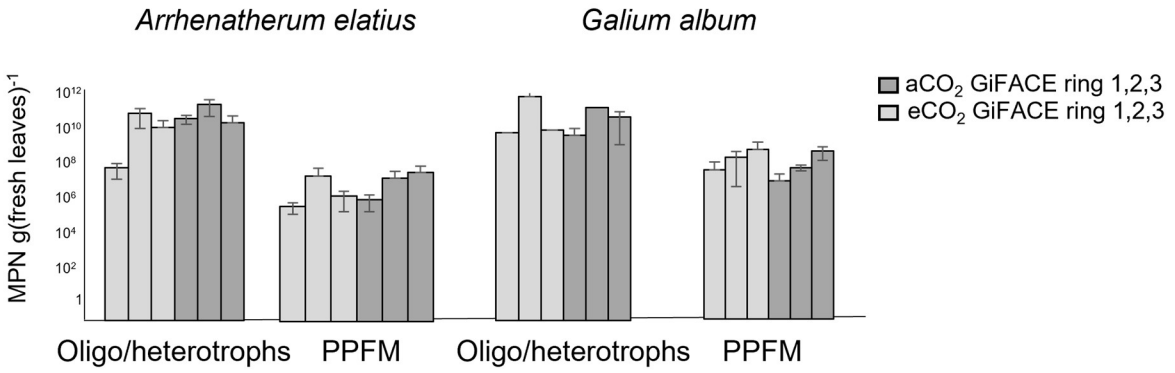

Figure 6 MPN concentrations of enriched cultured oligo/heterotrophs and PPFMs based on spot assays. Concentrations are given as most probable numbers (MPN) per g fresh weight of leaves different plant species (*A. elatius* and *G. album*) grown under ambient CO_2 (aCO_2) and elevated atmospheric CO_2 (eCO_2) conditions in the GiFACE ring 1-3. MPNs were calculated using MPN calculation program version 4 published by Jarvis et al. (2010). One-way ANOVA test for significant differences was performed in Sigma Plot (Applied Maths).

3.1.2 Shift in assemblages of the most abundant oligo/heterotrophs and methylotrophs

The influence of eCO_2 effect on the highest enriched bacterial assemblages as oligo/heterotrophs and methylotrophs on *A. elatius* and *G. album* leaves were studied by DGGE. For this purpose, bacterial assemblages of the last three highest positive dilutions (I, II, III), enriched by dilution to extinction, were analyzed. DGGE fingerprint patterns and their cluster analysis of the abundant bacterial assemblages (oligo/heterotrophs and methylotrophs grown on *A. elatius* and *G. album* leaves under aCO_2 and eCO_2 conditions in three rings) are presented in Fig. 7. The differences in DGGE fingerprint patterns between control rings (CC) and elevated CO_2 rings (CE) displayed significant differences in assemblages of most abundant oligo/heterotrophic and methylotrophic bacteria. Differences among treatments became more pronounced with increased serial dilution (II-III) of samples of the eCO_2 treatment.

Non-metric multidimensional scaling (NMDS) based on DGGE fingerprint patterns was used to compare the bacterial assemblages of abundant oligo/heterotrophic and methylotrophic bacteria and to show their correlation with environmental parameter (eCO_2). NMDS analysis showed the significant shift between CC and CE in the most abundant bacterial assemblages (oligo/heterotrophs and methylotrophs) enriched from both leaves (*A. elatius*, *G. album*), except methylotrophs of *A. elatius* leaves from ring 2 (One-way ANOSIMs, $P>0.081$, Fig. 8). In addition, NMDS revealed correlation of eCO_2 with significant differences of the assemblages.

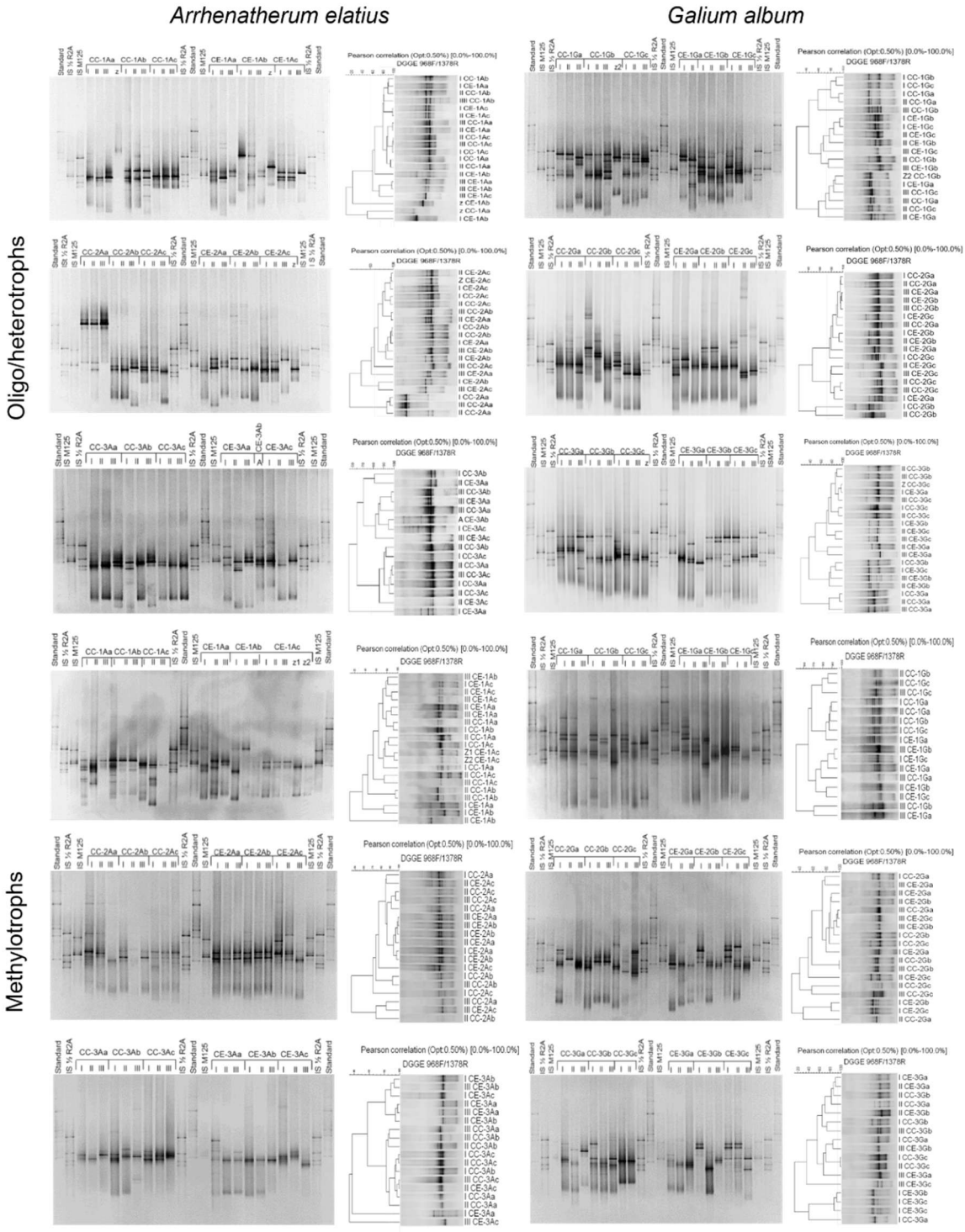

Figure 7 DGGE fingerprint patterns and cluster analysis of the abundant bacterial assemblages enriched oligo/heterotrophs and methylotrophs grown on the *A. elatius* and *G. album* leaves under control and elevated CO_2 conditions. Left: DGGE pattern based on bacterial 16S rRNA gene fragment amplified with primer system GC-968f/1378r (Nübel et al., 1996; Heuer et al., 1997) from the last three highest positive dilutions of the dilution-to-extinction cultivation. Right: Clustering based on DGGE fingerprint patterns were performed using unweighted pair-group method using arithmetic averages (UPGMA) with 1% position tolerance and 0.5% optimization in GelCompare II version 5.10 (Applied Meths, Sint-Martens-Latem, Belgium). A similarity matrix to compare the individual fingerprint pattern was calculated with the Pearson correlation which considered the presence/absence and intensity of DNA bands. CC: control CO_2 (with ambient atmospheric CO_2), CE: elevated CO_2 (approximately +20% CO_2 above ambient atmospheric CO_2 concentration), 1-3 rings of the GiFACE, A: *A. elatius*, G: *G. album* plant species, a, b, c: three defined collection points in each ring.

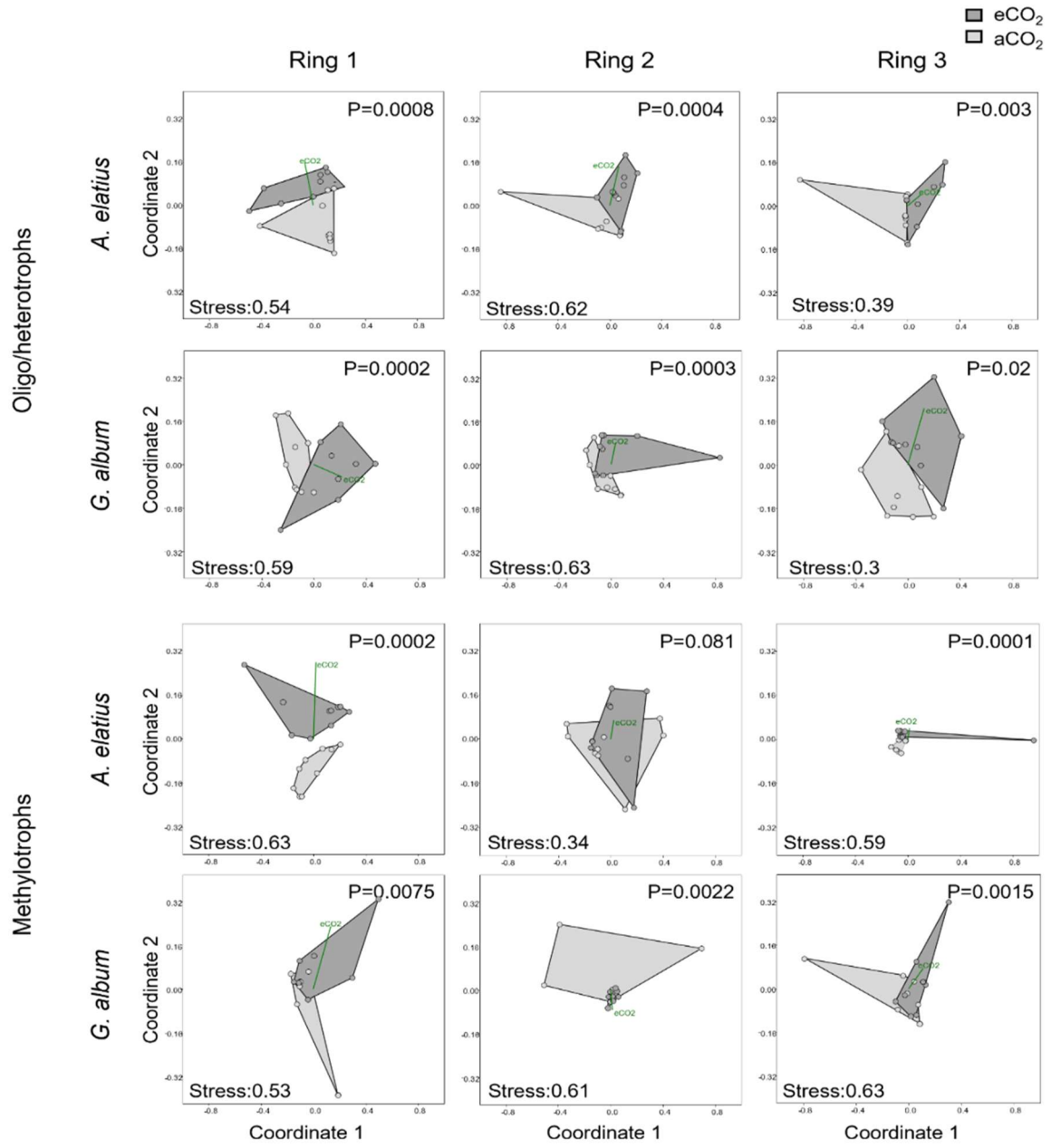

Figure 8 Non-metric multidimensional scaling (NMDS) analysis based on DGGE pattern of the three highest positive dilutions presenting the abundant enrichment cultured oligo/heterotrophs and methylotrophs grown on the leaves of *A. elatius* and *G. album* plant species under ambient CO_2 (CC) and elevated atmospheric CO_2 (CE) conditions in the GiFACE ring 1-3. DGGE pattern based on bacterial partial 16S rRNA gene fragments amplified from cell lysates generated from pool of the three highest positive dilutions (I-III) triplicates for each sample (a, b, c). NMDS analyses were carried out in PAST3 version 3.11 (Hammer et al., 2001) with similarity index Bray-Curtes. Statistical analysis was performed in PAST3 using One-way ANOSIM test (significance p-value <0.05). Light gray colored field: ambient CO_2 (CC), dark gray colored field: elevated CO_2 (CE).

The box plots were calculated using the interpolated quantile method. The box plots revealed the differences of variability in between CC and CE ring (Fig. 9). Ranked distance in the box plots showed the intra sample variability. Under CE, the variability of oligo/heterotrophic and methylotrophic bacteria on both plants is significantly lower than under CC, expect methylotrophs on *A. elatius* leaves from ring 2.

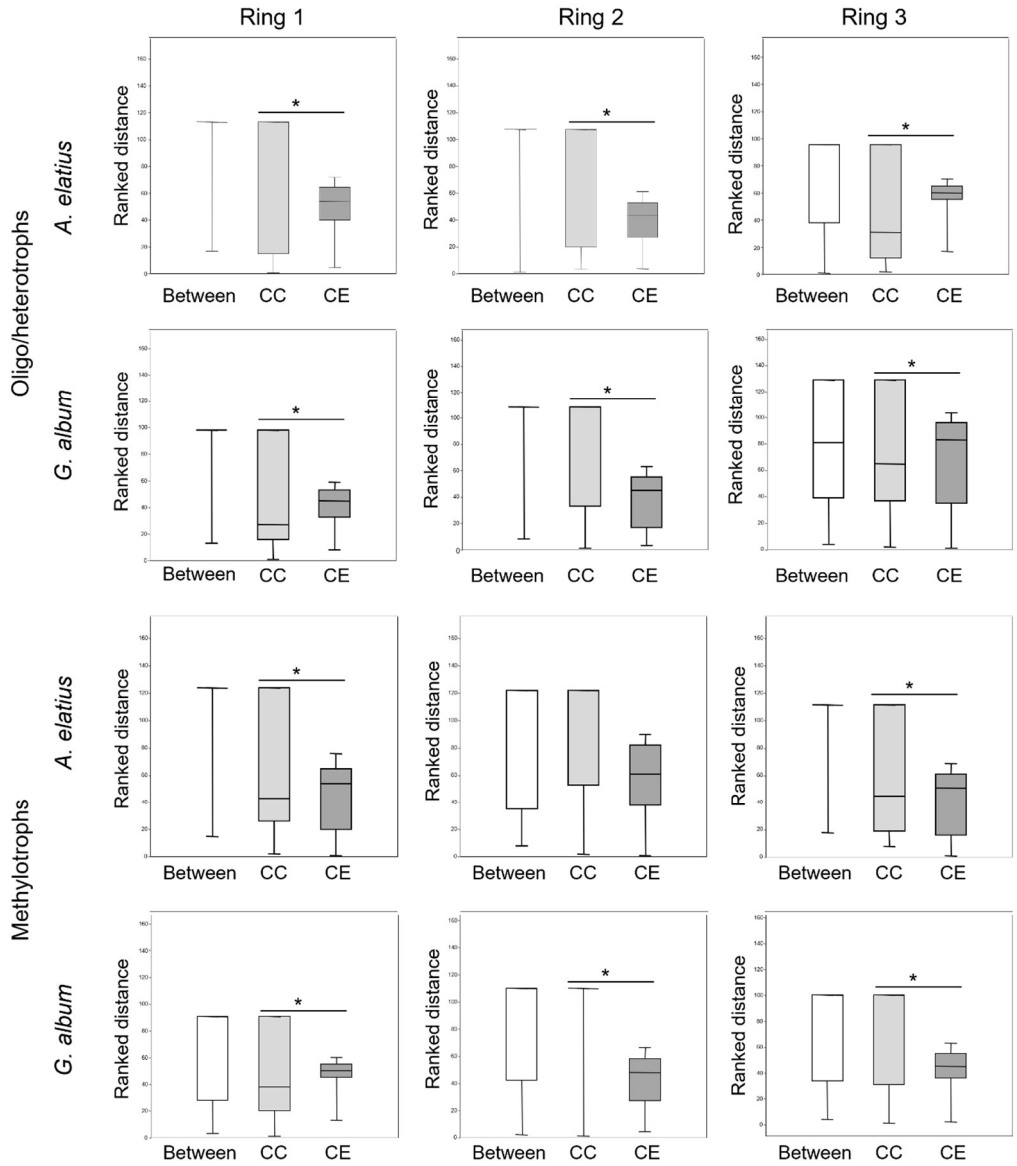

Figure 9 Box plots of the abundant bacterial assemblages enriched oligo/heterotrophs and methylotrophs grown on the leaves of *A. elatius* and *G. album* plant species under control and elevated CO_2 concentrations. The interpolation comparison of CC and CE groups based on quantile method and significant differences were computed using one-way ANOSIM test based on Bray-Curtis similarity index in PAST version 3.11 (Hammer et al., 2013). CC: control rings, CE: rings with elevated CO_2.

3.1.3 CO_2 effects abundance and diversity of different phylotypes

To determine how eCO_2 concentration affected the abundance and diversity of oligo/heterotrophic and methylotrophic phylotypes, pure cultures were obtained from the last three highest positive dilutions cultured using extinction-to-dilution. To clarify which of the isolates were specifically adapted to eCO_2 conditions and/or to plant species (*A. elatius, G. album*), isolates were differentiated into phylotypes. Phylotypes differentiation is based on partial 16S rRNA gene sequence similarity with >98.65% into monophyletic clusters in a calculated phylogenetic tree.

A total of 452 isolates (220 oligo/heterotrophs, 232 methylotrophs) were isolated and identified at genus level using partial 16S rRNA gene sequencing (Fig. 10a). All isolates were assigned to 38 genera belonging to the classes *Alpha-* and *Gammaproteobacteria, Actinobacteria, Bacilli, Flavobacteria* and *Sphingobacteria*. The comparison of the oligo/heterotrophic and methylotrophic isolates of both plant species at the genus level showed changes in the number of genera and their diversity in response to eCO_2 concentration (Fig. 10 a, b). The number of oligo/heterotrophic genera of the two plant species was significantly lower under eCO_2 conditions than under aCO_2 conditions (*A. elatius*: 20/13 Genera from aCO_2/eCO_2, *G. album*: 19/12). In contrast, the number of methylotrophic genera remained unchanged on both plant species (*A. elatius*: 16/14 genera of aCO_2/eCO_2, *G. album*: 13/12). Seriation analysis shows the difference in the diversity of oligo/heterotrophic and methylotrophic genera between both plants (*A. elatius*, *G. album*) and between both treatments (aCO_2, eCO_2) more clearly (Fig. 10b). From *A. elatius* and *G. album* leaves grown under eCO_2 concentrations were isolated eCO_2 treatment specific oligo/heterotrophic and methylotrophic genera (oligo/heterotrophic *Terracoccus*, *Herbiconiux* of *G. album* and *Brevibacillus*, *Anthobacter, Rhizobium* of *G. album*, methylotrophic *Pseudathrobacter*, *Pantoea*, *Nocardioides*, *Massala* of *A. elatius* and *Sauguibacter*, *Ensifer*, and *Pedobacter* of *A. elatius*).

As expected, eCO_2 has not only shifted diversity, but also the abundance of bacterial communities (Fig. 10a, b). The abundance (at genera level) of oligo/heterotrophs and methylotrophs on *A. elatius* leaves, as well as methylotrophs on *G. album* leaves, was slightly altered under eCO_2 concentration, except oligo/heterotrophs on *G. album* leaves. The most frequent oligo/heterotrophs such as *Curtobacterium* and *Stenotrophomonas* isolates of *A. elatius* leaves grown under aCO_2 conditions were augmented by *Microbacterium* under eCO_2 conditions. The abundance of methylotrophs *Methylobacterium*, *Pseudomonas* and *Stenotrophomonas* of *A. elatius* leaves grown under aCO_2 conditions were altered by replacing *Stenotrophomonas* with *Microbacterium* under eCO_2 conditions. The eCO_2 concentration did not affect methylotrophic *Pseudomonas* (13/13 isolates of aCO_2/eCO_2) on *A. elatius* leaves. On *the G. album* leaves, the methylotrophic abundance of *Methylobacterium*, *Rhizobium* and *Pseudomonas* under aCO_2 conditions was enhanced under eCO_2 conditions by *Sphingomonas*. However, oligo/heterotrophic *Pseudomonas*, *Plantibacter*, and *Sphingomonas* remained the most abundant genera on *G. album* leaves in both treatments (aCO_2, eCO_2). As hypothesized

in this work, the abundance of oligo/heterotrophic *Sphingomonas* (6/9 isolates under aCO_2/eCO_2) and *Pseudomonas* (8/11), as well as methylotrophic *Sphingomonas* (2/7) and *Pseudomonas* (7/11), was positively affected by eCO_2 concentration, but only on *G. album* plant species. However, the hypothesis that the abundance of *Methylobacterium* increases under the eCO_2 concentration is refuted by our results. This hypothesis is contradicted because the same number of *Methylobacterium* isolates were found in both aCO_2 and eCO_2 treatments (18/16 on *A. elatius* leaves and 12/13 on *G. album* leaves).

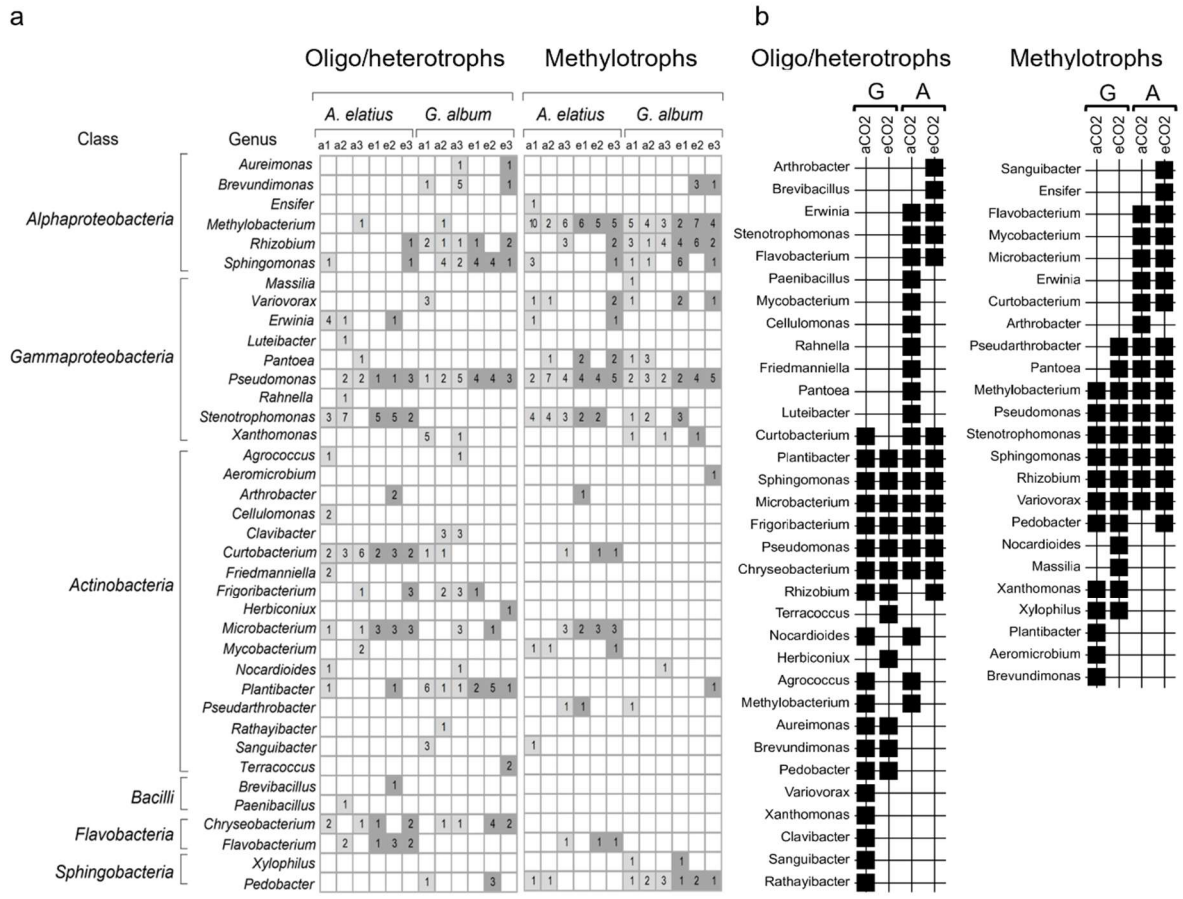

Figure 10 Representation of the most frequently cultivated oligo/heterotrophic and methylotrophic isolates at genus level, which were grown on the leaves of the plant species *A. elatius* and *G. album* under aCO_2 and eCO_2 concentrations. a: Numbers of isolates phylogenetically identified by 16S rRNA gene sequences at genus level using EzTaxon (Kim et al., 2014). The light gray squares content the number of isolates from control rings (a1, a2, a3) with ambient atmospheric CO_2 concentration. The dark gray squares contain the number of isolates from elevated rings (e1, e2, e3). b: Seriation analysis based on an absence or presence matrix shows the occurrence of isolated oligo/heterotrophic and methylotrophic genera from both CO_2 treatment (aCO_2, eCO_2) and from both plant species (*A. elatius*, *G. album*). aCO_2- ambient atmospheric CO_2 concentration conditions, eCO_2-eleveated CO_2 concentration conditions, A-leaves of *A. elatius* plant species, G-leaves of *G. album* plant species.

To determine which phylogenetic groups of oligo/heterotrophs and methylotrophs in the phyllosphere of two plant species were adapted to the eCO_2 concentration, a total of 452 isolates differentiated into 106 phylotypes (Fig. 11) were analyzed using a CCA. CCA showed a correlation between environmental factors, such as increased CO_2 (CO_2) and both plant

species (plant), and the identified phylotypes. The abundant genera, such as the total *Pseudomonas* (Fig. 12b), the total *Sphingomonas* (Fig. 12d) and PPFMs (Fig. 12c) were investigated separately from the total oligo/heterotrophs (Fig. 12a). Among oligo/heterotrophs (Fig. 12a), a single phylotype Chry 6 of the genus *Chryseobacterium* had a strong positive correlation with the eCO_2 factor. This phylotype was eCO_2 treatment specific since it was isolated from both plant species grown under eCO_2 conditions. Additionally, it could be a newly adapted ecotype of the *A. elatius* and *G. album* phyllosphere. Furthermore, CCA of oligo/heterotrophs revealed two groups of phylotypes that showcased specificity to eCO_2 treatment only on one plant species. One group of phylotypes (Art1, Brevi1, Chry3, Cu2, Micro1, Micro4, R7) was isolated from *A. elatius* leaves and the other group of phylotypes (P10, A1, H1, P9, Pe1, R2, R4, S3, S7, T1) was isolated from *G. album* leaves grown in eCO_2-rings. Of these groups, three phylotypes (Cu2, Micro1, Micro4) belonged to the abundant genera (*Curtobacterium* and *Microbacterium*) on *A. elatius* leaves, and three phylotypes (P9, P10, S7) belonged to the abundant *Pseudomonas* and *Sphingomonas* on *G. album* leaves. These results indicate that the adaptation can be found in both abundant and rare phylotypes at the genus level. The CCA of total *Pseudomonas* (Fig. 12b) shows no correlation between phylotypes of both plant species (*A. elatius*, *G. album*) and eCO_2 concentration. Instead, eCO_2 treatment specific phylotypes were observed for only one plant species (P10 for *G. album*, P6, P11, P15 for *A. elatius*). In addition, CCA shows a strong positive correlation of phylotype P9 with the plant vector, indicating plant specificity of the phylotype. This phylotype was isolated only from *G. album* leaves grown under both conditions (aCO_2, eCO_2). The CCA of total *Sphingomonas* (Fig. 12c) shows that the phylotypes S3 and S7, isolated from *G. album* leaves are positively correlated with the eCO_2 vector and can be identified as eCO_2 treatment-specific phylotypes for *G. album* leaves. Among the PPFM phylotypes (Fig. 12d), no phylotypes were found to be eCO_2 treatment-specific for both plant species. However, two PPFM phylotypes (M1c, M4) were isolated from the eCO_2 rings, each from different plant species, showing eCO_2 treatment specificity for one plant species (M1c for *G. album* and M4 for *A. elatius*).

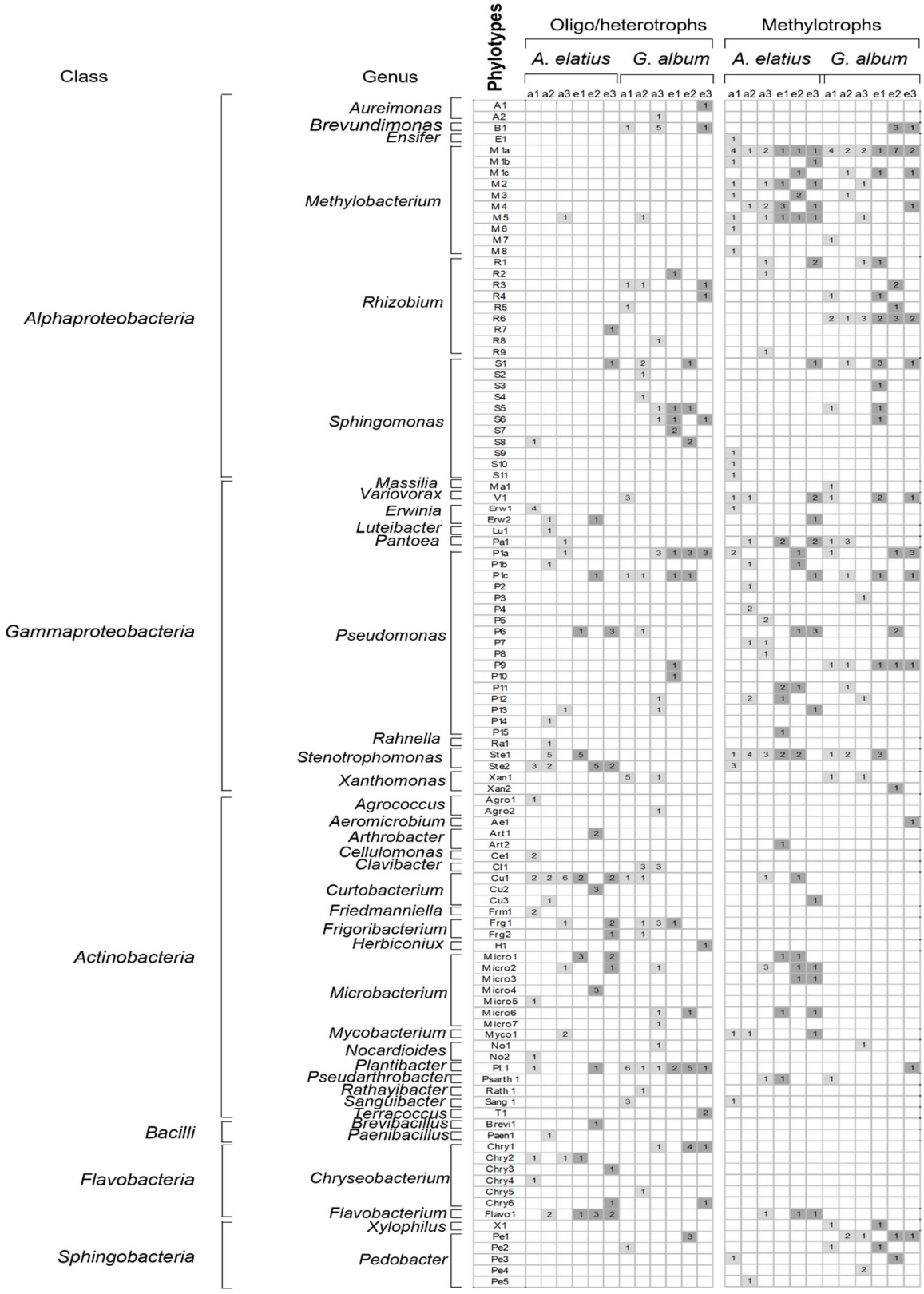

Figure 11 Isolates differentiated into different phylotypes representing frequently cultured oligo/heterotrophs and methylotrophs on the leaves of *A. elatius* and *G. album* plant species grown under control and elevated CO_2 conditions. The isolates with sequence similarity >98.65% within each genus were assigned to a phylotype using MEGA7 (Tamura et al., 2013). The light gray squares contain the number of isolated phylotypes from control rings (a1, a2, a3) with ambient atmospheric CO_2 concentration. The dark gray squares contain the number of isolated phylotypes from elevated rings (e1, e2, e3).

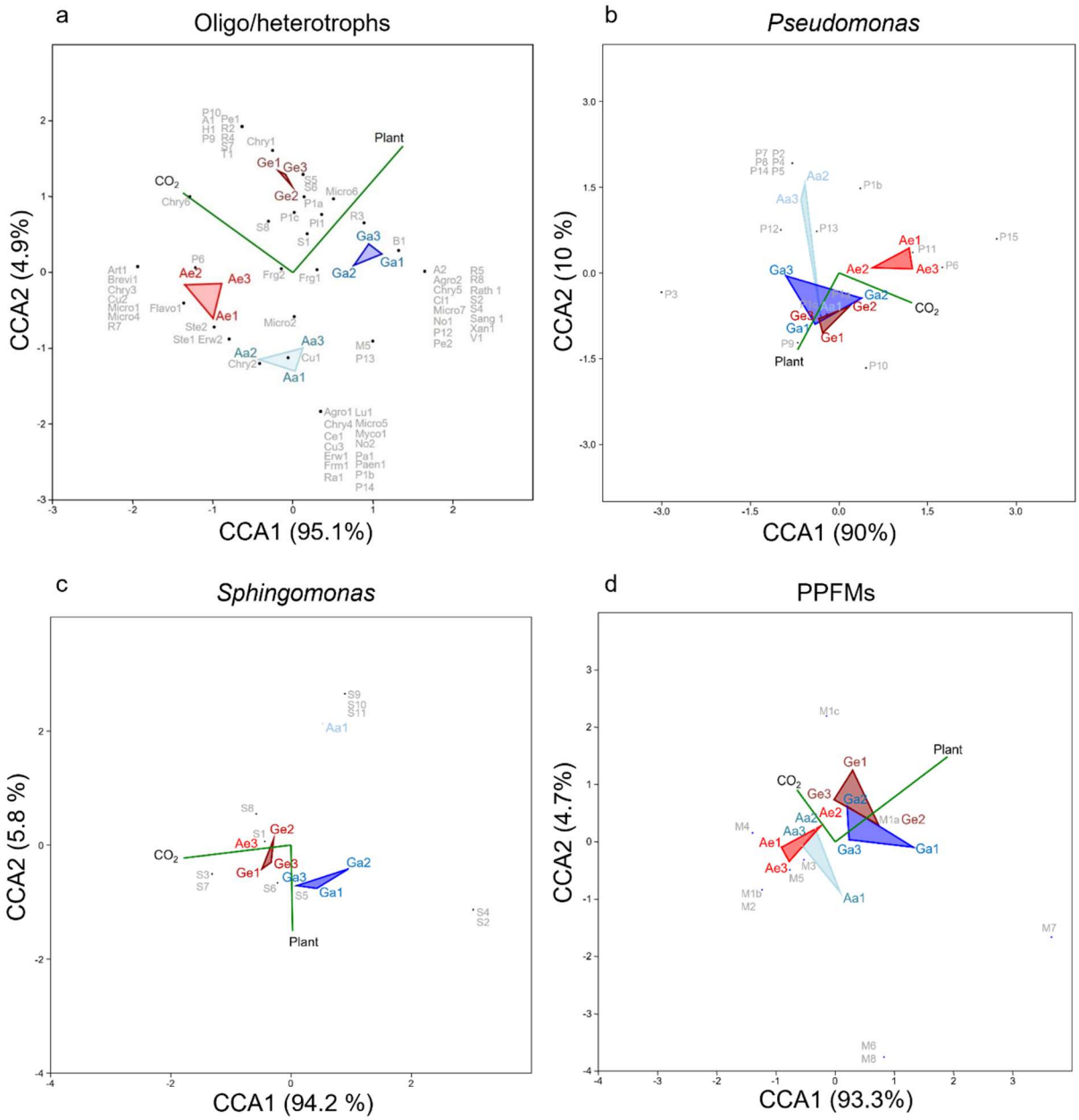

Figure 12 Canonical correspondence analysis (CCA) of different phylotypes: oligo/heterotophs (a), total *Pseudomonas* (b), PPFMs (c) and total *Sphingomonas* (d) phylotypes based on variable regions (V1-V6) of 16S rRNA gene sequences with correlation to environmental factor (CO_2) and plant species (*A. elatius*, *G. album*). CCA was performed in PAST3 version 3.11 (Hammer et al., 2001) with similarity index Bray-Curtis. The abbreviations of phylotypes are labeled with a gray color. Aa1, Aa2, Aa3 (light blue) are *A. elatius* leaves from control rings; Ae1, Ae2, Ae3 (light red) are *A. elatius* leaves from elevated CO_2 rings; Ga1, Ga2, Ga3 (blue) are *G. album* leaves from control rings; Ge1, Ge2, Ge3 are *G. album* leaves from elevated CO_2 rings.

3.1.4 Functional adaptation of PPFM

The frequently enriched PPFMs phylotypes were also analyzed for functional adaptation based on amino acid and nucleotide sequences of the *mxaF*-gene to determine whether their adaptation to eCO_2 concentration might be related to the evolution of specifically adapted ecotypes. A total of 63 PPFM isolates were isolated from both plants (*A. elatius*, *G. album*) and experiments (aCO_2, aCO_2) and differentiated into 10 PPFM phylotypes (F-M1a-c, F-M2 to F-M7) (Fig. 13a). The phylotype F-M1 consists of most isolates that have intra phylogenetic similarity (99.89-100%) and are closely related to *M. bullatum*. The other phylotypes, however, contain fewer isolates. Phylotypes F-M1b and F-M1c formed two separate clusters and shared the most closely related type of strain of *M. marchantiae*. The gene sequence similarity within the 16S rRNA genes of these phylotypes was between 99.22–99.78%. No eCO_2 treatment specific phylotype was detected between phylotypes for both plants. However, in each of the phylotypes F-M1c and F-M4, one PPFM isolate was found. The PPFM isolate found already responded specifically to the eCO_2 treatment in one plant. The PP-MPN-786c1-CE2bA isolate of phylotype F-M1c was isolated from *A. elatius* leaves and the PP-MPN-896-CE3cG isolate of phylotype F-M4 was isolated from *G. album* leaves which were grown under eCO_2 concentration for a long time. These two phylotypes are represented by an isolate from an eCO_2 treatment, which could indicate a plant species-specific adaptation. Furthermore, the isolates of the phylotypes were differentiated by the partial sequence of the *mxaF*-genes at the nucleotide (*mxaF*-type) and amino acid (MxaF-type) levels. The PPFMs phylotypes were differentiated into 10 MxaF-types (Fig. 13b) and 38 *mxaF*-types (Fig. 13c). Phylotypes (F-M2, F-M3, F-M5-8) showed only one phylotype specific MxaF amino acid sequences (MxaF-types 2, 5, 6-9,11) which contrasted to the phylotypes (F-M1a, F-M1c) that showed two different MxaF amino acid sequences (MxaF-type 3,4,10 of F-M1b, MxaF-type 4, 6). This difference MxaF-types indicates the presence of functional differences and similarities between phylotypes, but it does not showcase eCO_2 treatment specific adaptation on the two plants. Instead, adaptation of PPFM isolates to eCO_2 conditions was observed in plant-specific phylotypes (F-M1c and F-M4). In the phylotypes (F-M1c of *A. elatius* and F-M4 of *G. album*), only one isolate with amino acid sequence (MxaF-type 4, MxaF-type 6) was isolated from the eCO_2 treatment, which could indicate the functional plant-specific adaptation of the individual PPFM isolates to eCO_2 concentration. The *mxaF* nucleotide sequences show great diversity in

all MxaF-types, except for MxaF-type 2 (Fig. 13c). All *mxaF*-gene sequences represented one MxaF-type 2.

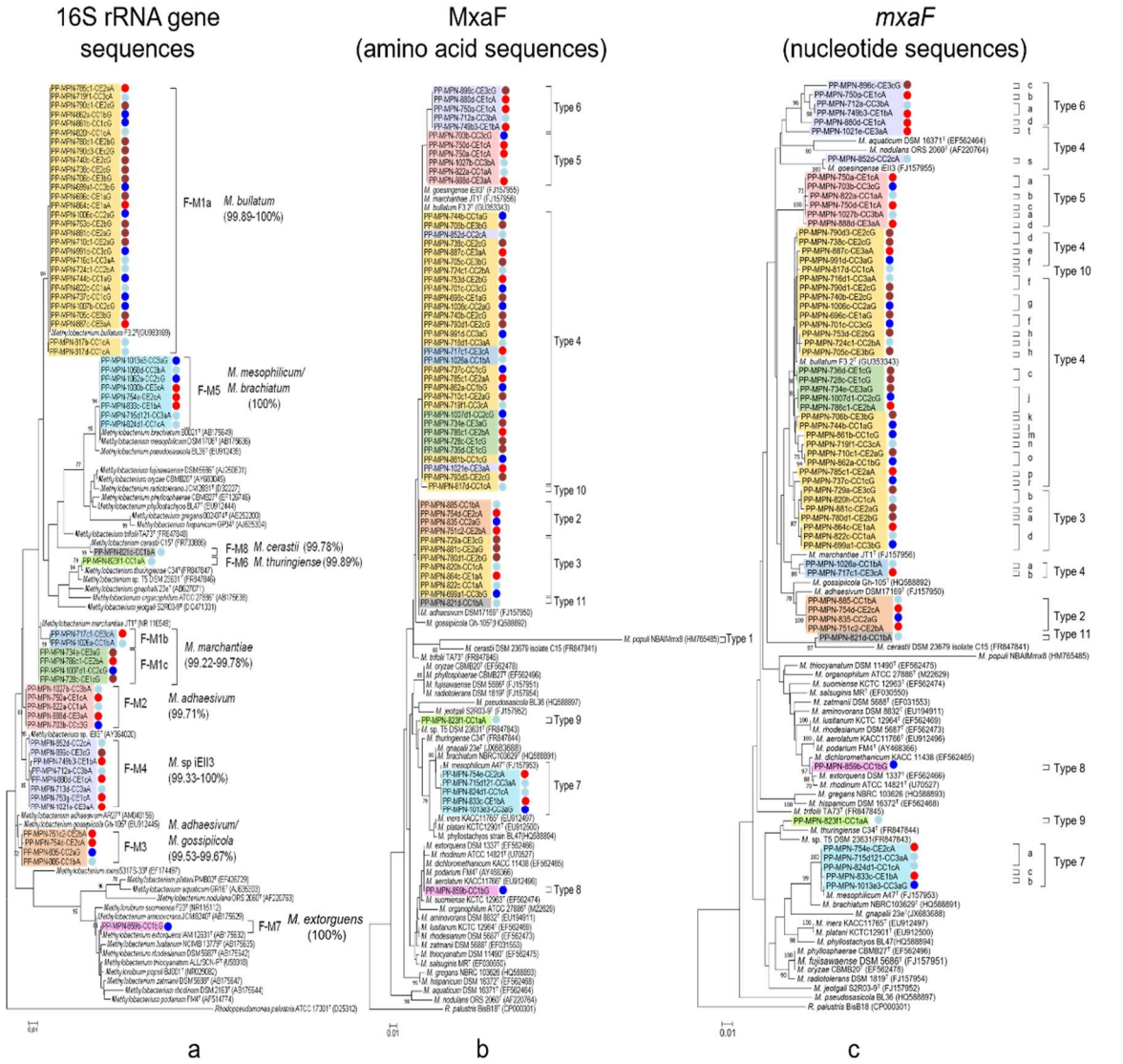

Figure 13 Phylogenetic resolution of 63 pink-pigmented facultative methylotrophs (PPFMs) based on 16S rRNA gene sequences (a), *mxaF*-gene sequences (c) and respective amino acid sequences (b). The phylogenetic tree (a) was constructed with Maximum-likelihood method using Kimura 2-parameter model with a discrete Gamma distribution. The phylogenetic tree (b) was constructed with the Neighbor-Joining method using the Jones-Thornton model (JTT; Jones et al., 1992). The phylogenetic tree (c) was constructed with the Neighbor-Joining method using the Jukes-Cantor model (Jukes and Cantor,1969). For *mxaF* and MxaF tree generation 117 sequences were used. The total length of these sequences was 429 nucleotides and 143 amino acids. All trees were constructed in MEGA7 (Tamura et al., 2013) and were based on bootstrap support (100 replicates). Numbers at notes show bootstrap value >70%. *Rhodopseudomonas palustris* strain ATCC 17001^T (D25312) and strain BisB18^T (CP000301) are out-groups. Abbreviations of samples name PP-MPN-896-CE3cG: PP-Plant phyllosphere; MPN-most probable number; followed by number of isolates e.g. 896; CC-control CO_2 or CE-elevated CO_2; 1, 2 or 3 indicates the number of rings; a, b or c-collection points in ring; A-*Arrhenatherum elatius*, G-*Galium album* plant species. Abbreviations of phylotypes name: F- Free Air Carbon dioxide Enrichment (FACE) system; M-*Methylobacterium*, followed by the number of phylotype. Each group of the same phylotype was colored by a different color (e.g. F-M1a is yellow). For a better overview, colorful circles represent an experiment: blue presents *A. elatius* leaves from control rings; red presents *A. elatius* leaves from elevated CO_2 rings; dark blue- *G. album* leaves from control rings and dark red, *G. album* leaves from elevated CO_2 rings.

3.1.5 Genotypes adaptation of PPFM

A phylogenetic analysis at genus level (based 16S rRNA gene sequence) paired with a function analysis (based on the *mxaF*-gene sequence) showed that two PPFM isolates might have a functional and plant-specific adaptation to the eCO_2 concentration (see section 3.1.4). To investigate the adaptation of all 63 PPFMs isolates at the genetic level, they were differentiated into 62 genotypes (based on genomic fingerprint patterns) (Fig. 14). All isolates represent different genotypes, except genotype 110 of phylotype F-M1a which has the same genomic fingerprint pattern. This is the same isolate that was grown on *G. album* leaves under eCO_2 concentration conditions in rings 2 (PP-MPN-740b-CE2cG) and 3 (PP-MPN-706b-CE3bG). Furthermore, the CCA showed how the genotypes of phylotype F-M1a correlated with environmental factors such as eCO_2 and plants (Fig. 14). The genotypes 150 and 130 (grown on *A. elatius* leaves under eCO_2 conditions) showed a strong positive correlation with the vector CO_2 which could be an indication of a genetic adaptation to eCO_2 concentration. In contrast, a weak positive correlation of genotype 800 (grown on *G. album* leaves under ambient CO_2 conditions), with the vector plant indicates a plant-specific genetic adaptation.

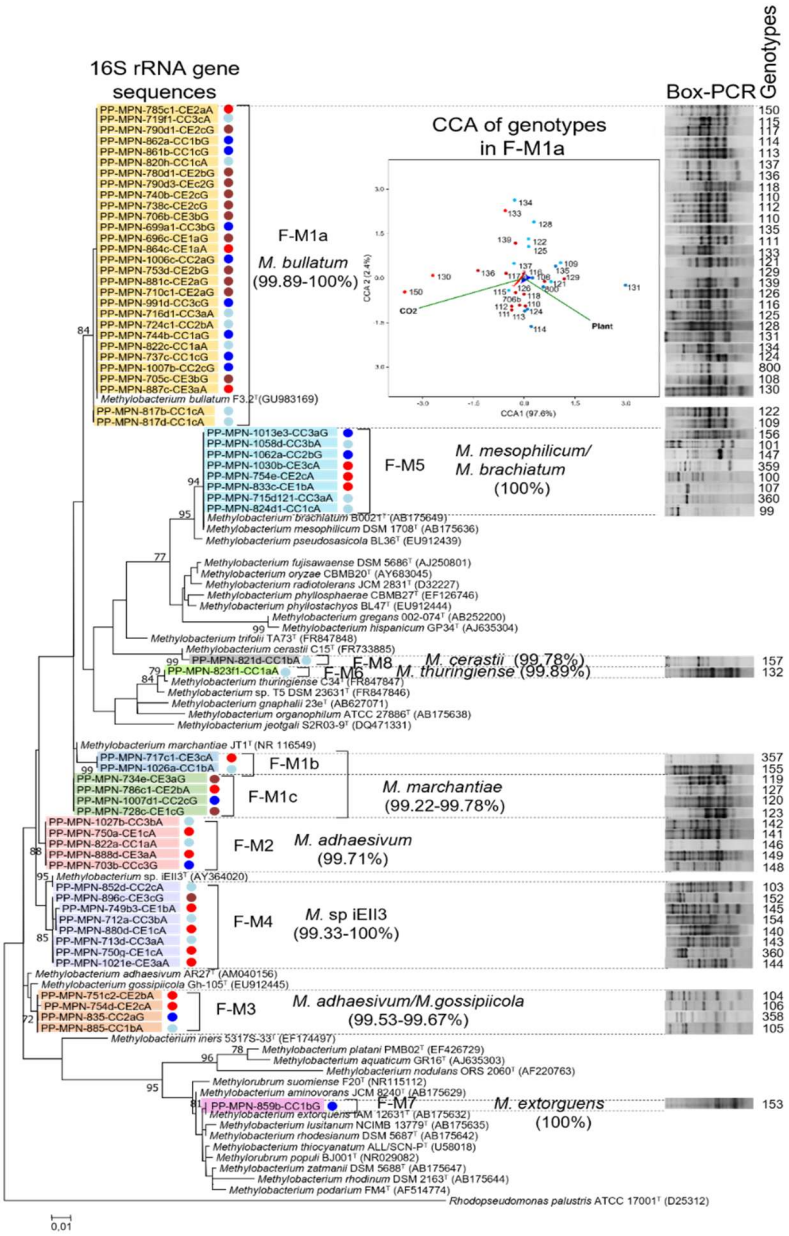

Figure 14 Genotyping of PPFM phylotypes cultured from *A. elatius* and *G. album* leaves grown under ambient atmospheric and elevated CO_2 conditions. On the left side: the phylogenetic tree of 61 PPFMs isolates, based on partial 16S rRNA gene sequences, was constructed with Maximum-likelihood method using Kimura 2-parameter model with a discrete Gamma distribution using MEGA7 (Tamura et al., 2013). The tree is based on bootstrap support (100 replicates). Numbers at notes show a bootstrap value of >70%. Sequences of the *Rhodopseudomonas palustris* strain ATCC 17001^T (D25312) were used as out-group. On the right side: Genomic fingerprint patterns of PPFMs isolates were analyzed based on BOX-PCR products generated by BOX1AR primer (Versalovic et al., 1994) and genotypes. A genotype with individual patterns represents a genotype. A cluster analysis was performed in BioNumerics (Applied Maths) using the Unweighted Pair Group Method Arithmetic Average (UPGMA) with 1% position tolerance and 0.5% optimization. A similarity matrix was calculated using the Pearson correlation coefficient, which considered the presence/absence of DNA bands and their intensity. Abbreviations of samples name: PP-Plant phyllosphere; MPN-most probable number; followed by number of isolates e.g. 859b; CC-control CO_2 or CE-elevated CO_2; 1, 2 or 3 are the number of rings; a, b or c are the collection points in the ring; A-*Arrhenatherum elatius*, G-*Galium album* plant species. Abbreviations of phylotypes name: F- Free Air Carbon dioxide Enrichment (FACE) system; M- *Methylobacterium*, followed by number of phylotype. Each group of the same phylotype was colored by different colors (e.g. F-M1a is yellow). For better overview, colorful circles represent an experiment: blue-*A. elatius* leaves from control rings, red-*A. elatius* leaves from elevated CO_2 rings, dark blue-*G. album* leaves from control rings, dark red-*G. album* leaves from elevated CO_2 rings.

3.2 Efficient DNA extraction methods for phyllosphere-associated bacteria of *A. elatius* and *G. album* plants

In this study, 27 different DNA extraction methods were tested and/or modified to find a DNA extraction method that enables an efficient bacterial DNA extraction from the phyllosphere without co-extracting high amounts of chloroplast and mitochondrial DNA. Subsequently, the aim was to use this method to study the microbiome of the phyllosphere of plant species (*A. elatius*, *G. album*) grown under ambient atmosphere and elevated CO_2.

3.2.1 Comparison of total DNA concentrations

Total DNA was extracted from frozen, frozen homogenized, freeze-dried and/or fresh leaves of *A. elatius* and *G. album* species using direct (CTAB methods, various commercial kits) and alternative (harvesting bacterial cells prior to DNA extraction) DNA extraction methods. The DNA concentration obtained using these methods are presented in Figure 15 and Table 13. Of all methods, the highest yield (1392–1814.5 ng/µl) of genomic DNA was obtained from frozen homogenized leaves (*A. elatius*, *G. album*) using the CTAB method for plant DNA. Using this method, the highest DNA concentrations were also extracted from the total frozen leaves, but the concentrations were lower (318.6, 432.4 ng/µl from *A. elatius*, 739.1, 785.8 ng/µl from *G. album*) than from the frozen homogenized leaves. The next best DNA yields were achieved with two direct methods, the modified Suda et al. (2008) method and a commercial kit (Fast DNA®Spin kit for Soil, MP Biomedicals). Using the modified Suda method 1 (Suda et al. 2008 mod 1), DNA was extracted from pool of total frozen and fresh leaves of *A. elatius* and *G. album*, with varying starting amounts (0.5 g, 3 g, 5 g). The highest yield was obtained from 3 g of total frozen leaves (182.4 ng/µl) and from 5 g of fresh leaves (254.6 ng/µl). Despite better yields from 5 g of leaf material, DNA was extracted from 0.5 g of the total frozen leaves of each plant (*A. elatius*, *G. album*) using the second modified Suda et al. (2008) method (Suda et al., (2008) mod 2) to compare with alternative methods. For example, the yield of 0.5 g leaves after the second modification from the total frozen leaves (Suda et al. (2008) mod 2) increased from 31.1/97.6 ng/µl (pool of A+G) to 211.2/332.2 ng/µl (pool of A1/A2) and 128.7,136.5 ng/µl (pool of G1/G2).

The Fast DNA®Spin kit for soil (MP Biomedicals) was used for DNA extraction from total frozen, frozen homogenized and freeze-dried leaves. The best DNA yields were obtained from both frozen, homogenized leaves (MP Bio) and from freeze-dried leaves after the third modification (MP Bio mod 3). The DNA concentrations were similar (e.g. frozen homogenized

A. elatius: 220.9 ng/µl, 247.6 ng/µl, freeze-dried *A. elatius*; 184.3 ng/µl, 239.8 ng/µl after the 2nd elution). Modifications of the Fast DNA®Spin kit for Soil (MP Biomedicals) using total frozen leaves (*A. elatius*, *G. album*) showed improved DNA yield with each subsequent modification. For *A. elatius* leaves it was 23.5 ng/µl (MP Bio), 156.9 /µl (MP Bio mod 1), 168.2 ng/µl (MP Bio mod 2) and for *G. album* leaves 11.5 /µl (MP Bio), 74.6 ng/µl (MP Bio mod 1), 181.1 ng/µl (MP Bio mod 2). In addition, the efficiency of the elution step was evaluated using this kit with modification 3 (MP Bio mod 3) by measuring the DNA yield after both the first, and second elution. After the first elution, the concentrations were 161.9 ng/µl, 193.8 ng/µl for *A. elatius* leaves and 93.7 ng/µl, 100.3 ng/µl for *G. album* leaves. After the second elution, the DNA concentrations were 22.4 ng/µl, 46 ng/µl for *A. elatius* leaves and 18.8 ng/µl, 28 ng/µl for *G. album* leaves. These values indicate large amounts of DNA loss during the elution step of the Fast DNA®Spin kit for Soil (MP Biomedicals).

The modified Griffiths et al. (2000) method, based on the use of zirconia-silica beats of different diameters (0.1 mm, 0.5 mm), was used for DNA extraction from the total frozen leaves of each pooled plant (*A. elatius*, *G. album*). The extraction was successful and showed an average DNA yield of approx. 100 ng/µl (Table 13). Furthermore, DNA extraction from the pool of total frozen leaves (pool A+G) without beads, with both beads (0.1 mm, 0.5 mm) or only with the smallest beads (0.1 mm) showed the highest DNA yield with the smallest beads and the lowest yield with both bead sizes. Alternative methods of DNA extraction, where bacterial cells were detached and collected by Sterivex filter or centrifugation or Percoll exhibited low concentrations of DNA ranging from 2.1-6.8 ng/µl. In contrast, the DNA concentrations (153 ng/µl from *A. elatius* leaves, 110.5 ng/µl from *G. album* leaves and 123.8 ng/µl from *A. elatius*+8 µm pore size of prefilter) extracted using the NucleoSpin®Soil kit (Macherey-Nagel) with modification 4, from bacterial cells detached from 10 g of leaves (*A. elatius*, *G. album*) and collected on a Sterivex filter (0.22 µm pore size), were much higher. Furthermore, the DNA concentrations of these methods were much higher than the yield of most direct extraction methods (MN plant, MN soil 96 mod 1, MN for soil mod 1, 2, Griffiths et al. (2000) mod, PowerSoil, PowerPlant).

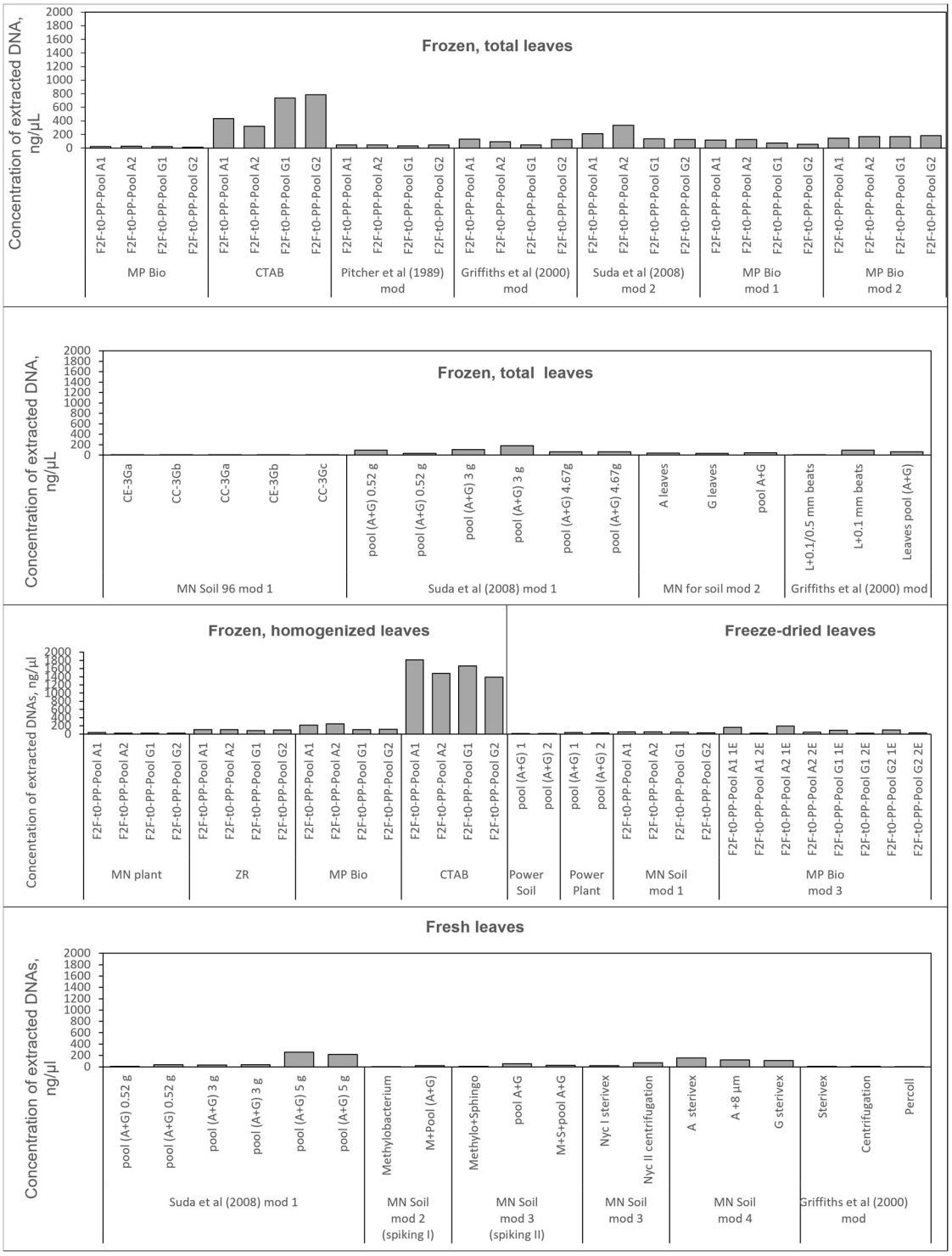

Figure 15 Total DNA concentrations from total frozen, frozen homogenized, freeze-dried and fresh leaves of *A. elatius* and *G. album* plant species after extraction by direct and alternative methods. Total DNA extraction methods: MP Bio- Fast DNA®Spin kit for Soil (MP Biomedicals); MP Bio mod 1, 2, 3- Fast DNA®Spin kit for Soil (MP Biomedicals) with modification 1 or 2 or 3; MN Soil 96 mod 1- NucleoSpin®96 Soil kit (Macherey-Nagel) with modification 1; MN plant- NucleoSpin®Plant II kit (Macherey-Nagel); ZR- ZR Plant/Seed MiniPrep™ kit (Zymo Research); PowerSoil- PowerSoil®DNA Isolation kit (MoBio); PowerPlant- PowerPlant®Pro DNA Isolation kit (MoBio); MN Soil mod 1, 2, 3- NucleoSpin®Soil kit (Macherey-Nagel) with modification 1 or2 or 3; MN Soil mod 3 (spiking I)- NucleoSpin®Soil kit (Macherey-Nagel) with modification 3 with addition of *Methylobacterium*; MN Soil mod 4 (spiking II)- NucleoSpin®Soil kit (Macherey-Nagel) with modification 4 addition of *Methylobacterium* isolate 1405 and *Sphingomonas* isolate 628; Pitcher et al. (1989) mod, Griffiths et al. (2000), Suda et al. (2008) mod-modified protocols of Pitcher et al. (1989), of Griffiths et al. (2000), of Suda et al. (2008); CTAB-method.

In addition, DNA extraction was tested on different leaf amounts (20-80 g, 200 mg, 500 mg, 1 g, 10 g). From the smallest extraction amount (20-80 g leaves), with the Fast DNA®Spin kits for Soil with modification 3 (MP Bio mod 3) from the freeze-dried leaves, the best DNA yields of 161.9-193.8 ng/µl were obtained. From the 200 mg of leaf material, extracted using the CTAB method from frozen homogenized leaves, the highest yield of 1484-1814.5 ng/µl was obtained. Additionally, for the same quantity of leaf material, extracted using the same method but from total frozen leaves, yielded 432.4 ng/µl. Finally, for the same quantity but using the Fast DNA®Spin kits for Soil (MP Bio) from frozen homogenized leaves, yielded 112-247.6 ng/µl. From the 500 mg of leaves, Suda et al. (2008) mod 2 showed the highest yield of 332.3 ng/µl, followed by the Fast DNA®Spin kit for Soil with modification 2 (MP Bio mod 2) with 181.1 ng/µl. High amounts of leaves (1 g, 10 g) were only used for alternative methods. The highest DNA concentration (153 ng/µl of *A. elatius*, 110.5 ng/µl of *G. album*) was obtained after extraction with the NucleoSpin®Soil kit (Macherey-Nagel) with modification 4 (MN soil mod 4) from cells detached and collected on the Sterivex filter. In the bacterial community analysis of the phyllosphere, not only is DNA concentration of great importance, but also the efficiency and reproducibility of the DNA extraction method. Similarity for the molecular analysis (e.g. PCR, sequencing) of the DNAs, both the DNAs concentration and the purity of the extracted DNAs are essential (see Chapter 2.2.3.4, Table 13). In this study, pure DNA was extracted from a sample of freeze-dried *A. elatius* leaves using only the NucleoSpin®Soil kit (Macherey-Nagel) after modification 1 (MN Soil mod 1, Table 13). The other DNA extracts were contaminated either with proteins ($A_{260}/_{280}$<1.8), RNAs ($A_{260}/_{280}$>2.0), chaotropic salts, polysaccharides ($A_{260}/_{230}$<2.0), or all the above. With the commercial kits there are two exceptions namely, Fast DNA®Spin Kit for Soil (MP Bio mod 2) and ZR Plant/Seed MiniPrep™ kit (Zymo Research). DNA was extracted without protein contamination, but with polysaccharides and chaotropic salts contaminations. Compared to all other methods, DNA could be extracted without chaotropic salts and polysaccharides using the CTAB method from frozen homogenized leaves using Griffiths et al. (2000) mod from total frozen leaves.

Table 13 The quantity and quality of extracted total DNA from total frozen, frozen homogenized, freeze-dried and fresh leaves of *A. elatius* and *G. album* species. Environmental samples: F2F-t0-PP-pool A1, F2F-t0-PP-pool A2 or F2F-t0-PP-pool G1, F2F-t0-PP-pool G2 are replicates pool samples of *A. elatius* or *G. album* leaves grown in FACE2FACE system (F2F). Sampling was at time point zero (t0) from plant phyllosphere (PP). CC-3Ga, CC-3Gb, CC-3Gc, CE-3Ga, CE-3Gb, CC-1Ga, CC-2Gb, CE-3G (2) are leaf samples from FACE system. G-leaves of *G. album* plant species, which were grown in the control ring with ambient atmospheric CO_2 (CC), in rings with elevated CO_2 (CE), or samples were collected from sampling points a, b, c in ring 2 or 3. In the sample CE-3G (2), the (2) refers to the same as "b" collected sampling point. A-*A. elatius* leaves, G-*G. album* leaves, L-leaves, M-*Methylobacterium*, S-*Sphingomonas*. F2F-to-PP-Pool A1 1E or 2E samples of F2F-to-PP-Pool A1 after 1 Elution (1E) or F2F-to-PP-Pool A1 after 2 Elution (2E), F2F-to-PP-Pool G1 1E or 2E samples of F2F-to-PP-Pool G1 after 1 Elution (1E) or F2F-to-PP-Pool G1 after 2 Elution (2E). Nyc I and Nyc II: phaseI and II after cell separation by gradient centrifugation with Nycodenz. A or G Sterivex: bacterial cells detached from *A. elatius* or *G. album* leaves collected by the Sterivex filter, A+8 µm: bacterial cells detached from *A. elatius* leaves filtrated with prefilter (membrane filter with pore size 8 µm). Percoll: after cells separation by Percoll. Green labeled values of A260/280 and A260/230 without contamination.

Leaves material	DNA extraction	Environmental samples	Weight, mg	Conc. of gDNA, ng/µl	$A_{260/280}$	$A_{260/230}$
frozen, total leaves	MP Bio	F2F-t0-PP-Pool A1	184	23.7	1.77	1.05
		F2F-t0-PP-Pool A2	168	25.7	1.96	1.05
		F2F-t0-PP-Pool G1	400	20.6	1.77	1.04
		F2F-t0-PP-Pool G2	202	11.5	2.2	0.83
	CTAB	F2F-t0-PP-Pool A1	200	432.4	2.1	1.52
		F2F-t0-PP-Pool A2	193	318.6	2.09	1.85
		F2F-t0-PP-Pool G1	332	739.1	2.4	1.87
		F2F-t0-PP-Pool G2	346	785.8	2.05	1.92
	Pitcher et al (1989) mod	F2F-t0-PP-Pool A1	544	47.5	0.76	0.21
		F2F-t0-PP-Pool A2	498	47.3	0.7	0.19
		F2F-t0-PP-Pool G1	562	33.5	0.69	0.18
		F2F-t0-PP-Pool G2	491	45.3	0.66	0.18
	Griffiths et al (2000) mod	F2F-t0-PP-Pool A1	197	129.4	2.08	1.81
		F2F-t0-PP-Pool A2	82	94.6	2.07	2.16
		F2F-t0-PP-Pool G1	190	44.5	2.13	1.99
		F2F-t0-PP-Pool G2	225	128	2.09	2.13
	Suda et al (2008) mod 2	F2F-t0-PP-Pool A1	505	211.2	2.57	0.57
		F2F-t0-PP-Pool A2	546	332.3	2.33	0.8
		F2F-t0-PP-Pool G1	573	136.5	3.68	0.31
		F2F-t0-PP-Pool G2	663	128.7	4.82	0.27
	MP Bio mod 1	F2F-t0-PP-Pool A1	476	115.7	1.97	0.16
		F2F-t0-PP-Pool A2	507	126.9	1.94	0.16
		F2F-t0-PP-Pool G1	522	74.6	1.74	0.1
		F2F-t0-PP-Pool G2	540	53.3	1.71	0.07
	MP Bio mod 2	F2F-t0-PP-Pool A1	296	144.3	2.88	1.83
		F2F-t0-PP-Pool A2	280	168.2	3.36	1.82
		F2F-t0-PP-Pool G1	428	181.1	3.62	1.76
		F2F-t0-PP-Pool G2	417	114	2.28	1.76
	MN Soil 96 mod 1	CE-3Ga	167	10.1	1.92	0.04
		CC-3Gb	168	8.8	1.91	0.3
		CC-3Ga	174	11.5	1.89	0.37
		CE-3Gb	167	6.8	1.93	0.24
		CC-3Gc	168	6.2	1.88	0.67
	Suda et al (2008) mod 1	pool (A+G) 0.52 g	520	97.6	2.44	0.64
		pool (A+G) 0.52 g	520	31.1	3.8	0.28
		pool (A+G) 3 g	3 060	109.1	2.47	0.65
		pool (A+G) 3 g	3 060	182.4	2.39	0.77
		pool (A+G) 4.67g	4 670	65.7	3.26	0.35
		pool (A+G) 4.67g	4 670	61.8	3.79	0.32
	MN Soil mod 2	*A leaves*	165	38.3		
		G leaves	169	33.1		
		pool A+G	168	44.6		
	Griffiths et al (2000) mod	L+0.1/0.5 mm beats	163	8.9	2.31	0.83
		L+0.1 mm beats	174	92.2	2.05	1.89
		Leaves pool (A+G)	175	61.7	2.13	1.52
frozen, homogenized leaves	MN plant	F2F-t0-PP-Pool A1	31	34.4	1.69	1.49
		F2F-t0-PP-Pool A2	34	20.8	1.9	1.48
		F2F-t0-PP-Pool G1	32	22.1	1.81	1.41
		F2F-t0-PP-Pool G2	29	20.5	1.84	1.49
	ZR	F2F-t0-PP-Pool A1	166	105.9	2.11	1.2
		F2F-t0-PP-Pool A2	165	106.1	2.15	1.9
		F2F-t0-PP-Pool G1	171	85.1	2.27	1.92
		F2F-t0-PP-Pool G2	170	98.1	2.13	0.81
	MP Bio	F2F-t0-PP-Pool A1	126	220.9	1.85	0.37
		F2F-t0-PP-Pool A2	176	247.6	1.85	0.41
		F2F-t0-PP-Pool G1	170	112	1.94	0.27
		F2F-t0-PP-Pool G2	179	113.6	1.83	0.22
	CTAB	F2F-t0-PP-Pool A1	211	1814.5	2.1	1.99
		F2F-t0-PP-Pool A2	184	1484	2.10	1.96
		F2F-t0-PP-Pool G1	249	1666	2.09	2.02
		F2F-t0-PP-Pool G2	182	1392	2.12	1.99
freeze-dried leaves	PowerSoil	pool (A+G) 1	53.7	12.7	1.56	0.94
		pool (A+G) 2	75.9	14.7	1.78	1.33
	PowerPlant	pool (A+G) 1	16.9	36.7	1.67	1.45
		pool (A+G) 2	16	30.8	1.71	1.09
	MN Soil mod 1	F2F-t0-PP-Pool A1	47	52.1	1.82	1.73
		F2F-t0-PP-Pool A2	38	56	1.83	2.16
		F2F-t0-PP-Pool G1	30	42.2	1.66	0.93
		F2F-t0-PP-Pool G2	27	26.5	1.86	0.95
	MP Bio mod 3	F2F-t0-PP-Pool A1 1E	72	161.9	1.82	0.51
		F2F-t0-PP-Pool A1 2E		22.4	1.89	0.17
		F2F-t0-PP-Pool A2 1E	65	193.8	1.83	0.3
		F2F-t0-PP-Pool A2 2E		46	1.82	0.28
		F2F-t0-PP-Pool G1 1E	55	93.7	1.8	0.19
		F2F-t0-PP-Pool G1 2E		18.8	1.88	0.12
		F2F-t0-PP-Pool G2 1E	59	100.3	1.8	0.17
		F2F-t0-PP-Pool G2 2E		28	1.77	0.14
fresh leaves	Suda et al (2008) mod 1	pool (A+G) 0.52 g	520	7.9	3.58	0.16
		pool (A+G) 0.52 g	520	35.4	2.28	0.39
		pool (A+G) 3 g	3 070	32.8	1.77	0.36
		pool (A+G) 3 g	3 070	36.9	1.79	0.36
		pool (A+G) 5 g	5 050	254.6	2.02	0.36
		pool (A+G) 5 g	5 050	219	2.01	0.36
	MN Soil mod 2 (spiking I)	M	$1.52*10^{7}$	1.4	2.59	0.72
		M+Pool (A+G)	140mg+M	20.3	1.94	1.23
	MN Soil mod 3 (spiking II)	M+S	$7.03*10^{7}$ /$3.44*10^{6}$	8.1	1.61	0.59
		pool A+G	168 mg	54.2	1.76	1.35
		pool A+G+M+S	175 mg+M+S	25.7	1.8	1.12
	MN Soil mod 3	NycI sterivex	1 200	19.4	1.65	0.56
		NycII centrifugation		68.8	1.64	0.67
	MN Soil mod 4	A sterivex	970	153	1.81	1.57
		A +8 µm		123.8	1.81	1.42
		G sterivex	10 400	110.5	1.8	1.46
	Griffiths et al (2000) mod	Sterivex	1 500	6.8	2.22	0.91
		Centrifugation	1 350	6.8	2.45	1.2
		Percoll	2 260	2.1	2.37	0.64

3.2.2 Comparison of DNA extractions with MN, ZR kits and their bacterial community analysis using DGGE method

To demonstrate the efficacy and reproducibility of DNA extraction using the NucleoSpin® Plant II kit (Macherey-Nagel) and TR Plant/Seed MiniPrep™ kit (Zymo Research), the DNA extracts from frozen homogenized *A. elatius* and *G. album* leaves were first checked for presence of DNA and co-amplicons, such as chloroplast and mitochondria. Subsequently, fingerprint analysis of the bacterial community was tested using denaturing gradient gel electrophoresis (DGGE) based on partial 16S rRNA gene fragments. In Figure 16, both kits showed no efficacy (presence of chloroplast and mitochondrial DNA) and good reproducibility. PCR products amplified with universal Bacteria targeting primer system GC-339f/907r (Fig. 16a) showed an amplification of bacterial partially of 16S rRNA genes and homologous partially of 16S rRNA genes from chloroplasts as a strong band (B+C, 568 bp) and as an amplification of mitochondrial partially of 16S RNA genes as a weak band (M, approx. 800 bp) in all samples. To exclude the chloroplasts of bacteria 16S rRNA gene, bacterial primer 799f (Chelius&Triplett, 2001) and primer 1492r (Lane, 1991) were used. Primer 799f binds to both the bacterial 16S rRNA and mitochondrial 16S rRNA gene sequences (Chelius&Triplett, 2001). In combination with primer 1492r (Fig. 16b), PCR amplified bacterial DNA (B) with a length of 693 bp, as well as mitochondrial DNA (M) with a length of 1025 bp from *A. elatius* leaves and 930 bp from *G. album* leaves, were amplified. The results showed clear amplification of mitochondrial DNA fragments, but absence of bacterial DNA fragments.

DGGE analysis of the generated PCR products with the primer system GC-339f/907r (Fig. 16c) showed the abundance of chloroplasts in all samples. The fingerprint profiles were dominated by one band which was sequenced from two of the samples. The two sequences showed 100% sequence identity to chloroplast sequences of *Cistanche phelypaea* (HG515538) (band 1) und of *Triticum macha* (LC005978) (band 2). A further DGGE band 3, which only occurs in the fingerprint patterns of *G. album* samples, represented mitochondrial DNA, showed 98% sequence identity to a mitochondrial small subunit ribosomal RNA gene sequence of *Lardizabala biternata* (DQ008710). The abundance of the thick DNA bands of chloroplasts made the analysis of diversity and abundance of the bacterial community practically impossible. Both DNA extraction kits were inefficient methods for extraction of bacterial DNA from frozen, homogenized leaves of *A. elatius* und *G. album* leaves.

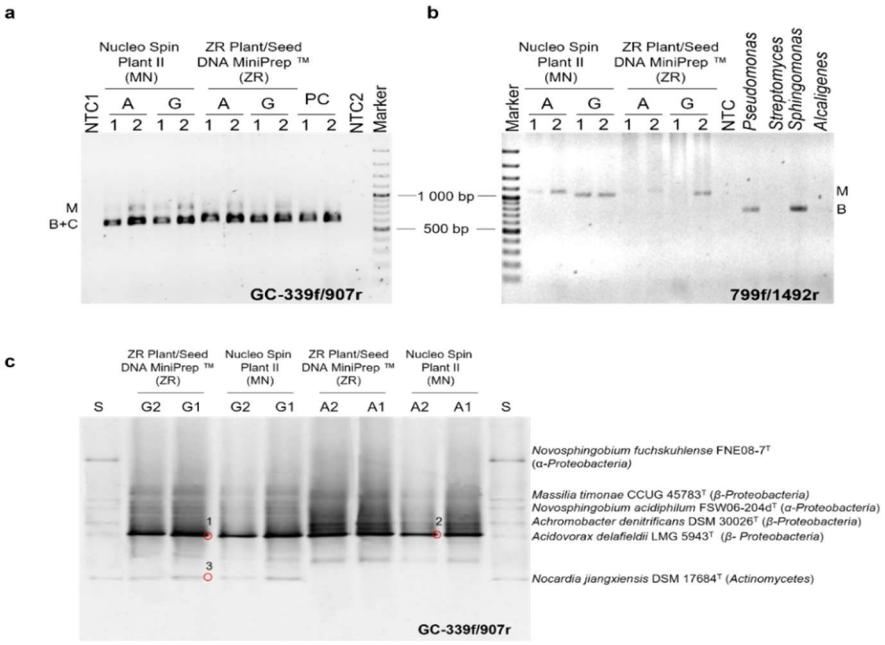

Figure 16 Comparison of DNA extractions using Nucleo Spin Plant II kit (Macherey-Nagel) and ZR Plant/Seed DNA MiniPrep ™ kit (Zymo Research) from frozen, homogenized leaves samples of *A. elatius* and *G. album* plant species. a: PCR detection of partial bacterial 16S rRNA gene targeting with universal bacterial primer systems GC339f-907r, b: PCR detection of partial bacterial 16S rRNA gene targeting with universal bacterial primer systems 799f-1492r, c: DGGE gel of community profile based on 16S rRNA gene targeting amplified from DNA extracted with both kits and universal bacterial primer systems GC339f-907r. 1.4% agarose gels (a, b) loaded with marker GeneRuler 100 bp Plus DNA Ladder (Fermentas). A1, A2 are two replicates of F2F-t0-PP-pool A sample; G1, G2 are two replicates of F2F-t0-PP-pool G sample, where F2F is FACE2FACE system, t0-time point zero samples, PP-plant phyllosphere, A-leaves of *A. elatius* plant species, G-leaves of *G. album* plant species. NTC, NTC1, NTC2-no template controls. a: M bands are mitochondrial PCR amplifications; B+C bands are bacterial and chloroplast PCR amplifications. PC1, PC2: positive controls with template as total DNA of *Novosphingobium rhizosphaerae* strain JM-1^T (DSM 29344™) extracted by Pitcher et al. (1989) modified and a Gen Elute Plant Genom DNA Miniprep kit (Sigma-Aldrich). Positive controls (b): *Pseudomonas extremaustralis* DSM 17835^T (*Gammaprotebacteria*), *Streptomyces* sp. St105671 (*Actinobacteria*), *Sphingomonas* sp. 449 (*Alphaproteobacteria)* and *Alcaligenes defragrans* DSM 12141^T (*Betaproteobacteria).* c: 7.5% polyacrylamide gel with denaturing gradient (70-40%). S- DGGE standard. DGGE bands 1, 2, 3 were identified: band 1 is 100% identical to a chloroplast sequence of the genome sequence of *Cistanche phelypaea* (HG515538); band 2 is 100% identical to a chloroplast sequence of *Triticum macha* (LC005978); band 3 showed with 98% highest sequence identity to a mitochondrial small subunit ribosomal RNA gene, partial sequence of *Lardizabala biternata* (DQ008710).

3.2.3 Comparative analysis of direct DNA extractions from different material leaves

Total bacterial DNA was extracted by direct extraction methods from different *A. elatius* and *G. album* leaves (total frozen leaves, frozen homogenized leaves, total freeze-dried leaves, total fresh leaves). Among them, both CTAB methods (Griffiths et al., 2000, Suda et al. , 2008, Pitcher et al. 1989) and the commercial kits (MP Bio- Fast DNA®Spin kit for Soil (MP Biomedicals), NucleoSpin®Soil kit, NucleoSpin®Plant II kit, NucleoSpin®96 Soil kit (Macherey-Nagel), ZR Plant/Seed MiniPrep™ kit (Zymo Research), PowerSoil®DNA Isolation kit (MoBio), PowerPlant®Pro DNA Isolation kit (MoBio)) with or without modifications based on co-extracts (chloroplasts, mitochondria) using the PCR method were investigated and compared. The

bacterial DNA (band 693 bp, 799f/1492r) could not be amplified from all DNA extracts, but only from DNA extracted using the following methods: the Fast DNA®Spin kit for Soil (MP Biomedicals) with modification 1-3; the NucleoSpin®Soil kit (Macherey-Nagel) with modification 2; the NucleoSpin®96 Soil kit (Macherey-Nagel) and Suda et al. (2008) with modification 1 from the completely frozen leaves of *G. album* or a pool of both plants (*A. elatius*, *G. album*) (Fig. 17). Additionally, amplification was successful from the DNA extracted using the NucleoSpin®Soil kit (Macherey-Nagel) with modification 1, the Fast DNA®Spin kit for Soil (MP Biomedicals) with modification 2, and from the completely freeze-dried leaves of *G. album* leaves (Fig. 19b). Furthermore, DNA extracted using Suda et al. (2008) with Modification 1, the NucleoSpin®Soil kit extracted DNA (Macherey-Nagel) with modification 2, and spinking I with modification 3 or spiking II from total fresh leaves (*A. elatius*, *G. album*) also showed successful amplification (Fig. 19c). The strongest bacterial bands were amplified from DNA extracted with the NucleoSpin®Soil kit (Macherey-Nagel) with modification 2, from the total frozen *G. album* leaves and a pool of *A. elatius* and *G. album* leaves. However, chloroplast (band 568 bp, 339f/907r) and mitochondrial DNA (band 1025 bp, 799f/1492r) were amplified from all DNA extracts, except those extracted with the modified Pitcher et al. (1989) and the modified Griffiths et al. (2000) methods, from the pool of *A. elatius* and *G. album* leaves with beads (0.1 mm and 0.5 mm, 0.1 mm) (Fig. 17). The absence of PCR products from DNA extracted from total frozen *A. elatius* and *G. album* leaves, using the modified Pitcher et al. (1989) method, indicates either the co-extraction of PCR-inhibiting compounds or that no DNA was extracted. Different amounts (0.52 g, 3.07 g, 5.05 g) of the total frozen and total fresh pools of *A. elatius* and *G. album* leaves were tested using the modified method of Suda et al. (2008). Amplification of DNA extracted from 0.52 g of total frozen leaves contained the most bacterial DNA (strong band, 693 bp, 799f/1492r) and the least bacterial DNA (weak band, 693 bp, 799f/1492r) from 0.52 g of fresh leaves (Figs. 17, 19). These results show that the method of Suda et al. (2008) could extract most of the bacterial DNA from the 0.5 g total frozen leaves, but together with the mitochondrial and chloroplast DNA. Unfortunately, the Suda et al. (2008) method proved to be non-reproducible. Using the Fast DNA® Spin kit for Soil (MP Biomedicals) with modification 3, mitochondrial DNA was extracted from the total freeze-dried *G. album* leaves in addition to bacterial DNA. The bacterial and mitochondrial DNA demonstrated the amplification of bacterial DNA (weak band, 693 bp, 799f/1492r) and mitochondrial DNA (strong band, 1025 bp, 799f/1942r) after the 1st and 2nd

elution. In addition, the bands containing bacterial DNA after the second elution indicated that some of the extracted DNA remained on the binding matrix after the first elution (Fig. 19b, Tab. 13).

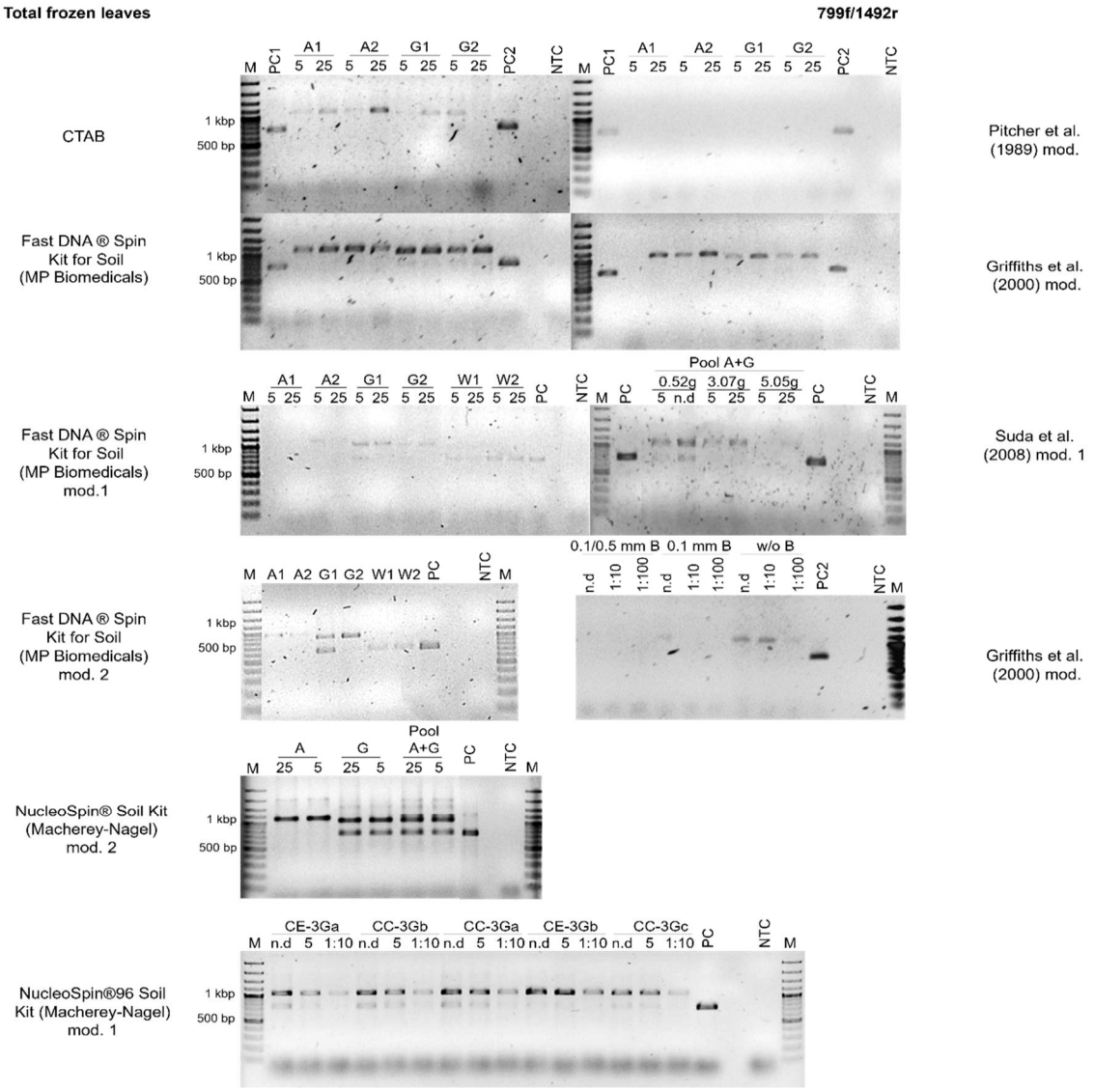

Figure 17 PCR-amplifications of 16S rRNA gene target with universal bacterial primer system 799f-1492r from the total DNA templates extracted by different approaches from total frozen leaves of *A. elatius* and *G. album* plant species. All PCR amplifications were tested on 1.4% agarose gel with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). PC, PC1, PC2- positive controls contain the DNA of *Novosphingobium rhizosphaerae* strain JM-1^T (DSM 29344™) extracted by modified protocol of Pitcher et al. (1989) and a Gen Elute Plant Genom DNA Miniprep kit (Sigma-Aldrich); W1, W2-positive control samples with DNA templates extracted from frozen leaves by Wellner et al. (2011) using Fast DNA® Spin Kit for Soil (MP Biomedicals LLC, Germany) from of *Trifolium repens* and *Cerastium holosteoides* leaves. NTC-no template control. 5 and 25 are adjusted concentrations (5 ng/µl and 25 ng/µl) of DNA templates used for PCRs. A1 and A2 are two replicates of F2F-t0-PP-pool A sample; G1, G2 are two replicates of F2F-t0-PP-pool G sample, where F2F is FACE2FACE system, t0-time point zero sample, PP-plant phyllosphere, A-leaves of *A. elatius* plant species, G-leaves of *G. album* plant species. Agarose gels with PCR products (length 693bp) were amplified with primer system 799f/1492r (Lane 1991). n.d -not diluted, L-leaves, mod-modification. CC-3Ga, b, c and CE-3Ga, b, c are samples from ring 3 of control (CC) and of elevated CO_2 (CE) collected from sampling points a, b, c in the GiFACE system (Linden, Germany). 1:10, 1:100 are dilutions.

The spiking test I showed a weak PCR band with the bacterial DNAs (693 bp, 799f/1492r) (Fig. 19c). The weak bands of the bacterial controls (*Methylobacterium* and *Sphingomonas* spp. (M+S)) and a stronger band from the leaf control (pool A+G) indicate a low efficiency of DNA extraction from the *Methylobacterium* and *Sphingomonas* sp. or co-extraction of PCR inhibitors.

DNA extraction from the *Methylobacterium* and in combination with the fresh leaves (pool of *A. elatius* and *G. album*) was tested using the NucleoSpin®Soil kit (Macherey-Nagel) with modification 3 (Fig. 19c, spiking test II). Amplification of bacterial DNAs (thick bands, 693 bp, 799f/1492r) from *Methylobacterium* (M) and from spiking II samples (M+Pool A+G) showed that *Methylobacterium* DNA could be efficiently extracted using this kit.

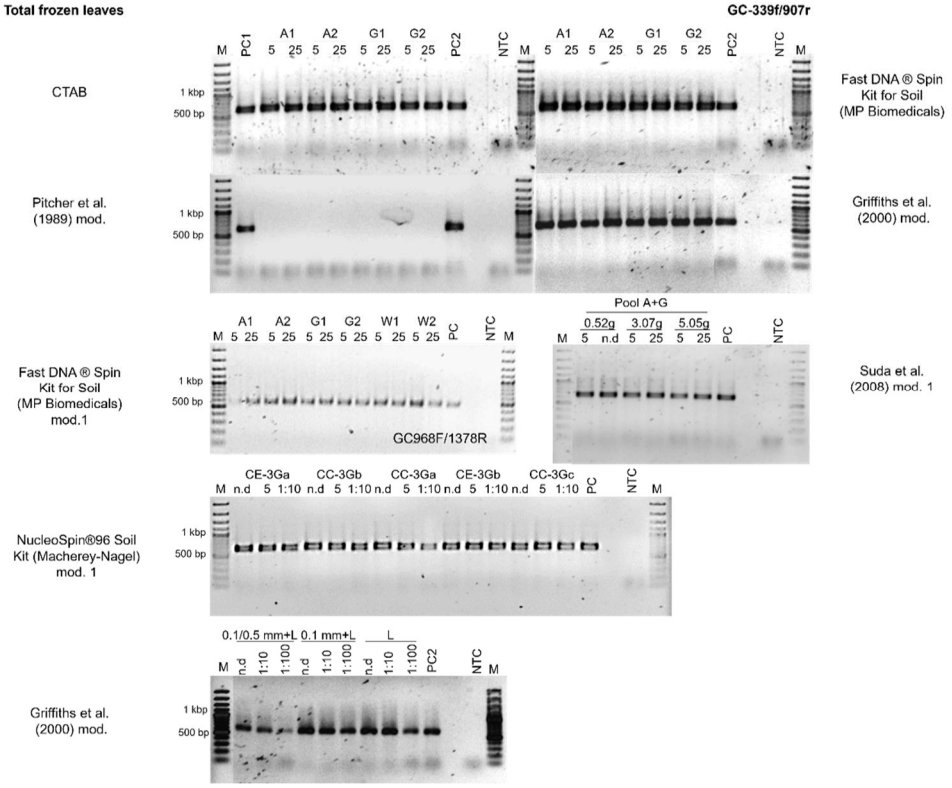

Figure 18 PCR-amplifications of 16S rRNA gene target with universal bacterial primer system GC-339f/907r or GC-968f/1378r from DNA templates extracted by different approaches from total frozen leaves of *A. elatius* and *G. album* plant species. All PCR amplifications were tested on 1.4% agarose gel with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). PC, PC1, PC2 are positive controls with a template as genomic DNA of *Novosphingobium rhizosphaerae* strain JM-1^T (DSM 29344^T) extracted by modified protocol of Pitcher et al. (1989) and a Gen Elute Plant Genom DNA Miniprep kit (Sigma-Aldrich); W1, W2-positive control samples with DNA templates were extracted by Wellner et al. (2011) from of *Trifolium repens* and *Cerastium holosteoides* leaves. NTC-no template control. 5 and 25 are adjusted concentrations (5 ng/µl and 25 ng/µl) of DNA templates used for PCRs. A1 and A2 are two replicates of F2F-t0-PP-pool A sample; G1, G2 are two replicates of F2F-t0-PP-pool G sample, where F2F is FACE2FACE system, t0-time point zero sample, PP-plant phyllosphere, A-leaves of *A. elatius* plant species, G-leaves of *G. album* plant species. Agarose gel with PCR products (length 568/410bp) were amplified with primer systems GC-339f/907f (Muyzer et al. 1993) and GC-968f/1378r (Heuer et al., 1997). n.d -not diluted, L-leaves, mod-modification. CC-3Ga, b, c and CE-3Ga, b, c are samples from ring 3 of control (CC) and of elevated CO_2 (CE) collected from sampling points a, b, c in the GiFACE system (Linden, Germany). 1:10, 1:100 are dilutions.

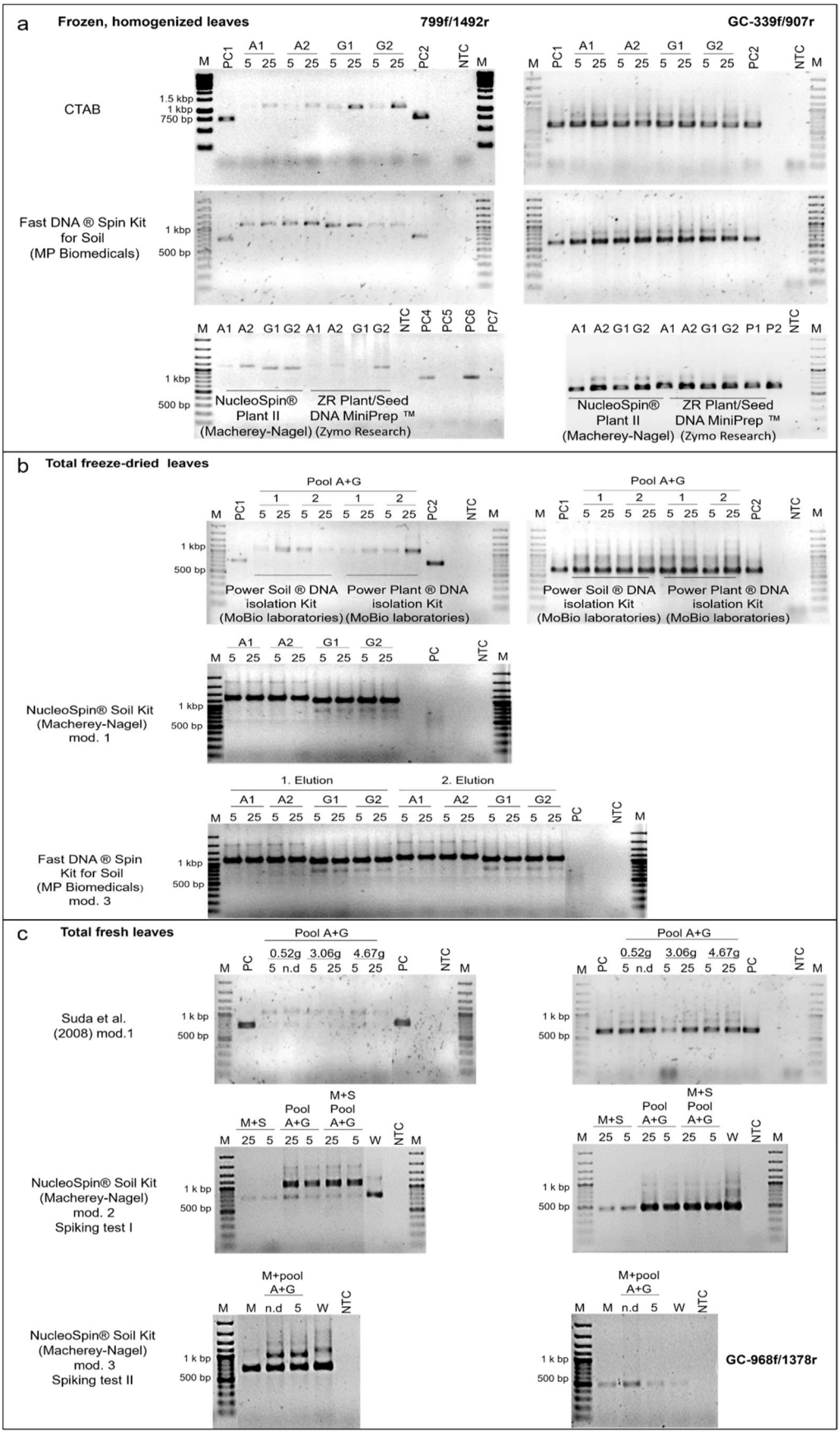

Figure 19 PCR-amplifications comparison of 16S rRNA gene target with universal bacterial primer system 799f-1492r and GC-339f/907r from total DNA templates extracted by different approaches from total frozen homogenized leaves (a), total freeze-dried leaves (b) and total fresh leaves (c) of *A. elatius* and *G. album* plant species.

All PCR amplifications were tested on 1.4% agarose gel with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). PC, PC1, PC2- positive controls with template as genomic DNA of *Novosphingobium rhizosphaerae* strain JM-1^T (DSM 29344™) extracted by Pitcher et al. (1989) modified and/or by a Gen Elute Plant Genom DNA Miniprep kit (Sigma-Aldrich); W1, W2-positive control samples with DNA templates extracted by Wellner et al., (2011) from of *Trifolium repens* and *Cerastium holosteoides* leaves. NTC-no template control. 5 and 25 are adjusted concentrations (5 ng/µl and 25 ng/µl) of DNA templates used for PCRs. A1 and A2 are two replicates of F2F-t0-PP-pool A sample; G1, G2 are two replicates of F2F-t0-PP-pool G sample, where F2F is FACE2FACE system, t0-time point zero sample, PP-plant phyllosphere, A-leaves of *A. elatius* plant species, G-leaves of *G. album* plant species. On the left side are agarose gels with PCR products (length 693bp) were amplified with primer system 799f/1492r (Lane 1991). On the right side are agarose gel with PCR products (length 568/410bp) were amplified with primer systems GC-339f/907f (Muyzer et al. 1993). As PCR templates were total DNA extracted by a: CTAB, Fast DNA®Spin kit for Soil (MP Biomedicals), NucleoSpinPlant II (Macherey Nagel), ZR Plant/Seed DNA Mini/Prep™ kit (Zymo Research) from frozen homogenized leaves, b: PowerSoil®DNA isolation kit (MoBio laboratories), PowerPlant®DNA isolation kit (MoBio laboratories), NucleoSpin®Soil kit (Macherey Nagel), Fast DNA®Spin kit for Soil (MP Biomedicals) modified from total freeze-dried leaves, c: Suda et al. (2008) from total fresh leaves.

3.2.4 Comparative analysis of modified Griffiths et al. (2000) DNA extraction after different cell collection methods (Sterivex, centrifugation, Percoll)

Before DNA extraction, cells were first detached from a 1-2 g pool of *A. elatius* and *G. album* leaves by shaking in PBS buffer and sonication, then collected by three different methods (Sterivex filtration, centrifugation, Percoll density gradient centrifugation) (Fig. 20a) to reduce co-amplification of chloroplasts and mitochondria. DNA extracted using the modified protocol of Griffiths et al. (2000) (chapter 2.2.3.2.8) was checked for the presence of the partial bacterial 16S rRNA gene using the universal bacterial primer system 799f/1492r, and for the amplification of the partial chloroplast 16S rRNA gene with bacterial DNA using the universal bacterial primer system-GC-339f/907r (Fig 20B). In all samples, no bands containing bacterial DNA (693 bp, 799f/1942r) were observed. Instead, strong bands containing chloroplast DNA (568 bp, GC-339f/907r) were amplified from cells collected on a Sterivex filter, and a weak band from the cells collected by centrifugation (Fig. 20b). These results indicate that cells detached using PBS buffer by shaking could be collected by both Sterivex filtration and centrifugation, however, the ratio of bacterial cells to chloroplasts was very low. Furthermore, the weak chloroplast band after centrifugation suggests that not all cells were collected, unlike with Sterivex filtration. The absence of bacterial and chloroplast DNA amplification after Percoll centrifugation indicates that this method was unsuccessful for separating and collecting cells. No amplification of bacterial and chloroplast DNA after Percoll centrifugation indicates that separation and collection by this method was not successful.

a

Pool of A+G		
1.5 g	1 g	2 g
Detachment by shaken in PBS buffer		
Sonification		
2x30 ml	2x30 ml	30 ml
Sterivex	**Centrifugation**	**Percoll**
DNA extraction using modified protocol of Griffiths et al. (2000)		

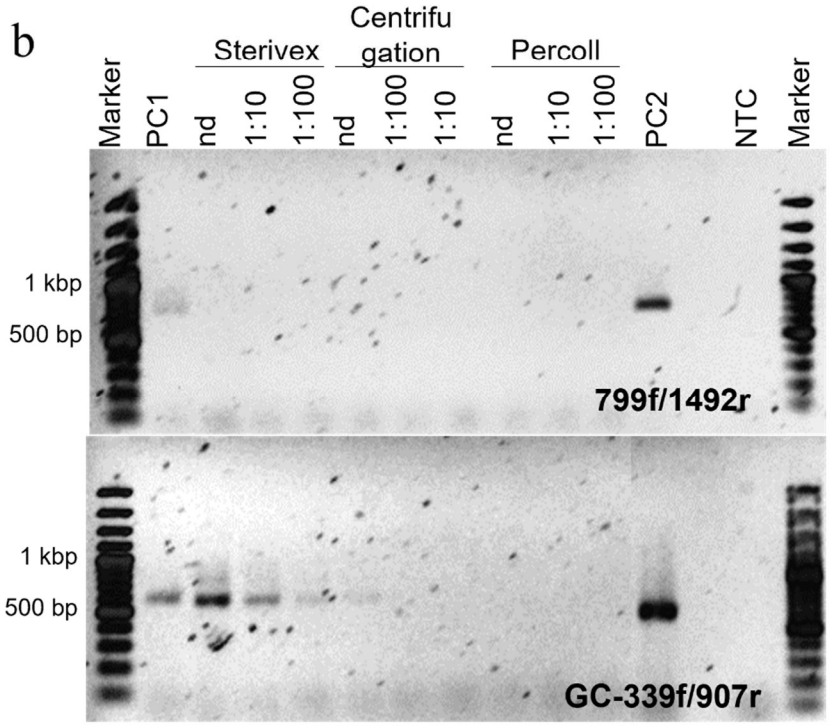

Figure 20 Comparison of DNA extractions using the modified method of Griffiths et al. (2000) after detachment and cells collection using different methods (Sterivex, centrifugation, Percoll). a: Brief overview of cells detachment and three collection methods (Sterivex, centrifugation, Percoll). b: PCR-amplifications of partially 16S rRNA gene targeting used universal bacterial primer system 799f/1492r (Chelius and Triplett, 2001; Lane, 1991) and GC-339f/907r (Muyzer et al. 1993) from DNA templates extracted using modified Griffiths et al. (2000) method after detachments and cells collection by Sterivex filtration, centrifugation and cells separation by Percoll gradient density centrifugation. All PCR products were tested on 1.4% agarose gel with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). PC1, 2- positive controls with template as genomic DNA of *Novosphingobium rhizosphaerae* strain JM-1^{T} (DSM 29344™) extracted by modified Pitcher et al. (1989); NTC-no template control. N.d- not diluted, 1:10 and 1:100 is adjusted dilutions of PCR templates.

3.2.5 Comparative analysis of DNA extractions using NucleoSpin® Soil kit (Macherey-Nagel) after different cells detachments, Nycodenz separation, collecting methods

Before DNA extraction with the NucleoSpin®Soil kit (Macherey-Nagel), leaf-associated bacterial cells were isolated from fresh leaves using two methods to reduce co-amplification of chloroplasts and mitochondria (Fig. 21A). In the first method, 1 g *A. elatius* leaves were first homogenized and the cells were separated from the leaf material using Nycodenz then collected by centrifugation or Sterivex filtration. In the second method, leaf-associated bacteria from *A. elatius* and *G. album* leaves were detached from 10 g leaf material, filtered (with and without a prefilter), and collected by Sterivex (treatment 1, 2).

Separation of the homogenate of *A. elatius* leaves containing 80% Nycodenz showed three defined phases (phase I, phase II and Nycodenz) (Fig. 21B). Bacterial cells were detected not only in phase (I) (Fig. 21B, a, c), but also in phase (II) with plant debris (Fig. 21B, e, g) by SYBR Green I staining (Fig. 21B, a, b). After collecting cells from the two phases by centrifugation, cells were still present in the supernatant after centrifugation (Fig. 21B, a-h). Thereafter, the cells from phase I were collected on Sterivex filters (0.22 µm pore size). PCR amplification, using the universal bacterial primer system (799f/1492r) of DNA extracted with the NucleoSpin®Soil kit (Macherey-Nagel) with modification 3, showed very weak amplification of bacterial DNA (band 693 bp) and a strong amplification of mitochondrial DNA (band 1025 bp) in both phases (I, II). In contrast to the other method which the bacterial cells were detached from 10 g of fresh leaves (*A. elatius*, *G. album*), filtered by nylon mesh filter and collected using a Sterivex filter. After rinsing with PBS buffer (treatment 1: double, 100 ml PBS, treatment 2: single, 50 ml PBS) and filtration using a nylon mesh filter, each plant showed different colorations due to different content of released chloroplasts and chlorophyll molecules *A. elatius* leaves (green) and *G. album* leaves (yellow green) (Fig. 21C). Prefilters (5 µm, 8 µm) used for removing chloroplasts became clogged by chloroplast and bacterial aggregates after 50 ml and 210 ml. PCR amplification with the universal bacterial primer (799f/1492r) of DNA extracted with the NucleoSpin®Soil kit (Macherey-Nagel) with modification 3 (treatment 1) and with modification 4 (treatment 2) showed stronger bands with the bacterial DNA (693 bp) compared to the bands from Nycodenz method. After single rinsing and filtration (treatment 2) and cells collection by sterile filter without prefilter, stronger bands with bacterial DNA (693 bp) and weaker bands with mitochondrial DNA (1025 bp) were amplified after double rinsing and filtration (treatment 2). Furthermore, comparison

of the PCR amplification of DNA extracts of *A. elatius* obtained after simple rinsing with filtration per nylon mesh filter with and without prefilter (treatment 2) showed that the PCR band with the mitochondrial DNA (1025 bp) was stronger without prefilter than with prefilter. Comparison of the amplified mitochondrial DNA of all methods showed that only the method with detachment, single rinsing, filtration and with cells collection via Sterivex filtration without prefilter, led to the greatest reduction of mitochondrial DNA of the *G. album* leaves.

A

Nycodenz		Treatment 1		Treatment 2	
A		A	G	A	G
1 g		10 g	10 g	10 g	10 g
50 mM Tris-HCl pH 7.5+1% Triton X-100		Detachment by stomacher in PBS buffer+silwet-77			
Homogenisation		Sonification			
80% Nycodenz		Filtration by nylon mesh filter			
		Rinse of leaves 2 times with 100 ml PBS buffer		Rinse of leaves1 time with 50 ml PBS buffer	
Centrifugation		Sterivex filtration			
32 ml (phase I)	2 ml (phase II)	50 ml prefilter 5 µm, 150 ml	380 ml	210 ml prefilter 8 µm, 130 ml	330 ml
Sterivex filtration	Cetnrifugation	Sterivex filtration			
Extraction using NucleoSpin® for Soil Kit (Macherey-Nagel) manufacturing protocol using					
modification 3				modification 4	

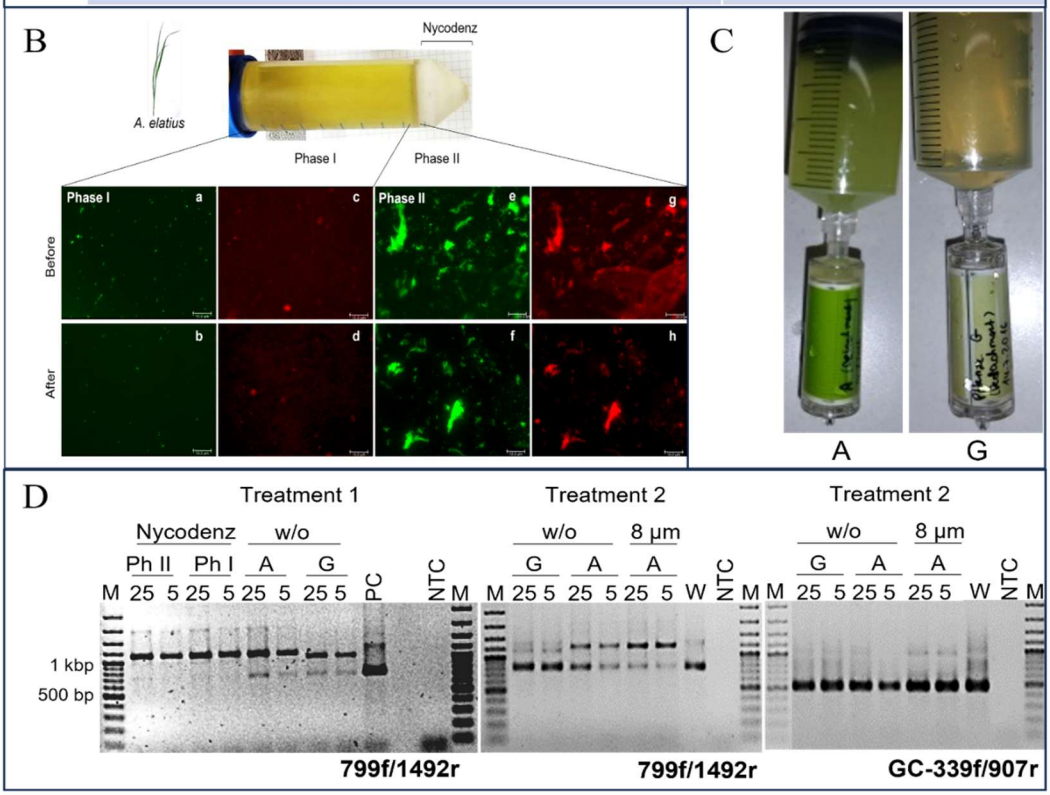

Figure 21 Comparison of DNA extraction using NucleoSpin®Soil kit (Macherey-Nagel) after bacterial cells separation, detachment and different filtration methods.

A: Brief overview of the Nycodenz test and treatments (1, 2). B: A photograph of 50 ml falcons with phases I, II, Nycodenz after centrifugation using the nonionic iodinated density gradient Nycodenz and pictures of green fluorescence of SYBR Green I stained cells in phasis I, II before (a, e) and after (b, f) centrifugation and auto-fluorescence in phasis I, II before (c, g) and after (d, h). C: A photograph of Sterivex filter and syringe with cell suspension from *A. elatius* leaves (A) and *G. album leaves* (G) after filtration with a nylon mesh filter in (treatment 2). D: PCR-amplifications of partial 16S rRNA gene targeting with universal bacterial primer system 799f/1492r (Chelius and Triplett, 2001; Lane, 1991) and GC-339f/907r (Muyzer et al. 1993) from DNA templates extracted using NucleoSpin® Soil kit (Macherey-Nagel) with modification 3 and 5 after cells separation (Nycodenz), detachments, different filtration methods (treatments 1, 2). All PCR amplifications were tested on 1.4% agarose gel with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). PC- positive controls with template as genomic DNA of *Novosphingobium rhizosphaerae* strain JM-1^T (DSM 29344™) extracted by modified Pitcher et al. (1989); W-positive control samples with DNA templates extracted by Wellner et al., (2011) from of *Trifolium repens* and *Cerastium holosteoides* leaves. NTC-no template control. 5 and 25 are adjusted concentrations (5 ng/µl and 25 ng/µl) of DNA templates used for PCRs. Ph I-phase I after separation by Nycodenz centrifugation and after Sterivex centrifugation, Ph II-phase II after separation by Nycodenz centrifugation and cells collection by centrifugation. A- *A. elatius* fresh leaves, G- *G. album* fresh leaves. Prefilter with 8 µm pore size. w/o-without prefilter.

4 Discussion

4.1 eCO_2 effect on cultured oligo/heterotrophs and methylotrophs

Global climate changes due to increased CO_2 concentrations in the atmosphere (IPCC, 2007) have an impact on plants (Ainsworth&Rogers, 2007, Jones et al., 2014; Ainsworth&Long, 2021) and bacterial communities on the phyllosphere and plant-microbe and microbe-microbe interactions (Ren et al., 2014, Ikeda et al., 2015, Christian et al., 2021). It is expected that CO_2 concentrations will continue to rise rapidly (Dyson et al., 2005, Canadell et al., 2007, Hansen et al., 2008). Since the phyllosphere microbiota plays an important role in plant health and growth making it of great importance for agriculture and the environment (Lugtenberg et al., 2002, Lindow&Brandl, 2003, Rasche et al., 2006, Delmotte et al., 2009, Zhou et al., 2011, Innerebner et al., 2011, Knief et al., 2011, Ali et al., 2012, Bulgarelli et al., 2013), it is of great interest to understand the adaptation of bacterial communities to the new climatic conditions. This study is the first to demonstrate the influence of elevated CO_2 concentration on the oligo/heterotrophic and methylotrophic bacteria of the phyllosphere of abundant plants (*A. elatius*, *G. album*) in permanent grassland in the long-term GiFACE system.

4.1.1 Concentration of oligo/heterotrophs and PPFMs

This study found that long growth (17 years) of *A. elatius* and *G. album* species under elevated CO_2 concentration in the GiFACE system had no influence on the concentration of their oligo/heterotrophic and PPFM inhabitants. The same concentration of oligo/heterotrophic and methylotrophic bacteria grown under aCO_2 and eCO_2 conditions might indicate that eCO_2 concentration altered community composition, abundance and diversity, while unaffecting population size. Changes in the communities were demonstrated in the eCO_2 studies (Ren et al., 2014). The concentration differences in the PPFMs between the two plant species *A. elatius* (grass, monocot) and *G. album* (herb, dicotyl) indicate a host plant specificity. The influence of plant species on the community size of PPFMs has been reported in previous studies (Omer et al., 2004, Knief et al., 2008, 2010, Wellner et al., 2011, Mizuno et al., 2012). A highlight of this study was that phyllosphere-associated bacteria (oligo/heterotrophic, methylotrophic) were cultured for the first time using the dilution to extinction method. Significantly more oligo-/heterotrophs (10^{10}–10^{11} MPN per g fresh leaves) were cultivated in this study, with the dilution-to-extinction method, than in previous studies (Jackson et al., 2013, Aydogan et al., 2020) using a CFU method. Jackson et al. (2013) found the highest mean value of 5.5×10^8 CFU per g of baby spinach and a similar range of 10^7 CFUs per g of leaf FW

was found on the *G. album* plant species by Aydogan et al. (2020). The concentrations of culturable oligo/heterotrophs, after cultivation by the dilution-to-extinction method, were higher than in previous studies. This increase is because the small, slow-growing abundant cells were not overgrown by the fast-growing cells in the liquid medium, which is the case on agar plates (Button et al., 1993). Although the amount of PPFMs (10^6–10^8 MPN per g fresh leaves) from this study was consistent with the highest PPFM amount found in the *Bellis perennis* herb (10^8 CFU per FW) and in *Arabidopsis thaliana* (10^8 CFU per g) from other studies (Knief et al., 2008, 2010), the PPFM amount (10^2–10^5 CFU per g leaf FW) obtained from the same plant species from the control samples, outlined in the Aydogan et al. (2020) study, was lower. This indicates the improved cultivation of PPFMs by the dilution-to-extinction method than by CFU method.

4.1.2 Effect of eCO_2 concentration on composition of bacterial assemblages

This study showed that permanent aeration with elevated CO_2 concentrations led to changes in the structure and composition of the most abundant enriched oligo/heterotrophic and methylotrophic bacteria on both plant species (*A. elatius*, *G. album*). The methylotrophic bacteria include the non-methylotrophic bacteria (*Sphingomonas*, *Pseudomonas*) that grew under methylotrophic conditions due to the low concentrations of sugar, organic and amino acid in the plant inoculum (Aydogan et al., 2020). This result was consistent with other studies (Ren et al., 2014; Ikeda et al., 2015) that had also investigated the structure and composition of bacterial communities on rice leaves from the rice-FACE system using culture-independent methods. The decrease in variability and diversity of the most enriched oligo/heterotrophic and methylotrophic communities on both plants under eCO_2 concentration was consistent with the result of Ikeda et al. (2015). Ikeda et al. (2015) reported the decrease in diversity of rice leaf speckle-associated bacteria grown under low-nitrogen eCO_2 conditions. In addition to this, the study also highlights how diversity depended on the physiological state of the rice plant and leaf metabolites (e.g., various sugars) rather than on eCO_2 concentration.

4.1.3 Elevated CO_2 effect diversity and abundance of oligo/heterotrophs and methylotrophs

The most frequently enriched members of *Pseudomonas*, *Sphingomonas*, *Plantibacter*, *Methylobacterium* genera were the most abundant inhabitants on the *G. album* plant leaves (herb) grown under eCO_2 concentration. The abundance of these genera has also been observed on other herbaceous plants such as the *Arabidopsis thaliana*, *Glycine max* (soybean)

and *Trifolium repens* (clover) (Delmotte et al., 2009, Innerbener et al., 2011, Knief et al., 2012, Bodenhausen et al., 2013, Jackson et al., 2013, Lumactud et al., 2016). The genera *Curtobacterium*, *Stenotrophomonas*, *Microbacterium*, *Pseudomonas* and *Methylobacterium*, which are most abundant on the leaves of *A. elatius* (grass), have also been found as common inhabitants on the phyllosphere of other grasses such as rice, *Poa* spp. (bluegrass), *Festuca* sp. (fescue) and *Agropyron repens* (cushion grass) (Behrendt et al., 1997, Ferrando et al., 2012, Knief et al., 2012; Sun et al., 2008; Ren et al., 2015). The differences in abundance and diversity found between these two plant species are plant specific. The structure of the bacterial communities is determined by the genotype of the plant (Whipps et al., 2008) and physiological status of the plant (Ikeda et al., 2015).

It is known that elevated CO_2 concentrations lead to an increase in non-structural soluble carbohydrates (sugars, starch) of leaves via increased photosynthesis (Ainsworth& Rogers 2007, Xu et al., 2013, Ainsworth&Long 2005, Watanabe et al., 2014, Dong et al., 2018). Ikeda et al. (2015) reported an increase in plant metabolites (e.g. sucrose content) under eCO_2 concentration when determining the metabolic profile of rice leaves grown under ambient and elevated CO_2 conditions. Increases in non-structural carbohydrates were also observed in the other short and long-term FACE studies (Campbell&Stafford Smith, 2000; Dumont et al., 2015; Picon-Cochard et al., 2004).

Therefore, I assume that the increase in the most abundant oligo/heterotrophic *Pseudomonas*, *Sphingomonas* and *Microbacterium* from this study was a response to the eCO_2 concentration and the subsequent increased availability of plant metabolites. Most phyllosphere bacteria can access plant metabolites (e.g. saccharides) by producing IAA (Goldberg, 1980, Fry, 1989; Vanderhoef&Dute, 1981) or by utilizing carbohydrates, amino and organic acids passively, which is all released directly by the plant (Tukey, 1970; Leveau&Lindow, 2001; Van der Wal&Leveau, 2011). The most frequently enriched *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas* and *Microbacterium* isolates from this study may be IAA producers. These genera are already known by many studies as dominant IAA producers (Madhaiyan et al., 2005, 2015; Ryu et al., 2006, Vorholt 2012, Kembel et al., 2014, Khan et al., 2014, Akter et al., 2016, Puri et al., 2016a, Krishnamoorthy et al., 2020, Abadi et al., 2020, Ulrich et al., 2021, Zhu et al., 2022).

It could be assumed that an increased supply of mono- and disaccharides and amino acids on *G. album* leaves influences the proliferation of *Pseudomonas*, this is because, firstly,

more *Pseudomonas* were isolated under eCO_2 conditions, and they are known to be specialists in the utilization of mono- and disaccharides and in an uptake of amino acids (Delmotte et al., 2009). The abundance and number of *Sphingomonas* isolates under eCO_2 conditions might also depend on the plant substrate supply, as seen with *Pseudomonas*. *Sphingomonas* can uptake and utilize many different carbon substrates in small quantities (Delmotte et al., 2009). The plant produces sugars (mono-, disaccharide such sucrose, trehalose, raffinose or galactinol) as signaling molecules for activating the defense response and increasing plant resistance against pathogens and/or producing disaccharides such as sucrose and trehalose as a response to abiotic stress (Trouvelot et al., 2014). These substrates can be used as nutrients by *Sphingomonas*, *Pseudomonas* and *Plantibacter*.

Another selection factor is mobility. Mobility is necessary for survival and growth in the phyllosphere, because the nutrient resources on leaves are limited and spatially heterogeneously distributed (Lindow&Brandl, 2003). The most isolated species of *Pseudomonas*, *Stenotrophomonas*, *Microbacterium* and *Methylobacterium* may include isolates that possess flagellin or numerous fimbrial appendages to quickly access nutrients (Haefele&Lindow, 1987, Schauer et al., 2011, Ulrich et al., 2021).

The increased abundance of *Pseudomonas*, *Sphingomonas* and *Microbacterium* under eCO_2 concentration could lead to improved plant growth due to their plant growth-promoting properties. Among these genera, more isolates were isolated from leaves grown under eCO_2 concentration than from aCO_2 concentration. The plant growth-promoting properties of these genera have been reported in many studies (Delmotte et al., 2009, Vorholt, 2011, Innerebner et al., 2011, Mayer et al., 2019, Abadi et al., 2020, Gayathry et al., 2024). *Pseudomonas*, *Sphingomonas* and *Microbacterium* can produce growth hormones such as auxin (Madhaiyan et al., 2005, 2015; Ryu et al.,2006; Vorholt, 2012, Kembel et al., 2014; Puri et al., 2016a; Abadi et al., 2020; Zhu et al., 2022) to improve plant growth. The certain that the *Pseudomonas* and *Sphingomonas* isolates may have led to improved growth of *G. album* plant species through their plant growth-promoting properties is supported by findings from Andresen et al. (2018) and Seibert et al. (2021) studies. During the 17-year study, using the GiFACE system, an increasing growth in only herbs (including *G. album* plant species) was observed. The increase in abundance of the *Microbacterium* isolates on the *A. elatius* leaves grown under eCO_2 conditions, could lead to improved plant nutrition due to the plant growth-promoting property of N_2 fixation (Knief et al., 2011; Mwajita et al., 2013; Batool et al., 2016). Two years

ago, Andresen et al. (2018) and Seibert et al. (2021) observed a decline in biomass and a reduced nitrogen concentration in the leaves of grasses (including A. *elatius* plant species) from the same Giessen-FACE system.

The increased abundance of the most frequently enriched *Sphingomonas*, *Pseudomonas* and *Microbacterium* under eCO_2 concentrations could have a protective effect against pathogenic bacteria on the plants. The protective effects of *Sphingomonas* (Innerebner et al., 2011), *Pseudomonas* (Baltrus et al., 2017) and *Microbacterium* (Lopes et al., 2015) against pathogens are known. However, it is known that there are both pathogenic and non-pathogenic *Pseudomonas* (Baltrus et al., 2017). Alegre et al. (2013) and Mikicinski et al. (2016) reported on the protective effects of the strain *Pseudomonas graminis* DSM 11363 against the pathogen *Erwinia amylovora* in the phyllosphere of apples. In this study, no *Erwinia* isolates were isolated from the phyllosphere of *the A. elatius* plant species grown under elevated and ambient CO_2 concentrations. The presence of keystone taxa such as *Sphingomonas* may have been a factor in stabilizing the bacterial community on the *G. album* leaves. Agler et al. (2016) reported on the "sub" or keystone taxa that play a disproportionate role in shaping microbial communities on host plants. The increased abundance of keystone taxa *Sphingonmonas*, could be a reason for the lower variability or high stability of the phyllosphere microbiota of *G. album* plants grown under elevated CO_2 concentrations. The increased abundance of *Microbacterium* under eCO_2 concentrations could decrease the abundance of genus *Curtobacterium*. The known pathogens *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, are inhibited under oligotrophic growth conditions by the plant growth-protecting *Microbacterium testaceum* (Lopes et al., 2015). Despite the decrease in *Curtobacterium* isolates under eCO_2 concentrations, *Cutrobacterium* genera was still abundant on *A. elatius* leaves. The abundance of *Cutrobacterium* could be because in addition to pathogenic species, there are also plant-protective species (Mayer et al., 2019) such as *C. albidium* (Vimal et al., 2019), *C. herbarum* (Diez-Mendez&Rivas, 2017, Mayer et al., 2019), *C. oceanosedimentum* (Khan et al., 2020), *C. plantarum* (Samain et al., 2017) present.

An interesting finding of this study was that abundant *Plantibacter*, *Stenotrophomonas*, *Methylobacterium* as plant growth promoting genera (Behrendt et al., 2002, Lumactud et al., 2017, Mayer et al., 2019, Innerebner et al., 2011, Ulrich et al., 2021) were not affected by eCO_2 concentrations. I suspect that in addition to their plant growth-promoting properties, they have other abilities that help them quickly adapt to changing

environments, e.g. increased CO_2 concentration. *Plantibacter flavus* strains and *Methylobacterium* contain not only genes for growth hormones but also genes for aminocyclopropane-1-carboxylate (ACC) deaminase, which can degrade ACC as a precursor of the stress hormone (ethylene, which triggers senescence in plants) (Madhaiyan et al., 2007, Lumactud et al., 2017, Mayer et al., 2019). *Plantivacter flavus* and *Methylobacterium* possess genes for ABC transporters for mono- and disaccharide utilization (Lumactud et al., 2017), which utilizes sugars produced by the plant during abiotic stress, which is a defense response to pathogens (Trouvelot et al., 2014) and by photosynthesis (Ainsworth&Long 2005, Watanabe et al., 2014, Dong et al., 2018). *Stenotrophomonas* can synthesize the disaccharide trehalose from glucose itself (Paul et al., 2008) and thereby indirectly increase the host plant´s immunity against abiotic stress factors such as eCO_2 concentration, drought and high temperatures (Ulrich et al., 2021).

This current study showed no indirect effect of eCO_2 concentration on *Methylobacterium* instead showed a positive effect on non-methylotrophic bacteria such as *Pseudomonas*, *Rhizobium* and *Sphingomonas* of *G. album*, and *Microbacterium* of *A. elatius*. An increase in the abundance of the genus *Methylobacterium* due to the enhanced response of plant methanol to eCO_2 concentration, which was hypothesized in this study, was not observed. Which is not consistent with any of the results from other studies. The results of several studies are contradictory and show both negative and positive effects of eCO_2 concentration. Ikeda et al. (2015) showed increase of cultivated *Methylobacterium* on rice leaves under eCO_2 conditions and Christian et al. (2021) showed a decrease of *Methylobacterium* sp. under eCO_2 concentration on soybean leaves due to their antagonistic abilities against fungi. It was initially assumed that methanol production is highly stimulated by eCO_2 concentration and leads to an increase in biomass, due to this assumption. Andresen et al. (2018) and Seibert et al. (2021) showed different responses of biomass in herbs and grasses to eCO_2 concentration. They observed an increase in biomass of herbs (*G. album*) and a decrease in biomass of grasses (*A. elatius*) in the GiFACE system during the study period of 17 years.

Since methanol is released through the stomata, the stomatal behavior under eCO_2 concentration should be considered. Holohan et al. (2015) found that stomatal density in *A. elatius* plant species was lower than in *G. album* plant species but showed no significant effect of eCO_2 concentration. Differences in stomatal density have been reported in several studies

(Ainsworth&Rogers, 2007; Haworth et al., 2013; Rodrigues et al., 2016) and are believed to be in response to CO_2 concentration which may vary depending on experimental duration, species, genotypes, and other factors (Ainsworth&Rogers, 2007; Haworth et al., 2013; Rodrigues et al., 2016). Gas exchange (e.g. methanol, CO_2) can be controlled by different regulatory mechanisms e.g. stomatal conductivity, stomatal size, stomatal density. These regulatory mechanisms can adapt to eCO_2 concentration over a long period of time (Konrad et al., 2008), depending on the plant species or genotype. I assume that the growth of methylotrophic bacteria on the plants (*A. elatius*, *G. album*) adapted to released methanol under eCO_2 concentration and that plant growth was influenced by plant growth-promoting bacteria or pathogens. The increase in *G. album* biomass (Andresen et al., 2018, Seibert et al., 2021) could be explained by an increased abundance of non-methylotrophic *Pseudomonas*, *Rhizobium*, *Sphingomonas* under eCO_2 conditions. Plant growth promoting effects of non-methylotrophic *Pseudomonas* and *Rhizobium* are known (Krishnamoorthy et al., 2020). The methylotrophic *Pseudomonas* and *Rhizobium* possess capabilities such as ACC reduction (Win et al., 2018, Saikia et al., 2018); high EPS and IAA production (Akter et al., 2016); N_2 fixation (Madhaiyan et al., 2015) and *mxaF*-gene presentation (Lau et al., 2013, Meena et al., 2012, Alibrandi et al., 2018, Subhaswaraj et al., 2017).

The strong increase of only non-methylotrophic *Microbacterium* on the *A. elatius* leaves, grown under eCO_2 concentration, indicated adaptability to changed environmental conditions. Mandakovic et al., (2020) reported on *Microbacterium* spp. that survived in soil with extreme environmental conditions due to their ability to utilize various substrates (e.g. methanol, carbohydrates). In addition, the increase in methylotrophic *Microbacterium* under eCO_2 conditions could be due to plant growth-promoting genus with the ability to fix N_2 (Knief et al. 2011; Mwajita et al. 2013; Batool et al. 2016) and produce IAA (Vanderhoef&Dute, 1981). In contrast, the decrease in abundance of plant growth promoting *Stenotrophomonas* (Ulrich et al., 2021) could have a stronger impact on the decrease in Biomass of the *A. elatius* plant species under eCO_2 concentrations during the period of 1998 until 2015 (Andresen et al., 2018, Seibert et al., 2021). The *Stenotrophomonas*, which are under control conditions, may be the key taxa of *A. elatius* phyllosphere microbiota.

4.1.3.1 Elevated CO_2 effect on specific phylotypes and genotypes of PPFM-isolates

In this study, no eCO_2 phylotypes of PPFMs (based on partial 16S rRNA gene sequences) and specific phylotypes (based on *mxaF*-gene nucleotide and amino acid sequences) were found on both plant species (*A. elatius*, *G. album*). However, under eCO_2 conditions, one isolate of each phylotypes (F-M1c or F-M4) was found on one plant leaf of *A. elatius* or *G. album* which indicates a plant specific functional adaptation. It suggests that eCO_2 can lead these phylotypes to plant-specific adaptations. Van der Walt et al. (2016) previously reported the unique habitat-specific phylotype found in the soil microbiota of fairy circles surrounded by grasses. In the study by Aydogan et al. (2020), on the leaves of the same plant species, specific *Methylobacterium* spp. phylotypes were found that seemed to be functionally adapted to increased temperatures.

The heterogeneity of PPFMs phylotypes both inter and intra at the strain-level (genotyping) indicated genetic adaptation to eCO_2 concentrations and plant species. However, two specific genotypes on *A. elatius* leaves indicated specific genetic adaptation to increased CO_2 concentration, while the other genotypes were influenced by both factors such as eCO_2 concentration and plant species. Ellis et al. (1999) also found that specific *Pseudomonas* genotypes could survive and dynamically adapt to changing environmental conditions.

4.2 Comparison with the effects of elevated temperature on phyllosphere microbiota of *A. elatius* and *G. album* plant species

To assess the impacts of global climate change, not only the effects of the eCO_2 factor on microbial communities must be considered, but also other environmental factors such as elevated temperatures. Besides the CO_2 factor, temperature is one of the most important environmental factors. Temperature is related to global climate change and is expected to continue to increase (Schütz et al., 1990). The effects of elevated temperatures (eT) on the two abundant plants of permanent grassland (*A. elatius* and *G. album* plant species) and their microbiota were also investigated by our group, Aydogan et al. (2020). A comparison of both effects (eCO_2 and eT) showed that elevated temperature had a much stronger effect on the most abundant cultured heterotrophic and methylotrophic bacteria associated with *A. elatius* and *G. album* than eCO_2 concentrations. Some isolates from the warming experiment were able to adapt better to the altered metabolites and the elevated temperature. Elevated temperature had a strong influence on the metabolite profile of both plants, which significantly shifted malic acid, ethanol and homoserine levels in *G. album* leaves while sucrose

and hydroxyproline levels were significantly shifted in *A. elatius* leaves (Fig. 22). Ikeda et al. (2015) reported that increased eCO_2 concentration also affects the metabolite profile in rice leaves through shifting sucrose and glycerolipids. These metabolic shifts led to changes in the composition and diversity of bacterial communities (Ikeda et al., 2015, Aydogan et al., 2020). The abundance of culturable *Sphingomonas* was significantly reduced under eT. However, some *Sphingomonas* isolates of different phylotypes showed better adaptation to the changed substrate supply (Fig. 23a); efficient growth at elevated temperature of 37°C (Fig. 23b); and indication on genetic adaptation to eT (genotyping, Fig. 24). Elevated temperature had a slightly reduced effect on the abundance of *Methylobacterium* spp. on *A. elatius* leaves but led to the appearance of new phylotypes with functional (alpha subunit of methanol dehydrogenase, *mxaF*) and genetic adaptation (Fig. 25).

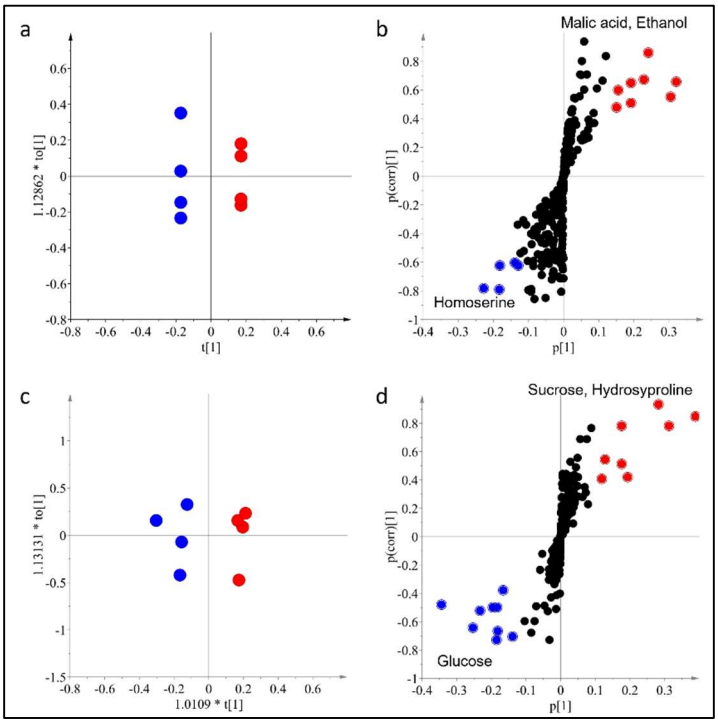

Figure 22 Nuclear magnetic resonance (NMR) analysis of leaf metabolites. a, c; Score plot of Orthogonal projections to latent structures discriminant analysis based on ^{1}H NMR data of (a) *Galium album* and (c) *Arrhenatherum elatius* (t1/to1). Blue: Samples from control (C) plots. Red: samples from elevated temperature (T) plots. b, d; S-plot of Orthogonal projections to latent structures discriminant analysis based on ^{1}H NMR data of (b) *Galium album* and (d) *Arrhenatherum elatius*. Blue: ^{1}H NMR signals higher in control samples, Red: ^{1}H NMR signals higher in elevated samples. (Aydogan et al., 2020).

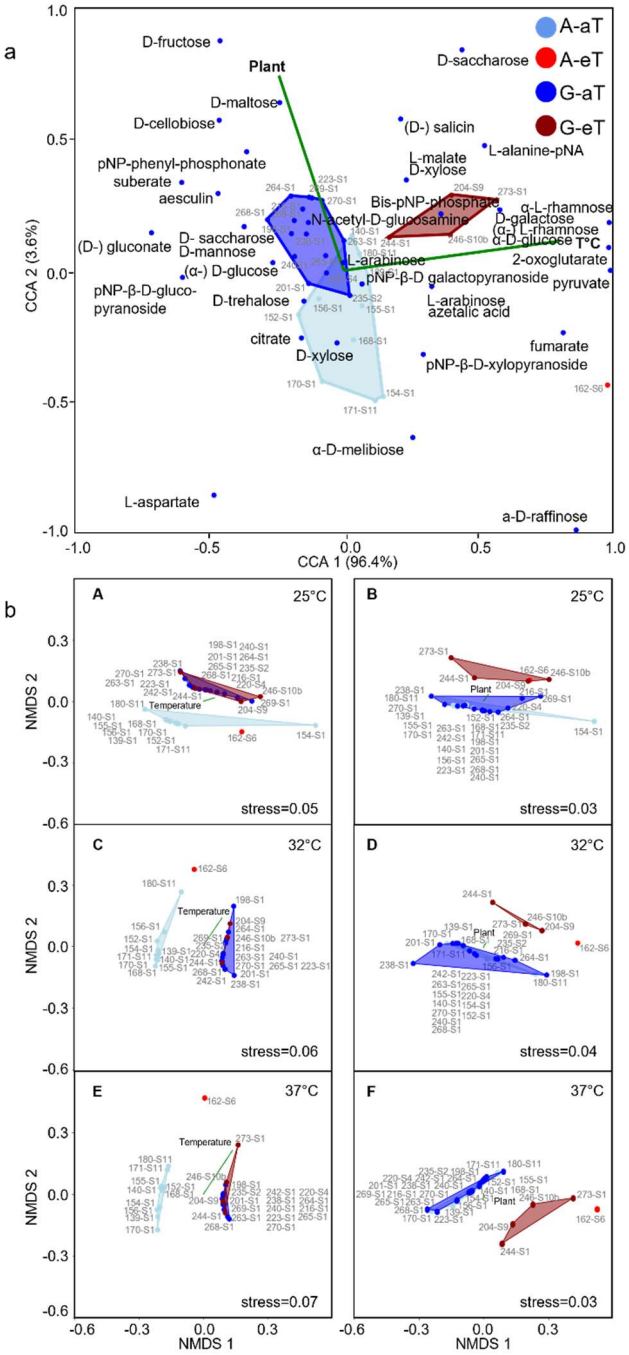

Figure 23 Physiological characterizations of specific *Sphingomonas* isolates. (A) CCA of specific *Sphingomonas* phylotypes: S1, S2, S4, S6, S9, S10b, S11 from C and T plots for two plant species, *A. elatius* and *G. album*, including the environmental factors (temperature, plant species) which are based on the results of carbon substrate utilization and qualitative enzyme test (Kämpfer et al. 1991). The positive/negative matrix of the tests were applied for CCA in PAST3. aT: ambient surface temperature (control plots); eT: plots with elevated surface temperature +2◦C. (B) Effect of different temperatures 25◦C (a, b), 32◦C (c, d) and 37◦C (e, f) on average doubling time of the growth. *Sphingomonas* phylotypes: S1, S2, S4, S6, S9, S10b, S11. The average doubling time (in hours) of those phylotypes presented in NMDS using the PAST3 based on a distance matrix calculated with the Bray–Curtis similarity index. The growth of each *Sphingomonas* phylotype was measured in three replicates for each temperature. Scatter plots (a, c, e) present average doubling time and plant species *A. elatius* and *G. album* as environmental factors; scatter plots (b, d, f) present average doubling time and temperature as the second environmental factor. A: *A. elatius*; G: *G. album*. NMDS scatter plots were performed using PAST3. The significance between C and T plots was determined by one-way ANOSIM test (Bray–Curtis similarity index) (Aydogan et al., 2020).

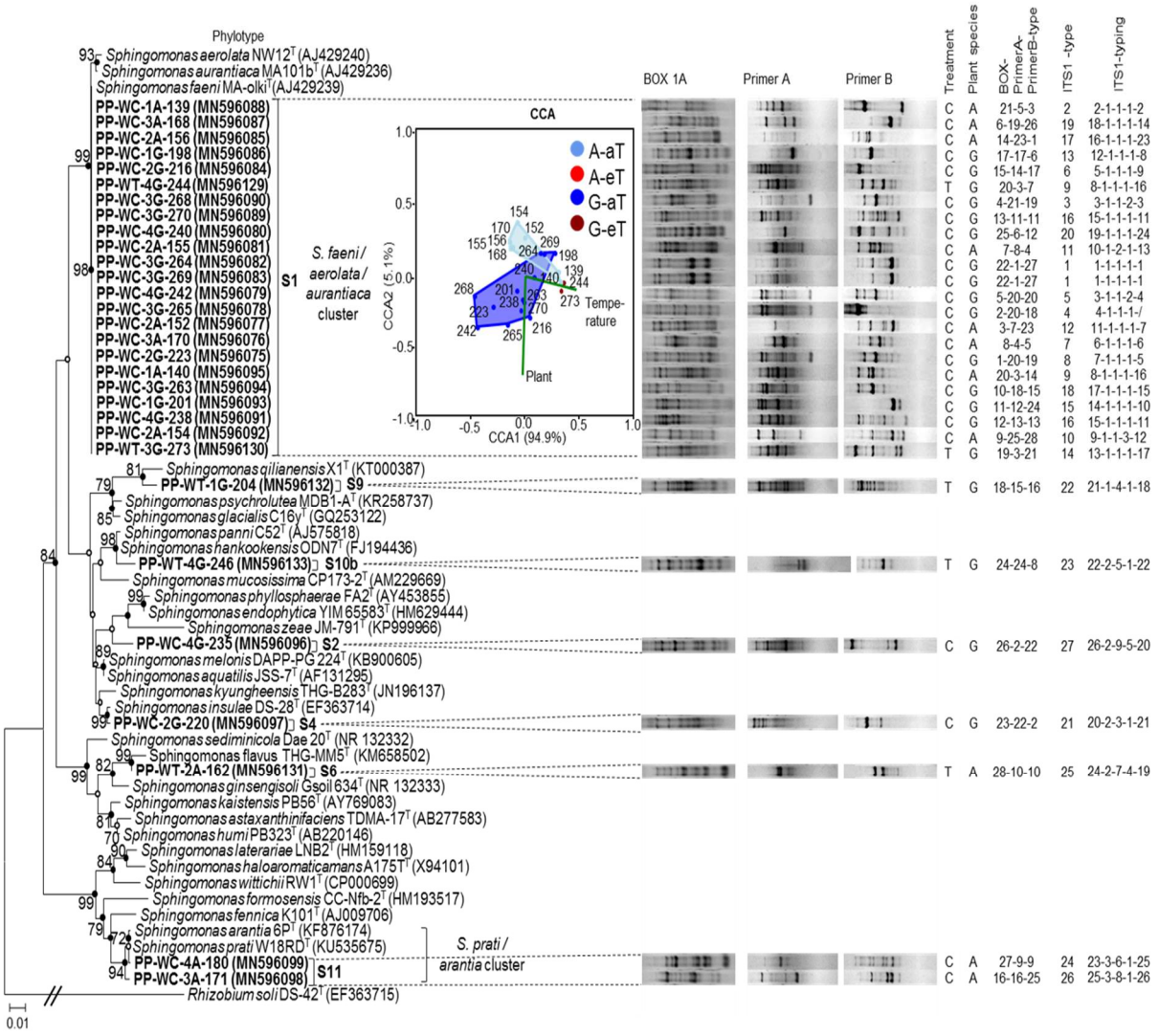

Figure 24 Phylogenetic tree based on nearly full-length 16S rRNA gene sequences showing the phylogenetic relationship of cultured *Sphingomonas* isolates (assigned to different phylotypes: S1, S2, S4, S6, S9, S10b, S11) among each other and to the next related *Sphingomonas* type strains. GenBank accession numbers are given in brackets. The phylogenetic tree was generated with the maximum-likelihood method using Kimura two-parameter model with Gamma distribution and based on 100 replications (bootstrap support) and 1 242 nucleotides. Bootstrap values >70% are represented by numbers and branch nodes. Nodes marked with a filled circle were supported with high bootstrap values (>70%), with an unfilled circle were supported with low bootstrap values (Aydogan et al., 2020).

The stronger effects of eT than eCO_2 indicate that temperature may be a dominant factor that most strongly regulates bacterial community composition and abundance via altered plant physiology. The metabolic profiles of *A. elatius* and *G. album* leaves, from the treatments with elevated CO_2 concentrations, were not investigated in the current study. The next question is what effects on bacterial communities would be expected if both environmental factors could be investigated together. Ikeda et al. (2015) showed a stronger effect of eT than of eCO_2 concentration as a single environmental factor, but in combination they resulted in similar community structures of rice leaf-associated bacteria as controls. In contrast, Ren et al. (2015)

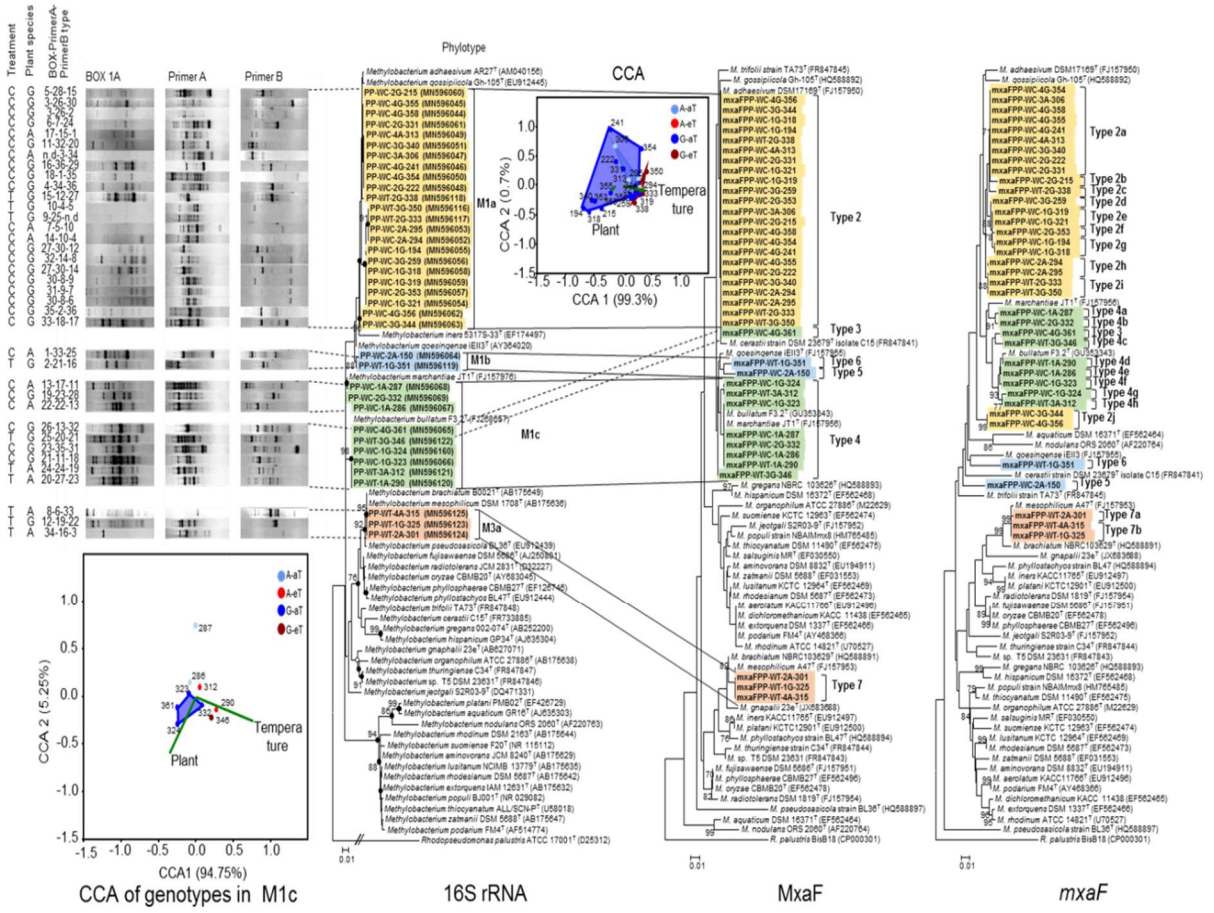

Figure 25 Phylogenetic trees based on nearly full-length 16S rRNA gene sequences and partial mxaF nucleotide and amino acid sequences showing the phylogenetic relationships of cultured *Methylobacterium* isolates (assigned to different phylotypes: M1a, b, c, M3a) among each other and to the next related *Methylobacterium* type strains. GenBank accession numbers are given in brackets. The phylogenetic trees which were based on partial 16S rRNA gene sequences were generated with the maximum likelihood method using Kimura two-parameter model with Gamma distribution. The phylogenetic trees which were based on partial *mxaF*-gene nucleotide and amino acid sequence were generated with the neighbor-joining method using Jukes–Cantor correction (*mxaF*) and JTT correction (MxaF). All phylogenetic trees were calculated with 100 replications (bootstrap support) and were based on 870 nucleotides (16S rRNA gene sequences), 432 nucleotides (*mxaF* nucleotide sequences) and 141 amino acids (MxaF). Bootstrap values >70% are represented by numbers at branch nodes. Nodes marked with a filled circle indicated a high bootstrap value (>70%), while an unfilled circle indicated a low bootstrap value (Aydogan et al., 2020).

also observed no influence of eT and of eCO_2 as a single factor, but the combined treatment of eT and eCO_2 had significant effects on the structure, abundance and diversity of bacterial communities in rice leaves. Based on these results, it is difficult to predict whether the effects of eT will continue to dominate or if it will be cancelled out by the effects of eCO_2 concentrations. To gain more information about the impacts of these major drivers of global climate change on the phyllosphere microbiota of the plants *A. elatius* and *G. album*, experiments need to be conducted taking both factors into account.

4.3 Efficiency of DNA extraction methods for *A. elatius* and *G. album* phyllosphere-associated bacteria

For phyllosphere-associated bacterial community analysis using molecular methods (e.g. DGGE, NGS) based on 16S rRNA gene sequences, it is essential to obtain pure DNA extracts free of co-extracts (chloroplasts, mitochondria) as they can interfere with the analysis (Saito et al., 2007, Reisberg et al., 2012, Arenz et al., 2015, Lefevre et al., 2020). The problem with co-extracts stems from the homology of the chloroplast and mitochondrial 16S rRNA gene sequences with the bacterial 16S rRNA gene sequences (Madigan&Martinko, 2006), a similarity that arose through endosymbiosis (Schimper, 1883). In this study, PCR fragments of different sizes were amplified from the bacterial 16S rRNA gene sequences and from the mitochondrial 16S rRNA gene sequences using the universal bacterial primer systems 799f (Chelius & Triplett, 2001) and 1492r (Lane (1991)). However, PCR fragments of the bacterial and chloroplast 16S rRNA gene sequences amplified using the universal bacterial primer systems GC-339f and 907r (Muyzer et al., 1993) were of the same size. In this study, the community analysis of *A. elatius* and *G. album* leaf-associated bacteria performed using the DGGE method based on the identical PCR-fragment size of 16S rRNA gene sequences of bacteria and chloroplasts with GC-339f/907r primer system amplified from extracted DNA using two kits (Nucleo Spin Plant II kit (Macherey-Nagel), ZR Plant/Seed DNA MiniPrep™ kit (Zymo Research)) was hindered by the abundance of chloroplasts DNA sequences. Consequently, establishing a highly efficient DNA extraction method for *A. elatius* and *G. album*-associated bacteria that avoids co-extracts is crucial.

4.3.1 Efficiency of DNA extraction using direct methods

This study showed a comparison of direct DNA extraction methods with and without modifications used for DNA extraction from *A. elatius* and *G. album* leaf-associated bacteria. These methods were chosen due to their previous use in DNA extraction of leaf-associated bacteria (Suda et al., 2008, Verbylaite et al., 2010, Wellner et al., 2011, Bodenhausen et al., 2013, Drabkova et al., 2014, Bai et al., 2015) and their application in extracting DNA from another host (e.g. coral) which showed homology with the 16S rRNA gene (Griffiths et al., 2000, Baker et al., 2014). In addition, the Pitcher et al., (1989) method, which was used for DNA extraction from pure cultures of Gram-positive and Gram-negative bacteria, was used for environmental microbial DNA extraction.

Of these tested DNA extraction methods such as CTAB methods (Griffiths et al. 2000, Pitcher et al. 1989), Suda et al. 2008 and kits (Fast DNA®Spin kit for Soil (MP Biomedicals), PowerSoil®DNA Isolation kit (MoBio), PowerPlant®DNA Isolation kit (MoBio laboratories), NucleoSpin®Plant II (Macherey-Nagel) and ZR Plant/Seed DNA MiniPrep™ kit (Zymo Research), PowerSoil®DNA Isolation kit (MoBio)) with and without modification, only two soil DNA extraction kits (NucleoSpin®Soil kit (Macherey-Nagel) and Fast DNA®Spin kit for Soil (MP Biomedicals) and a modified Suda et al. (2008) method successfully extracted bacterial DNA from *G. album* leaves. This indicates that these soil kits could be adapted for DNA extraction of *G. album*, but not of *A. elatius* leaf-associated bacteria. The adaptation of soil DNA extraction methods to environmental microbial DNA from various biological materials, including plants, was reported by Ikeda et al. (2005). The application of soil DNA extraction methods for analyzing microbial communities in the phyllosphere and seeds has also been confirmed by other studies (Götz et al., 2006, Garbeva et al., 2001, Green et al., 2006, Ikeda et al., 2006, 2007). The bacterial DNA from *A. elatius* leaves could not be extracted or detected using soil DNA extraction methods compared to *G. album* leaves. The total DNA from *A. elatius* leaves could be extracted a much higher proportion of plant DNA (e.g. chloroplasts) compared to *G. album* leaves, resulting in the amplification of only the strong PCR bands corresponding to chloroplast and mitochondrial DNA. The very low ratio of bacterial biomass of the phyllosphere to plant material was already described by Saito et al. (2007). In the Fast DNA®Spin kit for Soil (MP Biomedicals) protocol, modified steps such as separation of insoluble cellular debris and lysing matrix by repeated centrifugation, prolonged homogenization time and repeated elution with small volumes at 55°C led to increased DNA yield and the first detection of weak bacterial DNA bands after PCR amplification with universal bacterial primer systems. Although prolonged homogenization time is known to not increase DNA extraction but rather cause cleavage of small DNA fragments (Fujimoto et al., 2004), it was nevertheless extended from 40 seconds to three minutes due to the large amount of material used. In this study, it was speculated that despite the prolonged lysis process, lysis was not effective. Therefore, homogenization and removal of plant debris and lysing matrix was repeated once, resulting in a multiple increase in total DNA yield and an increase in bacterial DNA. The significantly higher DNA concentrations from different soils using the Fast DNA®Spin kit for Soil (MP Biomedicals) is already known (Mahmoudi et al., 2011). When bacterial DNA was extracted from total freeze-dried leaves using this method,

an additional centrifugation step was required to remove residual plant debris. However, this did not lead to the reduction of plant DNAs. Furthermore, DNA elution from the thick binding matrix was critically evaluated by measuring DNA concentrations after the second elution. DNA loss was observed after the first elution, which may be detrimental to the molecular characterization of the bacterial communities. Because this kit was able to extract and detect bacterial DNA and was shown to be reliable, it was used for DNA extraction of the oral microbiota of dolphins and seals to identify the pathogen *Oceanivirga salmonicida* using bacterial 16S rRNA gene amplification Illumina MiSeq sequencing (Roy et al., 2020).

In the NucleoSpin®Soil kit (Macherey-Nagel) protocol, DNA extraction with Lysis Buffer 1, which was developed for the mineral-rich soils by the manufacturer, resulted in the lower extraction of the bacterial DNA of *G. album* with the high proportion of plant DNA. The possible extraction of the bacterial DNA could be due to the mineral-rich *G. album* leaves. It is well known that herbs are richer in minerals than grasses (McDowell&Valle, 2000, McDowell&Arthington, 2005). Because the second lysis buffer was designed by the manufacturer for soils with organic carbon, this lysis buffer could release additional bacterial DNA that was not digested the first time with Lysis Buffer 1. DNA extraction with both lysis buffers and double DNA elution resulted in lower DNA yields, but high-quality DNA was obtained based on the successful amplification of bacterial DNA of *G. album*. Because Reisberg et al. (2012) reported the difficulty of DNA extraction from *Methylobacterium* species and the low DNA detectability, this kit was tested for its efficiency in extracting DNA from *Methylobacterium* both as pure culture and in combination with the leaves (*A. elatius*, *G. album*). In this study, this kit was able to efficiently extract and detect total DNA from *Methylobacterium* and from the combination with leaves. In addition, this method provided the best detection of extracted bacterial DNA from *G. album* leaves in the presence of co-extracts and was shown to be reliable. Furthermore, the kit was one of the few kits with an integrated step for the removal of chloroplasts and mitochondrial DNA (Arenz et al., 2015), which was used in our group to investigate the effect of elevated temperature on the composition of bacterial communities in the phyllosphere first of *G. album* plant and then of *G. album* and *A. elatius* plants using bacterial 16S rRNA gene amplicon Illumina sequencing (Aydogan et al., 2018, 2020). Additionally, this kit was used by our group to efficiently extract total bacterial DNAs from various matrices as well as skin and ear canal of German shepherd dogs (Apostolopoulos et al., 2023).

It is known that PCR inhibition can occur when whole cells are used as templates in PCR and DNA bound with proteins is released during PCR denaturation (Wilson, 1997). The high protein contamination of DNA extraction could be an indication of non-functioning cell lysis of the DNA extraction method. This could be the case for DNA extraction using methods such as the modified Pitcher et al. (1989) method, PowerSoil®DNA Isolation kit (MoBio) and PowerPlant®DNA Isolation kit (MoBio). The DNA extracts from these methods showed protein contamination and PCR inhibition in all samples.

Using the modified method of Suda et al. (2008), bacterial DNA could be extracted by carefully separating bacterial cells from plant surfaces using benzyl chloride. The plant DNA extracted using this method does not agree with the results of Suda et al. (2008). A possible reason for this could be the cuts that occurred during sampling by cutting out the middle part of the leaf of *A. elatius* and the hypocotyl of *G. album* plants and/or by the damage to the leaves due to freezing and storage at -80 °C. More damage is caused by freezing, as confirmed by the stronger PCR bands with the mitochondrial DNA from the frozen leaves and the weaker bands from fresh leaves. The results of this study are consistent with the results of the previous study by Zhu et al. (1993). Compared with Suda et al. (2008), Zhu et al. (1993) extracted the bacterial DNA from the cut leaves rather than from the total leaves, which resulted in the extraction of plant DNA.

Because in addition to the selection of DNA extraction method for leaf-associated bacteria, the quantity and plant material is also of great importance, small amounts of sample material are an advantage, especially given the large scale of environmental sampling. Therefore, the modified Suda et al. (2008) method was used to test the smaller amounts of *A. elatius* and *G. album* leaves. Bacterial DNA was extracted from 5 g of fresh leaves as in the study by (Suda et al., 2008), from 0.5 g of leaves as recommended in the soil kits (NucleoSpin®Soil kit (Macherey-Nagel), Fast DNA®Spin kit for Soil (MP Biomedicals)), and from 3 g of both totally fresh and totally frozen leaves. In this study, this method was shown to be suitable for 0.5 g of both fresh and frozen leaves. Unfortunately, this method was not reproducible in further tests. Of all leaf material (total frozen leaves, frozen homogenized leaves, total freeze-dried and fresh leaves), DNA extractions from frozen homogenized leaves performed the worst, showing no amplification of bacterial DNA and a strong amplification of mitochondrial and chloroplast DNA. The reason for this is the strong destruction of plant cells by homogenizing leaves in a mortar under liquid nitrogen, resulting in a dominant amount of

plant DNA after extraction, which was reflected in the PCR amplification. The very low ratio of bacterial biomass to plant material in the phyllosphere to plant material has been previously reported (Saito et al., 2007).

4.3.2 Efficiency of DNA-extraction methods from harvested phyllosphere-associated cells

My results demonstrated the most efficient DNA extraction method for leaf-associated bacteria from harvested A. *elatius* and *G. album* leaves-associated bacteria. Cells from 10 g of leaves were first mechanically separated using Stomacher (in PBS buffer supplemented with Silwet L-77), combined with ultrasonication, rinsed once, separated from plant debris using a nylon mesh filter, collected with Sterivex, and then DNA was extracted using NucleoSphin® Soil kit (Macherey-Nagel) with modification 4. The efficiency of this method lies in several steps. First, it is the amounts of leaves from which the leaf-associated bacteria are detached. Only after increasing the leaf extraction amount to 10 g was, it possible to detect bacterial DNA. This shows that removing larger amounts of leaves can lead to a higher bacterial biomass. In previous studies, bacterial cells were detached from similar leaf quantities such as 5–10 g of *Arabidopsis* rosette leaves (Reisberg et al., 2012, 2013), 10 g of floating macrophytes (Xie et al., 2015) or even from larger leaf quantities, such as 15–20 g of various plants (O´Brien&Lindow, 1989), 25 g of spinach leaves (Solis et al., 2024) and 200–250 g of rice leaves (Knief et al., 2012).

The addition of Silwet L-77 may have also contributed to the efficiency of the detachment process, as it is a surfactant with physical properties such as low interfacial tension, coefficient of friction, and surface tension, which improves the leaching of bacteria from the leaves. Huang et al. (2018) reported increased removal of bacteria from inoculated leaf samples after addition of surfactant. In the present study, 0.1% Silwet was added to the wash buffer, as done in the analysis of microbial community composition in the rice phyllosphere (Knief et al., 2012), where 0.1% Silwet L-77 was added to the wash buffer and cells were detached by sonication and shaking. A higher concentration of 0.2% Silwet L-77 was also used in an earlier study by the same research group (Delmotte et al., 2009).

Mechanical treatment in a Stomacher in combination with sonification, may have further increased the detachment of bacterial biomass. The benefits of mechanical detachment with a Stomacher were also reported by comparing treatment with Stomacher and shaking (Aydogan et al., 2018), where more cells were identified after detachment with the Stomacher than after shaking. It is known that biofilms adhere very strongly to the leaf

surfaces and are not easily removed, especially not as individual cells (Vorholt, 2012). Sonification can destroy biofilms (Morris et al., 1998). Therefore, the leaves were sonicated to remove the biofilm-forming inhabitants of the phyllosphere.

Rinsing with buffer and subsequent filtration of the leaves could also be crucial for the amounts of bacterial cells washed away. A greater yield of bacterial cells was achieved after one rinse with a smaller volume. Whereas two rinses resulted in more chloroplasts and mitochondria. I demonstrated that the flushing of leaves can lead to an increase in either bacterial cells or chloroplasts and other organelles. This could be due to the filtration volume and organelle content. The organelles abundant in the cell suspension may shrink the nylon filters after filtering large volumes, causing the bacterial cells to be retained. The numerous organelles in the cell suspension could also cause the nylon filters to retain the bacterial cells after filtering large volumes. In this study, nylon mesh filters with a pore size of 100 µm instead of 200 µm as in the study (Delmotte et al., 2009) were employed to separate the cell suspension from the leaves. However, the chloroplasts could not be separated with the prefilters (5 µm and 8 µm). The chloroplasts of wheat are known to have a diameter of 5 µm and the thickness 1-2 mm (Ellis&Leech, 1985). These filters clogged after a short period and with small volumes, and after filtration with the 8 µm prefilter, less bacterial DNA was amplified from the filtrate compared to filtrate without a prefilter. Because bacteria often occur in aggregates (e.g. when biofilms are disrupted from the leaf surface), these aggregates were lost on the prefilters and not analyzed. Alternative methods for separating bacterial cells from organelles (e.g. chloroplasts) using Nycodenz or Percoll centrifugation density media were not effective in this study. After Nycodenz centrifugation, bacterial cells were detected in all phases due to the low centrifugal force (3 345 x g of the available centrifuge. Separation with Nycodenz requires a centrifugal force several times higher (10 000 x g) (Ikeda et al., 2009, Law et al., 2016). A disadvantage of this method is that the laboratory must be equipped with an appropriate centrifuge for Nycodenz separation. The use of Percoll for cell separation from mitochondria and chloroplasts was not successful due to undetectable fractions. This could be due to the small amount of detached cells. In comparison to the 15 plants of *A. thaliana* (Delmotte et al., 2009), cells in this study were detached from only 3 g of leaves of *A. elatius* and *G. album*.

The most efficient method for collecting detached cells from the cell suspension was using Sterivex filter units. The Sterivex filters effectively captured all detached cells from the

cell suspension on the filter membrane without cells loss. However, centrifugation did not capture all cells, as some remained in the supernatant. This loss of cells may impact the bacterial community structure. Tian et al. (2017) demonstrated clear differences between collection methods by filtration and centrifugation in the composition of bacterial communities.

Studies of leaf surfaces indicate that the particularly dense population of bacteria (epiphytes) (Remus-Emsermann et al., 2014), is more strongly influenced by extreme environmental factors than endophytic bacteria (colonists within leaf tissue) (Vorholt, 2012). The epiphytes are particularly interesting in research on the environmental impacts on the phyllosphere inhabitants. However, detaching epiphytes, which strongly adhere to the leaf surface as biofilms and aggregates (Vorholt, 2012), are difficult and certainly not achievable as individual cells. In addition, the cell aggregates are lost chloroplasts are removed using a prefilter. To avoid losing these bacterial cells and ensure their inclusion in the analysis, it is better to extract them from total leaves using direct DNA extraction methods (e.g. NucleoSpin®Soil kit, Macherey-Nagel). However, endophytic bacteria are only partially extracted because the leaves remain intact after the first step (preparation of the sample by adding buffer) (Aydogan et al., 2018).

5 Conclusion

The study provides clear evidence of the effects of long-term elevated CO_2 concentration on the oligo/heterotrophic and methylotrophic phyllosphere bacteria of *A. elatius* and *G. album* plant species, which were enriched for the first time through dilution-to-extinction approach. Elevated CO_2 concentration led to changes in the structure and assemblages of the frequently enriched oligo/heterotrophic and methylotrophic bacteria. The oligo/heterotrophic and methylotrophic abundance of the two plant species was plant genotype specific and had different effects of eCO_2 concentration. The reduced variability and diversity of these bacteria as well as changes in their abundance, could be attributed to alterations in plant physiology and metabolic profiles under eCO_2 conditions. Elevated CO_2 concentration led to changes in the metabolite profile (e.g. increase in sucrose) and plant physiology through increased photosynthesis (Ikeda et al., 2015). This study shows that the abundance of oligo/heterotrophic *Sphingomonas*, *Pseudomonas* of *G. album* and *Microbacterium* of *A. elatius* is associated with various adaptation strategies, such as utilization of different sugars (mono- and disaccharides) (Trouvelot et al., 2014), production of IAA (Goldberg, 1980, Fly, 1989; Vanderhoef&Dute, 1981), mobility via flagellin (Haefele&Lindow, 1987, Schauer et al., 2011, Ulrich et al., 2021), and plant growth promoting traits such as IAA production (Madhaiyan et al., 2005, 2015; Ruy et al.,2006; Vorholt, 2012, Kembel et al., 2014; Puri et al., 2016a; Abadi et al., 2020; Zhu et al., 2022). The oligo/heterotrophic *Sphingomonas* and *Pseudomonas* may have contributed to the enhanced growth of herbs (including *G. album*) in the GiFACE system (Andresen et al., 2018, Seibert et al., 2021). The increase of *Microbacterium* on *A. elatius* may have been related to their plant-promoting N_2 fixation ability as well as the low biomass and reduced nitrogen concentration in the leaves of grasses (including *A. elatius*) from the same Giessen-FACE system (Andresen et al., 2018, Seibert et al., 2021). The study shows a simultaneous increase in abundance of *Microbacterium* and a decrease in abundance of *Curtobacterium* from *A. elatius* leaves, with *Microbacterium* spp. having a protective effect against pathogenic *Curtobacterium* spp. This study provided evidence that eCO_2 has no effect on the abundance of pink-pigmented facultative *Methylobacteriums*, but lead to an increase in abundance of non-methylotrophic *Pseudomonas*, *Rhizobia*, *Sphingomonas* from *G. album*, and *Microbacterium* from *A. elatius*, which was supported by plant growth-promoting effects such as high production of EPS and IAA (Akter et al., 2016) and ACC reduction (Wim et al., 2018, Saikia et al., 2018). The strong increase in abundance of non-methylotrophic

Microbacterium indicates their ability to adapt to different substrates (methanol, carbohydrates) under eCO_2 conditions. This study also suggests plant specific functional and genetic adaptation of some PPFM-isolates of different phylotypes to eCO_2 concentrations. Further investigations, such as analyses of the plant physiological status (metabolite profiles) and the physiological properties of *Sphingomonas*, *Pseudomonas* and *Microbacterium* are necessary to obtain more information about the adaptation mechanisms of these cultured phylotypes to elevated CO_2 concentrations.

This study shows that eCO_2 concentration, as a single factor, has a weaker effect on microbial communities than elevated temperatures, with some isolates from warming experiments being better able to adapt functionally and genetically to the altered metabolites and elevated temperatures. Additionally, this study showed an increasing effect of eCO_2 concentration on *Sphingomonas*, while elevated temperature had a significant reducing effect on *Sphingomonas* (Aydogan et al., 2020). This indicates a balance between the two effects or a low dominance of the elevated temperature effect. To obtain a complete picture of the impacts of these two main drivers of global climate change on the phyllosphere microbiota of the plants *A. elatius* and *G. album*, field studies considering both factors would be beneficial.

Furthermore, this study provides a comparative analysis of direct and alternative DNA extraction methods for the analysis of phyllosphere-associated bacterial communities using molecular methods based on 16S rRNA gene sequences. No highly efficient method for bacterial DNA extraction from the phyllosphere without co-extractions could be established. However, two methods have been established that enable bacterial DNA extraction with reduced co-extracts. For the isolation of bacterial DNA from fresh leaves of *A. elatius* and *G. album*, the alternative method proved to be effective, whereby cells were detached using a Stomacher in PBS buffer supplemented with Silwet L-77 and sonication, rinsed one, filtered from plant residues, collected using Sterivex filter and DNA extracted using the modified NucleoSpin®Soil kit (Macherey-Nagel). This method greatly reduced co-extractions and increased the yield of bacterial DNA, especially from *G. album* leaves. This study also provides evidence that bacterial DNA could only be detected from the large amount of 10 g fresh leaves after washing, but not from 1 g. Among all direct DNA extraction methods, the modified NucleoSpin®Soil kit (Macherey-Nagel) was best suited for DNA extraction of phyllosphere-associated bacteria from 200 mg total frozen leaves, but only from *G. album* plants. However, with an integrated step to remove chloroplast and mitochondrial DNA (Arenz et al., 2015), *G.*

album and *A. elatius*-associated bacteria could be studied (Aydogan et al., 2018,2020). The difficulties reported by Reisberg et al. (2012) in DNA extraction of *Methylobacterium* from the phyllosphere of *Arabidopsis* could not be confirmed in this study. The total DNA of *Methylobacterium* species was effectively extracted from the phyllosphere of *A. elatius* and *G. album* plant species using NucleoSpin®Soil kit (Macherey-Nagel).

This method is quick and easy to use and even requires small amounts of plant material to be investigated. The DNA extracts obtained in this study require further metagenomic analyses to verify and improve the efficiency of the method for analyzing bacterial communities.

Publications

Parts of this work have been published in the following publications:

1. Aydogan, E. L., Budich, O., Hardt, M., Choi, Y. H., Jansen-Willems, A. B., Moser, G., ... & Glaeser, S. P. (2020). Global warming shifts the composition of the abundant bacterial phyllosphere microbiota as indicated by a cultivation-dependent and-independent study of the grassland phyllosphere of a long-term warming field experiment. *FEMS Microbiology Ecology*, *96*(8), fiaa087.

2. Palmer, R., Fleming, G. T., Glaeser, S., Semmler, T., Flamm, A., Ewers, C., ... & Eisenberg, T. (2020). Marine mammals are natural hosts of *Oceanivirga salmonicida*, a bacterial pathogen of Atlantic salmon. *Diseases of aquatic organisms*, *139*, 161-174.

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Figures

Tables

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