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Der Einfluss veränderter Wintertemperaturverläufe auf die mikrobielle C und N Nutzung aus Ernterückständen und die Überlebensrate pilzlicher Phytopathogene

Dissertation

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt und keine anderen als in dieser Arbeit angegebenen Hilfsmittel benutzt habe. Die wörtlich oder sinngemäß angeführten und aus Veröffentlichungen oder unveröffentlichtem Material entnommenen Zitate habe ich unter Angabe der Quellen kenntlich aufgeführt. Kein Teil dieser Arbeit liegt in einem anderen Promotions- oder Habilitationsverfahren vor.

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Vorwort

Die vorliegende Dissertation wurde im Rahmen des vom Niedersächsischen Ministerium für Wissenschaft und Kultur geförderten Forschungsverbundes „KLIFF – Klimafolgenforschung in Niedersachsen“ an der Universität Kassel im Fachbereich Ökologische Agrarwissenschaften im Fachgebiet Bodenbiologie und Pflanzenernährung angefertigt, um die Anforderungen des akademischen Grades des Doktors der Naturwissenschaften (Dr. rer. nat.) zu erfüllen. Die Arbeit wurde von der Universität Kassel gefördert, ist mit dem DFG-Graduiertenkolleg 1397 assoziiert und beinhaltet drei wissenschaftlichen Publikationen, von denen zwei bereits bei einer international, begutachteten Fachzeitschrift veröffentlicht wurden. Die Artikel sind in die Kapitel 3, 4 und 5 eingearbeitet. Kapitel 1 liefert eine generelle Einleitung zum Thema, während in Kapitel 2 die Ziele dieser Arbeit herausgestellt werden. In den Kapiteln 6 und 7 sind die Ergebnisse der Kapitel 3, 4 und 5 auf deutsch und englisch zusammengefasst, während Kapitel 8 einen Ausblick auf weitere Untersuchungen gibt.

Folgende Publikationen sind Bestandteil der vorliegenden Arbeit:

Kapitel 3

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Kapitel 4

Lukas, S., Abbas, S.J., Karlovsky, P., Potthoff, M., Joergensen, R.G., 2014. Substrate use and survival of fungal plant pathogens on maize residues at winter temperatures around freezing point. Soil Biology and Biochemistry 77, 141–149.

Kapitel 5

Lukas, S., Abbas, S.J., Karlovsky, P., Potthoff, M., Joergensen, R.G. Substrate use, survival and development of fungal plant pathogens on infected maize residues under field conditions in winter – A soil warming experiment. (submitted to Plant & Soil)

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Abkürzungsverzeichnis

^{13}C	Kohlenstoffisotop mit der Masse 13
^{15}N	Stickstoffisotop mit der Masse 15
AG	Anastomose-Gruppe
ANOVA	Varianzanalyse
C	Kohlenstoff
$CaCl_2$	Kalziumchlorid
CH_4	Methan
$CHCl_3$	Chloroform
cm	Zentimeter
C_{mic}	Mikrobiell gebundener Kohlenstoff
CO_2	Kohlenstoffdioxid
CTAB	Cetyltrimethylammoniumbromid
CV	Variationskoeffizient (engl. coefficient of variance)
d	Tag (engl. day)
$\delta^{13}C$	$^{13}C/^{12}C$ Verhältnis der Probe bezogen auf das $^{13}C/^{12}C$ Verhältnis des PDB Standards
DFG	Deutsche Forschungsgemeinschaft
DNA	Desoxyribonukleinsäure (engl. deoxyribonucleic acid**)**
dNTPs	Desoxyribonukleosidtriphosphate
DW	Trockengewicht (engl. dry weight)
E	Ost (engl. east)
EDTA	Ethylendiamintetraacetat
e.g.	Zum Beispiel (lat. exempli gratia)
FAO-WRB	Food and Agriculture Organisation of the United Nations – World Reference Base for Soil Resources
g	Gramm
g	Beschleunigung
GalN	Galaktosamin
Glc	Glucosamin
h	Stunde (engl. hour)
HCl	Salzsäure
hPa	Hektopascal

HPLC	Hochleistungsflüssigkeitschromatographie (engl. high performance liquid chromatography)
i.e.	das heißt (lat. id est)
IPCC	Zwischenstaatlicher Ausschuss für Klimaänderungen (engl. Intergovernmental Panel on Climate Change)
IRMS	Isotopenverhältnis-Massenspektrometrie (engl. isotope-ratio mass spectrometry)
K	Kelvin
K_2SO_4	Kaliumsulfat
k_{EC}, k_{EN}	Extrahierbarer Teil des Gesamtkohlenstoffes und -stickstoffes gebunden in der mikrobiellen Biomasse
km^2	Quadratkilometer
l	Liter
L.	Carl von Linné
LB	Netzbeutel (engl. litterbag)
m	Meter
M	Molar (mol/L)
m^2	Quadratmeter
mg	Milligramm
$MgCl_2$	Magnesiumchlorid
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimol
MurN	Muraminsäure
N	Nord
N_2	Stickstoff
N_2O	Distickstoffmonoxid (Lachgas)
NaCl	Natriumchlorid
NaOH	Natriumhydroxid (Natronlauge)
n.d.	nicht nachweisbar (engl. not detectable)
ng	Nanogramm
NH_4NO_3	Ammoniumnitrat
nm	Nanometer

N_{mic}	Mikrobiell gebundener Stickstoff
O_2	Sauerstoff
OPA	ortho-Phthaldialdehyd
P	Wahrscheinlichkeit
PCR	Polymerase-Kettenreaktion
PDA	Kartoffeldextroseagar (engl. potato dextrose agar)
PE	Priming Effekt (engl. priming effect)
PEG	Polyethylenglycol
pg	Pikogramm
pH	negativer dekadischer Logarithmus der Wasserstoffionenaktivität (lat. potentia Hydrogenii)
POM	Partikuläres organisches Material (engl. particulate organic matter)
PVC	Polyvinylchlorid
qPCR	quantitative Polymerase-Kettenreaktion
r	Korrelationskoeffizient
rev	Umdrehungen
rpm	Umdrehungen pro Minute
s	Sekunde
SOC	Organischer Kohlenstoff des Bodens (engl. soil organic carbon)
SOM	Organisches Material des Bodens (engl. soil organic matter)
Taq	*Thermus aquaticus*
vol%	Volumenprozent
VPDB	Vienna Pee Dee Belemnite (Standard für die Messung von δ^{13}C-Werten)
v/v	Volumen pro Volumen
w/v	Gewicht pro Volumen
z. B.	Zum Beispiel
%	Prozent
‰	Promille
µM	Mikromolar
Σ	Summe

1. Einleitung

Im Zuge des Klimawandels sind die Jahresmitteltemperaturen in Europa während des 20. Jahrhunderts um 0,8 °C angestiegen. Dabei war die Erwärmung im Winter deutlich stärker ausgeprägt als im Sommer (Alcamo et al., 2007), was speziell in Deutschland zu einer Abnahme der Tage mit Tiefsttemperaturen unter 0 °C geführt hat (Haberlandt et al., 2010). Verglichen mit der Referenzperiode von 1961-1990 sagen Simulationen des regionalen Klimamodells REMO weitere Klimaänderungen mit ansteigenden Temperaturen bis +2 °C um das Jahr 2050 und +4 °C bis 2100 vorher (Jacob und Podzun, 1997; Werner und Gerstengarbe, 2007). Neben den Auswirkungen auf verschiedenste Ökosysteme sind dadurch gerade landwirtschaftlich genutzte Böden besonders betroffen, da sich dort die Temperaturen im Winter um den Gefrierpunkt bewegen (Henry, 2008). Zusätzlich ist eine schützende Pflanzenbedeckung, im Gegensatz zu Grünlandflächen oder Waldökosystemen, im Winter meist nicht vorhanden. Daher wird das Bodenklima (d.h. die Bodentemperatur, der Wassergehalt und die Sauerstoffverfügbarkeit) auf Ackerflächen im Winter stark durch die Lufttemperatur und den Niederschlag beeinflusst. Dabei können ansteigende Temperaturen und abnehmende Niederschläge zu einer Reduzierung oder dem vollständigen Fehlen einer isolierenden Schneedecke und dadurch zu dem Phänomen der „kälteren Böden in einer wärmeren Welt“ führen (Isard und Schaetzl, 1998; Groffman et al., 2001). Folglich kann Frost tiefer und länger anhaltend in den Boden eindringen, es kann aber auch zu einem häufigeren Auftreten von Gefrier- und Tauereignissen kommen, da der Boden den tageszeitlichen Temperaturschwankungen ungeschützt ausgesetzt ist. Diese haben ähnliche Auswirkungen wie Trocken- oder Feuchtereignisse (Priemé und Christensen, 2001) und besitzen damit das Potential, Nährstoffkreisläufe, wie z. B. Kohlenstoff- und Stickstoffflüsse, im Boden zu verändern. In landwirtschaftlich genutzten Böden sind Frost- und Tauereignisse für einen Großteil der CO_2- und N_2O-Emissionen im Winter und Frühjahr verantwortlich, welche neben CH_4 als schädliche Klimagase bei der Erderwärmung von Bedeutung sind (Rodhe, 1990; Neilsen et al., 2001; Priemé und Christensen, 2001; Teepe et al., 2004). Im Zusammenhang mit Bodenfrost oder Frost-Tau-Ereignissen wurde in verschiedenen Studien außerdem eine erhöhte Auswaschung von Kohlenstoff, Stickstoff, Phosphor und Nitrat festgestellt (Brooks and Williams, 1999; Groffman et al., 2001; Fitzhugh et al., 2001), was hauptsächlich aus der Zerstörung der organischen Bodensubstanz und einer verringerten Aggregatstabilität aufgrund der Volumenänderung des Wassers beim Gefrieren resultiert (Oztas und Fayetorbay, 2003; Six et al., 2004). Neben C- und N-Verlusten beeinflussen wechselnde Bodentemperaturen in Kombination mit der Wasserverfügbarkeit ebenfalls den

mikrobiellen Abbau von Pflanzenresiduen, welche vielfach nach der Ernte auf den landwirtschaftlichen Flächen zurückbleiben und einen wichtigen Beitrag als Nährstoffreservoir für Bodenlebewesen leisten. Durch die enzymatische Zersetzung der Erntereste tragen Bodenmikroorganismen zur Mobilisierung vormals gebundener Nährstoffe bei, welche den Feldfrüchten im Frühjahr zur Verfügung stehen und deren Nährstoffversorgung verbessern. Durch die partielle Immobilisierung von Nährstoffen beim Aufbau eigener Biomasse wird außerdem einem Austrag aus dem Boden z. B. durch Auswaschung entgegengewirkt (Brooks, 2001). Bodenbürtige Mikroorganismen, speziell Bakterien und saprotrophe Pilze (Richards, 1987; Bowen und Harper, 1990; Cheshire et al., 1999), besitzen damit eine Schlüsselfunktion im Nährstoffkreislauf der Böden (Swift et al., 1979), welche von Jenkison (1977) mit „the eye of the needle through wich all the organic material must pass“ beschrieben wurde.

In der Vergangenheit wurden die Auswirkungen von Bodenfrost und veränderten Frost-Tau-Ereignissen auf Mikroorganismen kontrovers diskutiert. Dabei wurden in verschiedenen Untersuchungen sowohl keine (Koponen et al., 2006., Sharma et al., 2006; Dam et al., 2012), als auch stark negative Effekte (Pesaro et al., 2003; Dörsch et al., 2004; Feng et al., 2007), z. B. durch Frost-induzierten Trockenstress, auf die bakterielle und pilzliche Biomasse festgestellt (Jensen et al., 2003; Feng et al., 2007; Schmitt et al., 2008). Die Aktivität von Mikroorganismen in (temporär) gefrorenen Böden ist dabei zwar in zahlreichen Studien belegt (Mikan et al., 2002, Öquist et al., 2004, Kurganova et al., 2007; Öquist et al., 2009; Drotz et al., 2010). Der mikrobielle Abbau von komplexem, organischem Material und damit verbundene Nährstoffflüsse unter dem Gesichtspunkt sich wandelnder Winterklimate waren dagegen bisher kaum Gegenstand aktueller Forschung. Es wird jedoch angenommen, dass Frost- und Tauereignissen neben Makroaggregaten des Bodens auch pflanzliche Rückstände zerstören (Harris und Safford, 1996; Schimel und Clein, 1996; Herrmann und Witter, 2002; Six et al., 2004), wodurch zusätzliche Nährstoffe mikrobiell verfügbar werden (Bullock et al., 1988; Christensen und Christensen, 1991) und ihre Aktivität erhöhen können. Dabei ist unter Frostbedingungen nicht nur der mikrobielle Abbau organischer Substanzen, sondern auch der Transport und die Aufnahme der Nährstoffe stark von der Verfügbarkeit flüssigen Wassers limitiert (Romanovsky und Osterkamp, 2000; Öquist et al., 2009). Die Hauptprozesse des mikrobiellen Substratabbaus, die Mineralisation von C und N, finden jedoch auch bei Temperaturen unter 0 °C in geringen Mengen von ungefrorenem Wasser statt, welches den Bodenpartikeln anhaftet (Edwards und Cresser, 1972; Panikov et al., 2006). Dabei wurden die Abbauwege durch Mineralisation und Immobilisation bisher entweder mithilfe ungeeigneter

Substrate (Herrmann und Witter, 2002) oder unrealistischer Temperaturszenarien (Feng et al., 2007) untersucht.

Pflanzliche Ernterückstände können jedoch nicht nur zur Verbesserung der Bodenqualität und somit zur Produktivität im Pflanzenbau beitragen. Sie stellen ebenso ein Langzeitsubstrat für bodenbürtige, pilzliche Phytopathogene dar (Inch and Gilbert, 2003), die nach Tiedemann und Ulber (2008) zu den Hauptschaderregern in vielen wichtigen Ackerbaukulturen zählen. Dazu gehören (neben Raps) die am häufigsten angebauten Kulturpflanzen Weizen (*Triticum aestivum* L.), Mais (*Zea mays* L.) und Zuckerrübe (*Beta vulgaris* L.). Deren Hauptschaderreger sind vor allem in Europa und den USA Pilze der Gattung *Fusarium* sowie *Rhizoctonia solani* (Teleomorphe *Thanatephorus cucumeris* (Frank) Donk), welche als Standorttreue Erreger von Fruchtfolgekrankheiten angesehen werden. Die besonders durch *Fusarium culmorum* und *Fusarium graminearum* hervorgerufene partielle Taubährigkeit im Weizen und Kolbenfusariose im Mais führen dabei weltweit zu hohen Ernteverlusten (Parry et al., 1995; Mesterhazy et al., 1999; Popiel et al., 2008). Zusätzlich kann der Pathogenbefall eine Kontamination der für die Futter- und Lebensmittelherstellung relevanten Pflanzenorgane (z. B. Ähren) mit toxischen Sekundärmetaboliten, sog. Mykotoxinen, zur Folge haben (Magan et al., 20011, Obenauf, 2003). Dabei ist *F. culmorum* die vorherrschende Spezies in kühleren Regionen, während *F. graminearum* bei höheren Temperaturen dominiert (Parry et al., 1995; Champeil et al., 2004). Der bodenbürtige Pilz *R. solani* verursacht die „Späte Rübenfäule“, welche die Ernteerträge in Teilen Europas, Japan und den USA ebenfalls um bis zu 50% verringern kann (Sneh et al., 1991; Rieckmann und Steck, 1995; Kiewnick et al., 2001, Kühn et al., 2009). Daneben können bestimmte Stämme wie die AG-2-2 IIIB auch verschiedene andere Pflanzen wie z. B. Mais und Sonnenblumen befallen (Pfähler und Petersen, 2004). Veränderte Witterungsverläufe können somit zu veränderten Populationsdynamiken der Pathogene beitragen und den daraus resultierenden Schaderregerdruck entscheidend beeinflussen. Ausschlaggebend sind dabei kurze Generationszeiten der Pathogene, sowie effektive Verbreitungsmechanismen (Coakley et al., 1999; Scherm und Coakley, 2003), wobei der Lebenszyklus der Mikroorganismen hauptsächlich durch Luft- und Bodentemperaturen sowie Niederschlag und Bodenfeuchte gesteuert wird (Magan, 2007; Tiedemann und Ulber, 2008). Dabei können längere, mildere Perioden ohne Frost dazu führen, dass bereits während des Winters ein größeres Inokulum aufgebaut wird, von welchem Nutzpflanzen im Frühjahr befallen und infiziert werden können. Neben der Temperatur bestimmt zusätzlich die Feuchte die Konkurrenzfähigkeit der Pathogene. Trockene Bedingungen im Frühjahr begünstigen beispielsweise die Entwicklung von Krankheitssymptomen, die durch *F. culmorum* und *F. graminearum* hervorgerufen

werden (Champeil et al., 2004), während eine hohe Bodenfeuchte im Winter die Überlebensrate verringert (Sutton, 1982). Die Fruchtfolge, d.h. der Anbau von Zwischenfrüchten, ist für den Grad der Infektion ebenfalls von entscheidender Bedeutung und kann auch zur Regulierung des Schaderregerdrucks beitragen. Für Weizen wurde in einer Studie von Cromey et al. (2002) die höchste Befallsrate mit *F. graminearum* festgestellt, wenn die Pflanzen nach Mais als Vorfrucht angebaut wurden. Gerade große Mengen an Ernterückständen, die häufig nach dem Maisanbau zurückbleiben (Dill-Macky und Jones, 2000), können dabei das Infektionsrisiko für Folgefrüchte erhöhen, denn alle Spezies der Gattung *Fusarium* sind in der Lage, saprotroph auf Pflanzenresten zu überleben (Parry et al., 1995). Daher ist es wichtig, Nicht-Wirtspflanzen wie Flachs (*Linum usitatissimum*) oder Luzerne in die Fruchtfolge einzubringen, um das Infektionsrisiko für spätere Folgefrüchte zu minimieren (Champeil et al., 2004). Die Pathogene, speziell *F. culmorum* und *R. solani*, können aber auch in Form von langlebigen Chlamydosporen, Dauermycel, Diasporen oder Sklerotien mehrjährig im Boden überdauern und anfällige Pflanzen unter entsprechenden Bedingungen besiedeln (Cook, 1970; Cook, 1981a; Cook, 1981b; Sutton, 1982; Inglis und Cook, 1986; Parry et al., 1995). Die Dauerstadien von *R. solani* (Sklerotien oder Dauermycel) sind dabei häufig auch mit Pflanzenrückständen assoziiert (Cubeta und Vilgalys, 1997; Strausbaugh et al., 2011) und eine Infektion aus dem Boden wird insbesondere durch eine trockene und warme Witterung begünstigt (Schlüter et al., 2006). Pilzliche Pathogene, speziell die der Gattung *Fusarium*, können jedoch nicht unbegrenzt auf Ernteresten überdauern, ihr Fortbestand wird von der Zersetzbarkeit des organischen Materials (d.h. der chemischen Zusammensetzung) bestimmt und durch schwerer abbaubare Substrate begünstigt (Sutton, 1982). Gerade der hohe N-Gehalt von Maisrückständen (z. B. Blätter und Stängel) erlaubt ein längeres Überdauern der Pathogene und kann deren Population stabilisieren (Sutton, 1982; Champeil et al., 2004). Die Auswirkungen veränderter klimatischer Verhältnisse im Winter auf den mikrobiellen Abbau von Pflanzenresten im Hinblick auf das Überleben und Interaktionen zwischen Pathogenen sind bisher kaum erforscht.

2. Ziele der Arbeit

Der mikrobielle Abbau von komplexem, organischem Material und damit verbundene Nährstoffflüsse unter dem Gesichtspunkt sich wandelnder Winterklimate waren bisher kaum Gegenstand aktueller Forschung. Um die C- und N-Dynamiken durch die mikrobielle Aktivität bei Temperaturenszenarien um 0 °C zu bestimmen, wurde das natürliche ^{13}C-Isotopenverhältnis von Mais, welches sich als C_4-Pflanze von dem der organischen Bodenmaterie unter C_3-Vegetation unterscheidet (Ryan and Aravena, 1994; Rochette et al., 1999; Potthoff et al., 2005), mit einer künstlichen Anreicherung des ^{15}N-Isotopenverhältnisses kombiniert. Dadurch ist es möglich, die mikrobielle Nutzung von pflanzen- und bodenbürtigem C und N, sowie deren Einlagerung in verschiedene Kompartimente (mikrobielle Biomasse, CO_2, partikuläre organische Substanz, extrahierbarer C und N) gleichzeitig zu untersuchen. In einem ersten Inkubationsversuch, welcher in Kapitel 3 beschrieben ist, sollten folgende Forschungsfragen geklärt werden: (1) Beschleunigen Frost-Tau-Ereignisse den Abbau von streubürtigem C und N im Vergleich zu konstanten Temperaturen um 0 °C? (2) Sind Mineralisation und Immobilisation von streubürtigem C und N durch die Häufigkeit der Frost-Tau-Ereignisse reguliert?

Gegenwärtig gibt es kaum Informationen über den Einfluss von Wintertemperaturen um 0 °C oder von Frost-Tau-Ereignissen auf die Überlebensrate und Populationsdynamiken bodenbürtiger, pilzlicher Phytopathogene. Diese sind für die Abschätzung des Schaderregerdruckes und Infektionspotentials von Feldfrüchten jedoch von großer Bedeutung. Bisherige Studien nutzten entweder ungeeignete Temperaturen oder künstliche Substrate (Beyer et al., 2004). In einem zweiten Inkubationsversuch, welchen Kapitel 4 detailliert beschreibt, wurden die Auswirkungen der Inokulation dreier verschiedener Pathogene (*F. culmorum*, *F. graminearum*, *R. solani*), entweder einzeln oder in Kombination, auf den Abbau von Ernteresten, pathogene Populationsdynamiken und die mikrobielle Zersetzergemeinschaft untersucht. Dieser Versuch sollte folgende Forschungsfragen klären: (1) Steigern konstante Wintertemperaturen über 0 °C den Abbau von pathogen-infizierten Ernterückständen und damit die Inokulummenge? (2) Werden die Überlebensraten der eingesetzten Pathogen durch die Häufigkeit von Frost-Tau-Ereignissen reguliert?

In einem dritten Experiment, welches in Kapitel 5 dargestellt ist, wurde der Versuchsansatz der zweiten Studie auf einer Bodenerwärmungsanlage unter natürlichen Bedingungen wiederholt. Dabei kamen mit Mais gefüllte Netzbeutel zum Einsatz, die häufig zur Untersuchung von Abbauprozessen in Freilandstudien verwendet werden (Johansson et al., 1986; Knacker et al., 2003; Miura et al., 2008; Jacobs et al., 2011). Dieses

Temperaturmanipulationsexperiment wurde unter Feldbedingungen durchgeführt, um die im Laborversuch gefundenen Ergebnisse auf ihre Praxisrelevanz zu überprüfen und folgende Fragen zu beantworten: (1) In welchem Ausmaß werden die Dynamiken der eingesetzten Pathogene durch steigende Bodentemperaturen im Winter beeinflusst? (2) Wird die Menge des Inokulums durch den Zersetzungsgrad der pflanzlichen Residuen reguliert?

3. Microbial use of ^{15}N-labelled maize residues affected by winter temperature scenarios

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Abstract

A 56-day incubation experiment was carried out to investigate decomposition and microbial use of ^{15}N-labelled maize (*Zea mays* L.) residues incubated under four winter temperature scenarios. The residues were mixed to mesocosms equivalent to 1.2 mg C and 42.5 µg N g^{-1} dry soil, after which the samples were incubated at a constant temperature of +4 °C, a constant -3 °C, and under multiple and single freeze-thaw conditions. A constant +4 °C was most favourable for microbial substrate use, with 4- and 6-fold higher total and maize-C mineralization, respectively, in comparison with constant frost. The cumulative maize mineralization was not determined by the frequency of freeze-thaw events, but regulated by the overall time of frost and thaw conditions. The decomposition of maize straw significantly increased soil organic C mineralization (in all scenarios) and incorporation into microbial biomass (in the freeze-thaw scenarios only). The positive priming effects observed were equivalent to an additional loss of total soil organic C of between about 0.2 (continuous frost) and 0.8% (single freeze-thaw). Microbial biomass was significantly increased after maize straw amendment, with constant frost and freeze-thaw scenarios not having any negative effect on microbial biomass C compared with constant +4 °C. Highest fungal biomass was found after constant frost without fresh substrates and also after extended frost followed by a

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warm period when fresh plant residues were present. On average, 50% of the added maize N were recovered in the soil total N after 56 days of constant +4 °C and in the freeze-thaw scenarios, with the strongest effect after single freezing and thawing.

Keywords: Freeze-thaw cycles; Decomposition; CO_2; Microbial biomass C and N; Ergosterol; Extractable C and N; Soil total N; $\delta^{13}C$ and $\delta^{15}N$; Priming

3.1 Introduction

Although it is predicted that climate warming will be most pronounced at high latitudes (Houghton et al., 2001; IPCC, 2007), temperate soils may also be affected as they remain close to freezing point throughout the winter (Henry, 2008). In fact, trend analysis has shown an increase in mean annual temperature for Germany of about 0.8 to 1.1 °C from 1901 to 2000, with marked increases in winter precipitation (Schönwiese and Janoschitz, 2008). A positive trend for the mean annual temperature (increase of 1.3 °C from 1951 to 2005) and increased winter precipitation as well as a decrease in the number of days with minimum temperatures below 0 °C was also found by Haberlandt et al. (2010) for Lower Saxony, which is a large temperate area (357,000 km^2) in the north of Germany. Winter climate change may have stronger effects on microbial activity and C and N dynamics in fallow arable soils, due to a lack of protective plant cover in comparison with grassland or forest systems.

It is assumed that climate warming reduces the snow pack thickness, thus leading to lower soil temperatures or to higher frequencies of freezing and thawing, i.e. colder soils in a warmer world (Isard and Schaetzl, 1998; Groffman et al., 2001). Increased soil freezing can cause leaching losses of C and N (Groffman et al., 2001; Fitzhugh et al., 2001) as well as lower winter soil respiration (Monson et al., 2006). In addition, it has been suggested that soil freezing and thawing disrupts soil aggregates (Oztas and Fayetorbay, 2003; Six et al., 2004), plant material (Mellick and Seppelt, 1992; Harris and Safford, 1996) and microbial cells (Skogland et al., 1988; Yanai et al., 2004; Larsen et al., 2002). This enhances microbial activity upon thawing, due to increased availability of substrates or easily decomposable organic matter (Edwards and Cresser, 1992; Schimel and Clein, 1996; Lipson et al., 2000; Grogan et al., 2004; Sharma et al., 2006). Microorganisms are the main drivers of soil organic matter decomposition and nutrient cycles (Swift et al., 1979), with saprotrophic fungi being most important in decomposing plant residues in arable soils (Bowen and Harper, 1990; Cheshire et al., 1999). Contradictory impacts of freeze-thaw events on microorganisms have been reported, with either decreasing (Lipson et al., 1999; Pesaro et al., 2003) or no effects

(Grogan et al., 2004; Sharma et al., 2006) on microbial biomass. If temperature drops below 0 °C, shifts in microbial substrate use occur (Schimel and Mikan, 2005), which may be accompanied by shifts in microbial community composition from bacteria towards fungi (Lipson et al., 2002; Schadt et al., 2003; Lipson and Schmidt, 2004; Sjursen et al., 2005).

In order to follow microbial C and N dynamics around the freezing point, we combined the natural $\delta^{13}C$ value of maize straw, which is usually different from soil organic matter (Ryan and Aravena, 1994; Rochette et al., 1999; Potthoff et al., 2005), and an artificial enrichment in $\delta^{15}N$. To our knowledge, this is the first attempt to simultaneously follow microbial respiration and C and N sequestration into different fractions, such as microbial biomass, CO_2, particulate organic matter, extractable C and N, and soil total C and N, during the decomposition of a complex organic substrate at low temperatures. This incubation study addressed the following questions: (1) Do freeze-thaw scenarios accelerate the decomposition of straw-derived C and N in comparison with constant temperatures around 0 °C? (2) Is the microbial use, i.e. mineralization and immobilization, of straw-derived organic matter regulated by the frequency of freeze-thaw cycles?

3.2 Material and methods

3.2.1 Soil and plant material

The arable soil used for the experiment was taken from the upper 10 cm of an experimental site in Neu-Eichenberg near Witzenhausen (51°23’ N, 9°55’ E, Northern Hessia, Germany) in September 2009. The site is located at 240 m above sea level with a mean annual precipitation and temperature of 670 mm and 8.7 °C. The soil is classified as a Haplic Luvisol (FAO-WRB, 2006) with the following characteristics: 3.3% sand, 83.4% silt, 13.3% clay, a water holding capacity of 55%, a pH ($CaCl_2$) of 6.3, 1.4% total C, a $\delta^{13}C$ value of -26.4 ± 0.1‰, 0.14% N and a $\delta^{15}N$ value of 7.8 ± 0.3. Plant tissue, insects and stones were removed by hand and the soil was sieved (< 2 mm). The soil was incubated at 3 °C for 4 weeks before the experiment started. For ^{15}N labelling, maize was grown for six months in the greenhouse and fertilised once with 278 ml fertilizer solution containing 160 mg l^{-1} NH_4NO_3 enriched with 10 atom-% ^{15}N. Maize leaf residues were harvested in October 2009, air dried, chopped into pieces of 0.5 cm x 1 cm and stored in a paper bag at room temperature. The maize residues contained 43.7% (± 0.3) C with a $\delta^{13}C$ value of -12.4 ‰, 1.8% (± 0.01) N with a $\delta^{15}N$ value of 594.8‰ and had a C/N ratio of 24.7.

3.2.2 Incubation procedure

The incubation experiment was carried out in 2 l glass jars with 5 replicates per treatment, each containing soil with a water content of 20% on a dry weight basis (corresponding to 36% of the water holding capacity), equivalent to 300 g dry soil. After equilibration at 3°C, maize residues were mixed thoroughly into half of the soil samples at amounts of 1.2 mg C and 42.5 µg N g^{-1} dry soil, the remaining samples serving as non-amended controls. All samples were then incubated simultaneously by temperature treatment in two climate cabinets (MV 600, LinTek, Germany) for 56 days. To simulate winter climate regimes, four different temperature treatments were applied: (1) a constant +4 °C ($+4_{CON}$), (2) a constant -3 °C (-3_{CON}), (3) multiple freeze-thaw cycles of 48 h at +4 and -3 °C, respectively ($+4/-3_{MULTIPLE}$), and (4) a single freeze-thaw cycle of four weeks at -3 °C between two warm periods of two weeks each at +4 °C ($+4/-3_{SINGLE}$).

Air samples for CO_2 measurement were taken on days 1, 4, 8, 10, 16, 18, 22, 36, 43, 46, 50, 52, 54 and 56 after the incubation period started. For this purpose, two evacuated gas containers (50 ml) were connected to one of two ports on the PVC lid of each glass jar, which was attached with two rubber bands to achieve an airtight seal. The second port was used to connect a 50 l gas bottle with CO_2-free synthetic air (synthetic air 5.0, ≥ 99.999 vol% purity, Air Liquide, Germany, at 20.5 ± 0.5% O_2 in 79.5 ± 0.5% N_2). Each glass jar and the connections of the attached gas containers were flushed with synthetic air for about 2 min to remove the CO_2 before the first sampling. The headspace volume of each jar was homogenized by a fan affixed to the inside of the PVC lid. To take the temperature differences between the synthetic air and the refrigerators/freezers into account, the synthetic air was first cooled in a styrofoam box by passing it through a 10-meter flexible silicon tube covered with ice. After the flushing procedure, the first air sample (A_{T0}) was taken. The second air sample (A_{T1}) was taken after an accumulation period of 24 hours. CO_2 concentrations were measured using an automated gas chromatograph with an electron capture detector according to Loftfield et al. (1997). The volume of CO_2 in the headspace-volume of each jar under standard conditions for temperature and pressure was calculated as:

$$CO_{2_{Headspace}}\left(ml\right) = V_{Net} \text{ x } \Delta CO_2 \text{ x } \frac{p_n \text{ x } T_n}{p_n \text{ x } \left(T_n + IT\right)} \quad (1)$$

where V_{Net} is the headspace volume (ml), ΔCO_2 is the difference between the CO_2 concentrations A_{T1} and A_{T0} (expressed as %), p_n is the standard atmospheric pressure (hPa), T_n is the standard temperature (K) and IT is the temperature during the incubation (°C). Soil respiration expressed as CO_2-C was then calculated by the following equation:

$$CO_2\text{-C}\left(\mu g\ g^{-1}\ soil\ d^{-1}\right) = \frac{CO_{2_{Headspace}}\ x\ M_{CO_2}}{V_{m0}}\ x\ 0.2729\ x\ \frac{1000}{ds}\ x\ \frac{24}{\Delta t} \quad (2)$$

where $CO_{2\ Headspace}$ is the volume of CO_2 (ml) under normal conditions related to the respective incubation temperature, M_{CO2} is the molar mass of CO_2 (mg), V_{m0} is the volume of one mol of a gas under normal conditions (ml), 0.2729 is the mass fraction of C in CO_2, ds is the dry weight of the incubated soil sample (g) and Δt is the time between A_{T0} and A_{T1} (h). Air samples for $\delta^{13}C$ analysis of evolved CO_2 were taken with a syringe (35 ml) directly after A_{T1} and stored in 12 ml Labco Exetainer vials (Labco Limited, UK). CO_2-$\delta^{13}C$ analyses were performed on a Delta plus IRMS (Thermo Scientific, Bremen, Germany). Soil sub-samples for analysis were taken at the end of the incubation on day 56 after all visible particles of maize straw residues had been removed from the amended soil samples by sieving (2 mm).

3.2.3 Analytical procedures

Microbial biomass C and N were estimated by fumigation extraction (Brookes et al., 1985; Vance et al., 1987). A sub-sample of 20 g moist soil was taken and separated into two portions of 10 g. One portion was fumigated at 25 °C with ethanol-free $CHCl_3$, which was removed after 24 h. Fumigated and non-fumigated samples were extracted for 30 min with 40 ml of 0.05 M K_2SO_4 (Potthoff et al., 2003) by horizontal shaking at 200 rev min^{-1} and filtered (hw3, Sartorius Stedim Biotech, Göttingen, Germany). Organic C and total N in the extracts were measured after combustion at 850 °C using a Dimatoc 100 automatic analyser (Dimatec, Essen, Germany). Microbial biomass C was calculated as E_C/k_{EC}, where E_C = (organic C extracted from fumigated soil) – (organic C extracted from non-fumigated soil) and k_{EC} = 0.45 (Wu et al., 1990, Joergensen, 1996). Microbial biomass N was calculated as E_N/k_{EN}, where E_N = (total N extracted from fumigated soil) – (total N extracted from non-fumigated soil) and k_{EN} = 0.54 (Brookes et al., 1985, Joergensen and Mueller, 1996). The conversion values k_{EC} and k_{EN} have been repeatedly used in freeze-thaw cycle experiments (Larsen et al., 2002; Sjursen et al., 2005; Fan et al., 2012). For the determination of ^{13}C and ^{15}N-isotope composition of microbial biomass, 20 ml aliquots of 0.05 M K_2SO_4 extracts of fumigated and non-fumigated samples were freeze dried for about 3 days. The freeze-dried K_2SO_4 extracts were analysed for ^{13}C and ^{15}N-isotope composition by isotope-ratio mass spectrometry (Delta plus, Finnigan, Bremen).

The fungal cell-membrane component ergosterol was extracted from 2 g moist soil with 100 ml ethanol (96%) according to Djajakirana et al. (1996). Quantitative determination of

ergosterol was then performed by reversed-phase HPLC analysis with 100% methanol as the mobile phase and detected at a wavelength of 282 nm (Dionex UVD 170 L).

For determination of particulate organic matter (POM), the soil samples (250 g moist soil) were dispersed in 400 ml of saturated sodium chloride, shaken by hand and allowed to stand for 45 min (Magid and Kjærgaard, 2001; Muhammad et al., 2006). The samples were poured gradually onto two sieves of 0.4 mm and 0.063 mm mesh size and washed with tap water. The aggregates were destroyed by pushing the soil through the sieve during the washing procedure until the water passing through the sieve became clear. The material retained on the 0.4 mm sieve (POM 0.4-2 mm) as well as the washed POM > 2 mm fraction were then transferred into a crucible, dried at 60°C, weighed and milled for further analysis (total C, total N, $\delta^{13}C$, $\delta^{15}N$).

3.2.4 Calculations and statistical analysis

Isotope values are expressed in delta notation relative to VPDB and air for ^{13}C and ^{15}N, respectively. The $\delta^{13}C$ value of the microbial biomass ($\delta^{13}C_{MB}$) was calculated by the following equation (Ryan and Aravena, 1994; Potthoff et al., 2003):

$$\delta^{13}C_{MB}\ (‰) = \frac{\left(\delta^{13}C_{fum}\ x\ C_{fum}\right) - \left(\delta^{13}C_{nfum}\ x\ C_{nfum}\right)}{\left(C_{fum} - C_{nfum}\right)}$$

(3)

where C_{fum} and C_{nfum} represent the mass of C (µg g^{-1}) extracted from the fumigated and non-fumigated samples, respectively, and $\delta^{13}C_{fum}$ and $\delta^{13}C_{nfum}$ represent the corresponding $\delta^{13}C$ values. Accordingly, the $\delta^{15}N$ value of the microbial biomass ($\delta^{15}N_{MB}$) was calculated as follows (Dijkstra et al., 2006; Zareitalabad et al., 2010):

$$\delta^{15}N_{MB}\ (‰) = \frac{\left(\delta^{15}N_{fum}\ x\ N_{fum}\right) - \left(\delta^{15}N_{nfum}\ x\ N_{nfum}\right)}{\left(N_{fum} - N_{nfum}\right)}$$

(4)

The fraction of maize-derived C ($f_{maize-C}$) was calculated for each individual replicate of all treatments from the $\delta^{13}C$ data according to a two pool-mixing model with the following equation:

$$f_{maize-C}\ (\%) = \frac{\left(\delta^{13}C_{sample} - \delta^{13}C_{control}\right)}{\left(\delta^{13}C_{maize} - \delta^{13}C_{control}\right)}\ x\ 100 \qquad (5)$$

where $\delta^{13}C_{sample}$ represents the $\delta^{13}C$ value of SOC, POM-C (0.4-2 mm), microbial biomass C at day 56, and CO_2-C at each measurement day; $\delta^{13}C_{control}$ is the average $\delta^{13}C$ value of the non-amended control samples within each temperature treatment and $\delta^{13}C_{maize}$ is the $\delta^{13}C$ of the maize residues. Accordingly, the fraction of maize-derived N ($f_{maize\text{-}N}$) was calculated by the following equation:

$$f_{maize-N}\ (\%) = \frac{\left(\delta^{15}N_{sample} - \delta^{15}N_{control}\right)}{\left(\delta^{15}N_{maize} - \delta^{15}N_{control}\right)} \text{ x } 100 \tag{6}$$

where $\delta^{15}N_{sample}$ represents the $\delta^{15}N$ value of soil total N, POM-N (0.4-2 mm), microbial biomass N at day 56; $\delta^{15}N_{control}$ is the average $\delta^{15}N$ value of the non-amended control samples within each temperature treatment and $\delta^{15}N_{maize}$ is the $\delta^{15}N$ of the maize residues.

To determine priming effects, the amount of soil-derived C in the respective fractions of maize amended samples (C_3-C_{sample}) was obtained by subtracting the maize-derived C from the total amount of C. If the difference between C_3-C_{sample} and C_3-$C_{control}$ ($p \leq 0.05$, *t*-test) was significant, priming effects were calculated using the following equation:

$$PE\ (\%) = \frac{\left(C_3 - C_{sample} - C_3 - C_{control}\right)}{C_3 - C_{control}} \text{ x } 100 \tag{7}$$

where C_3-$C_{control}$ is the amount of SOC in each fraction of the non-amended control samples. Isotopic fractionation during microbial decomposition processes is still a controversial issue and often suggested to be negligible and of little importance (Ehleringer et al., 2000; Ekblad et al., 2002). Also, Collins et al. (2000) found no evidence of isotopic discrimination after incubating maize residues for 50 days. We therefore assume that no fractionation occurred during the incubation process (see also Rochette et al., 1999)

The data presented in tables and figures are arithmetic means expressed on an oven-dry basis (about 24 h at 105 °C), standard deviations are given in brackets. The Kolmogorov-Smirnoff test was used to check for normal distribution. Significance of treatment effects was tested by a one-way analysis of variance (ANOVA) using post hoc Tukey HSD and significant differences were determined at $P \leq 0.05$. All statistical calculations were performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, USA).

3.3 Results

3.3.1 Maize-derived C and N fractions

Application of maize residues increased ^{13}C in SOC on average by 0.46‰ and in K_2SO_4 extractable C on average by 0.50‰ (Fig. 1a, Table 1). However, this enrichment was only significant for the $+4/-3_{SINGLE}$ treatment. For microbial biomass C, the $\delta^{13}C$ values of the maize amended samples varied around -22.5‰ and were thus significantly enriched in ^{13}C by on average 3.35‰, with highest values in the $+4/-3_{SINGLE}$ treatment. In the POM 0.4-2 mm fraction, maize amendment increased ^{13}C by a mean of 4.7‰ in comparison with the control samples. The highest enrichment in this fraction was again found in the $+4/-3_{SINGLE}$ treatment. The application of maize residues also significantly increased ^{15}N in all compartments analysed (Table 1). The ^{15}N in soil total N, microbial biomass N and K_2SO_4 extractable N was increased by 9.8‰, 77‰ and 184‰, respectively (Fig. 1b, Table 1). Here, the highest values were found in the $+4/-3_{SINGLE}$ treatment, while samples under constant frost showed the lowest increases.

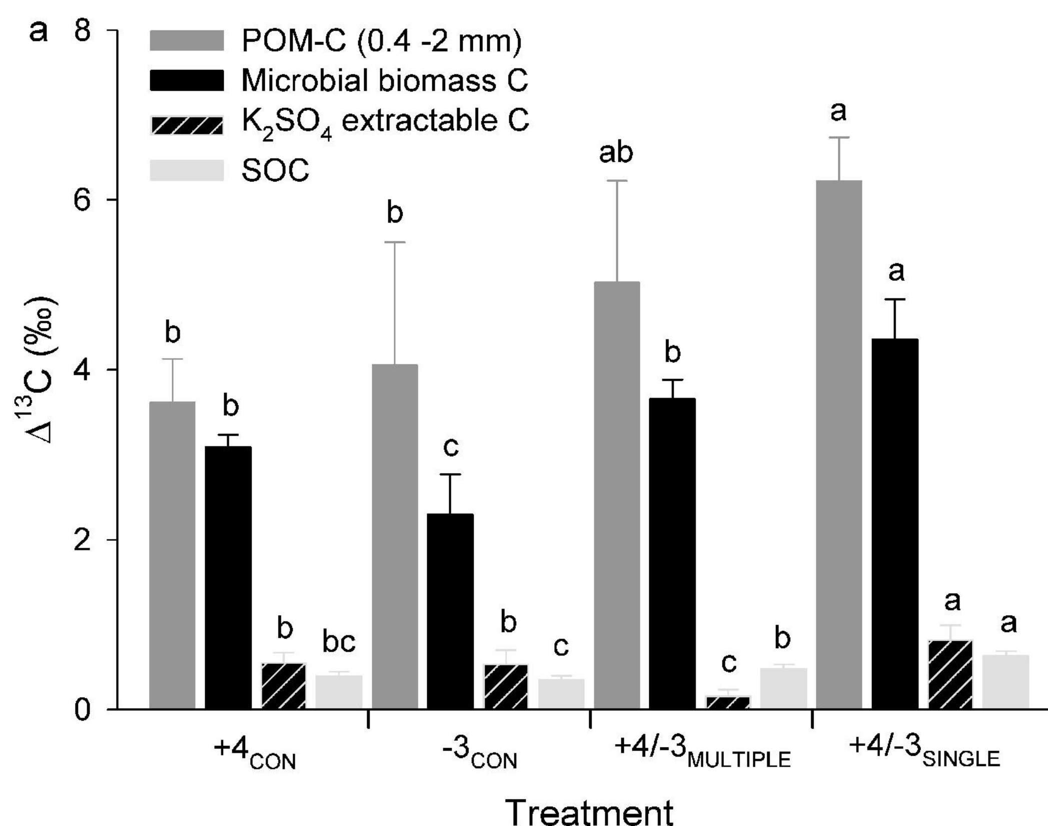

Fig. 1a. Isotopic enrichment in POM-C (0.4-2 mm), microbial biomass C, K_2SO_4 extractable C, soil organic C (SOC) in maize-amended samples compared with non amended controls at the end of the 56-day incubation; error bars show ± one standard deviation (n = 5); different letters above the columns indicate significant differences within fractions ($P < 0.05$).

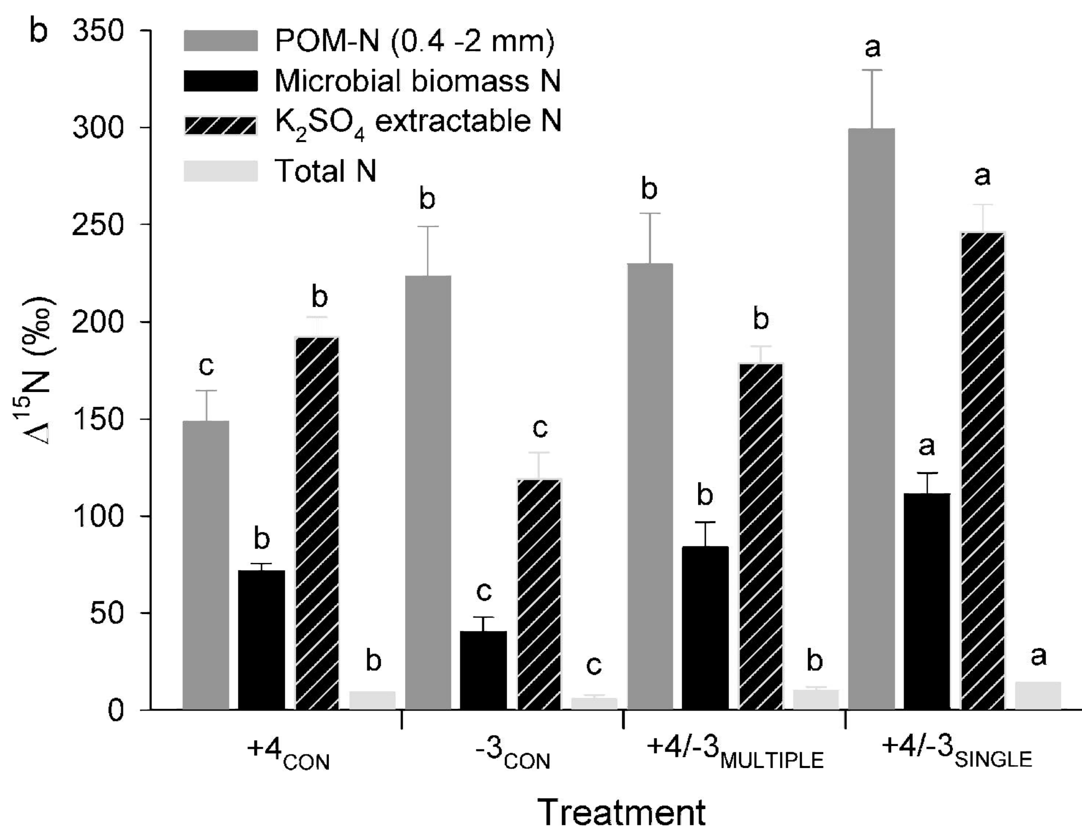

Fig. 1b. Isotopic enrichment in POM-N (0.4-2 mm), microbial biomass N, K_2SO_4 extractable N and soil total N (total N) in maize-amended samples compared with non amended controls at the end of the 56-day incubation; error bars show ± one standard deviation (n = 5); different letters above the columns indicate significant differences within fractions (P < 0.05).

On average, 99% of the maize C and 96% of maize N added were recovered in the different compartments (Fig. 2a+b, Table 2). About 36% of the maize C and 61% of the maize N were transferred to SOC and soil total N in the $+4/-3_{SINGLE}$ treatment. In the other treatments, only 25% of the maize C and 41% of the maize N were found on average in these compartments. Also, nearly 6% of the maize C and about 10% of the maize N were transferred to the POM 0.4-2 mm fraction in the $+4/-3_{SINGLE}$ treatment, whereas in the other treatments the recovery for this fraction was around 2% for maize C and nearly 4% for the added maize N.

Table 1. $\delta^{13}C$ values in soil organic C, K_2SO_4 extractable C, microbial biomass C, POM-C 0.4-2 mm and > 2 mm at the end of the 56-day incubation; $\delta^{15}N$ values in soil total N, K_2SO_4 extractable N, microbial biomass N, POM-N 0.4-2 mm and > 2 mm at the end of the 56-day incubation in control and maize amended samples of four temperature treatments.

Treatment	SOC	K_2SO_4 extractable C	Microbial biomass C	POM-C 0.4-2 mm	POM-C > 2 mm
	$\delta^{13}C$ (‰)				
Maize					
$+4_{CON}$	-26.4 abc	-24.4 ab	-22.7 bc	-25.4 b	-14.9 a
-3_{CON}	-26.5 abc	-24.3 ab	-23.3 c	-24.8 b	-15.3 a
$+4/-3_{MULTIPLE}$	-26.3 ab	-24.6 b	-22.2 ab	-23.6 ab	-15.5 a
$+4/-3_{SINGLE}$	-26.2 a	-23.7 a	-21.6 a	-22.6 a	-15.6 a
Control					
$+4_{CON}$	-26.8 bc	-24.8 b	-25.8 d	-29.0 c	-
-3_{CON}	-26.9 c	-24.9 b	-25.6 d	-28.7 c	-
$+4/-3_{MULTIPLE}$	-26.8 bc	-24.7 b	-25.9 d	-28.9 c	-
$+4/-3_{SINGLE}$	-26.9 c	-24.9 b	-26.0 d	-28.7 c	-
CV (±%)	0.8	1.2	1.2	1.9	3.6

Treatment	Total N	K_2SO_4 extractable N	Microbial biomass N	POM-N 0.4-2 mm	POM-N > 2 mm
	$\delta^{15}N$ (‰)				
Maize					
$+4_{CON}$	16.9 b	197 b	78 b	156.c	483 c
-3_{CON}	12.9 c	124 c	45 c	231 b	581 a
$+4/-3_{MULTIPLE}$	17.2 b	184 b	88 b	237 b	522 bc
$+4/-3_{SINGLE}$	22.0 a	251 a	116 a	307 a	537 ab
Control					
$+4_{CON}$	7.8 d	5.0 d	5.6 d	7.4 d	-
-3_{CON}	7.7 d	4.9 d	5.0 de	7.6 d	-
$+4/-3_{MULTIPLE}$	7.8 d	5.0 d	4.5 e	7.8 d	-
$+4/-3_{SINGLE}$	7.9 d	5.0 d	4.3 e	7.9 d	-
CV (±%)	3.0	6.1	11.2	8.0	4.8

Different letters within a column indicate a significant difference ($P < 0.05$; Tukey HSD, n = 5); CV = pooled coefficient of variation between replicate incubations (n = 5).

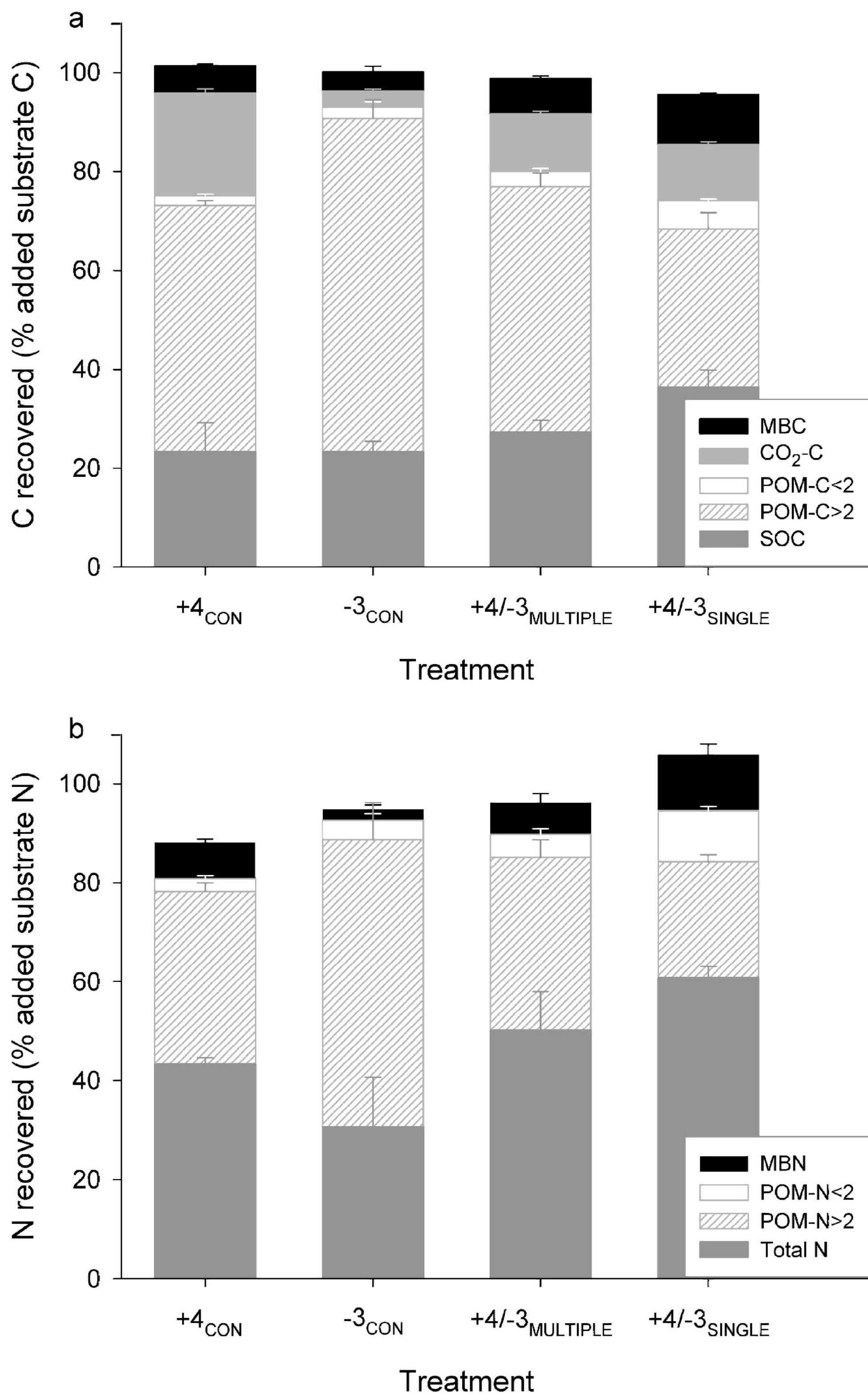

Fig. 2a+b. Recovery of the added maize straw C and N in the fractions (a) microbial biomass C (MBC), CO_2-C, POM-C < 2 mm, POM-C > 2 mm, soil organic C (SOC) and (b) microbial biomass N (MBN), POM-N < 2 mm, POM-N > 2 mm and soil total N (total N) at the end of the 56-day incubation; error bars show ± one standard deviation (n=3).

Table 2. Recovery of the added maize C in the cumulative CO_2-C production, soil organic C (SOC), microbial biomass C, POM-C 0.4-2 mm and > 2 mm at the end of the 56-day incubation; recovery of the added maize N in total N, microbial biomass N, POM-N 0.4-2 mm and > 2 mm at the end of the 56-day incubation in maize amended samples of four temperature treatments.

Treatment	ΣCO_2-C	SOC	Microbial biomass C	POM-C 0.4-2 mm	> 2 mm	Σ Recovery
	(µg g^{-1} soil)					(%)
$+4_{CON}$	246 a	278 b	65 c	22 b	591 b	101 a
-3_{CON}	39 c	275 b	46 d	27 b	792 a	100 a
$+4/-3_{MULTIPLE}$	136 b	320 ab	84 b	37 b	583 b	99 a
$+4/-3_{SINGLE}$	136 b	438 a	121 a	70 a	383 c	96 a
CV (±%)	7	13	11	24	6	3

Treatment	Total N	Microbial biomass N	POM-N 0.4-2 mm	> 2 mm	Σ Recovery
	(µg g^{-1} soil)				(%)
$+4_{CON}$	18.4 b	3.3 b	1.1 c	14.9 b	89 a
-3_{CON}	13.1 c	1.0 c	1.6 bc	24.5 a	95 a
$+4/-3_{MULTIPLE}$	21.0 b	2.8 b	2.0 b	14.6 b	96 a
$+4/-3_{SINGLE}$	26.2 a	5.1 a	4.4 a	10.1 c	106 a
CV (±%)	14	24	23	8	5

Different letters within a column indicate a significant difference ($P < 0.05$; Tukey HSD, n = 5); CV = pooled coefficient of variation between replicate incubations (n = 5).

3.3.2 C mineralization

Microbial activity was very low in all temperature scenarios without substrate addition. Lowest CO_2 emission was measured in the constant frost scenario (-3_{CON}), with a mean respiration rate of 0.6 µg CO_2-C g^{-1} soil d^{-1} (Fig. 3a). Mean heterotrophic respiration in the $+4_{CON}$ treatment was about 45% higher. Multiple freeze-thaw cycles increased the C mineralization over that of the $+4_{CON}$ and -3_{CON} treatments and averaged 1.0 µg CO_2-C g^{-1} soil d^{-1} (Fig. 3b). Here, mean respiration rates at +4 and -3 °C were about 21% and 40% higher than those of the $+4_{CON}$ and -3_{CON} samples, respectively. Mean respiration rate of the $+4/-3_{SINGLE}$ treated samples at -3 °C four days after the temperature change was 0.6 µg CO_2-C g^{-1}

soil d^{-1} and not significantly different to that of the -3_{CON} samples. Four days after the samples were thawed again, respiration rates doubled and decreased thereafter to the level of the first 14 days (Fig. 3b).

The addition of maize residues led to an immediate increase in CO_2 evolution, which was less pronounced under the constant frost scenario. Microbial respiration in the $+4_{CON}$ treatment peaked on day 4 and decreased continuously thereafter (Fig. 3c). Mean respiration rate was 6.6 µg CO_2-C g^{-1} soil d^{-1}. Lowest C mineralization was again observed in the -3_{CON} treatment and averaged 1.9 µg CO_2-C g^{-1} soil d^{-1}. Until the end of the experiment, no clear pattern of microbial substrate utilization was observed. Samples that were exposed to multiple freeze-thaw cycles had an average respiration rate of 5.2 µg CO_2-C g^{-1} soil d^{-1} (Fig. 3d). When measured at +4 °C the samples had 9.4% higher C mineralization rates than samples of the $+4_{CON}$ treatment on the same days. However, these were only minor and not significant. On the other hand, at -3 °C the respiration rates were about 81% higher than mineralization rates of the -3_{CON} samples on the same days. In the $+4/-3_{SINGLE}$ treatment, microbial respiration also peaked four days after application of maize residues, with a mean rate of 13.3 µg CO_2-C g^{-1} soil d^{-1}, and decreased sharply thereafter, especially when the incubation temperature was set to -3 °C (Fig. 3d). CO_2 evolution at -3 °C equilibrated four days after temperature change and was on average 16% higher than respiration rates of -3_{CON} samples on the same days. Thawing of the samples led to a 4.5 fold increase in respiration four days after the incubation temperature was set back to 4 °C. Thereafter, microbial respiration decreased again but was on average still 1.6 times higher than respiration rates of the $+4_{CON}$ samples at that time.

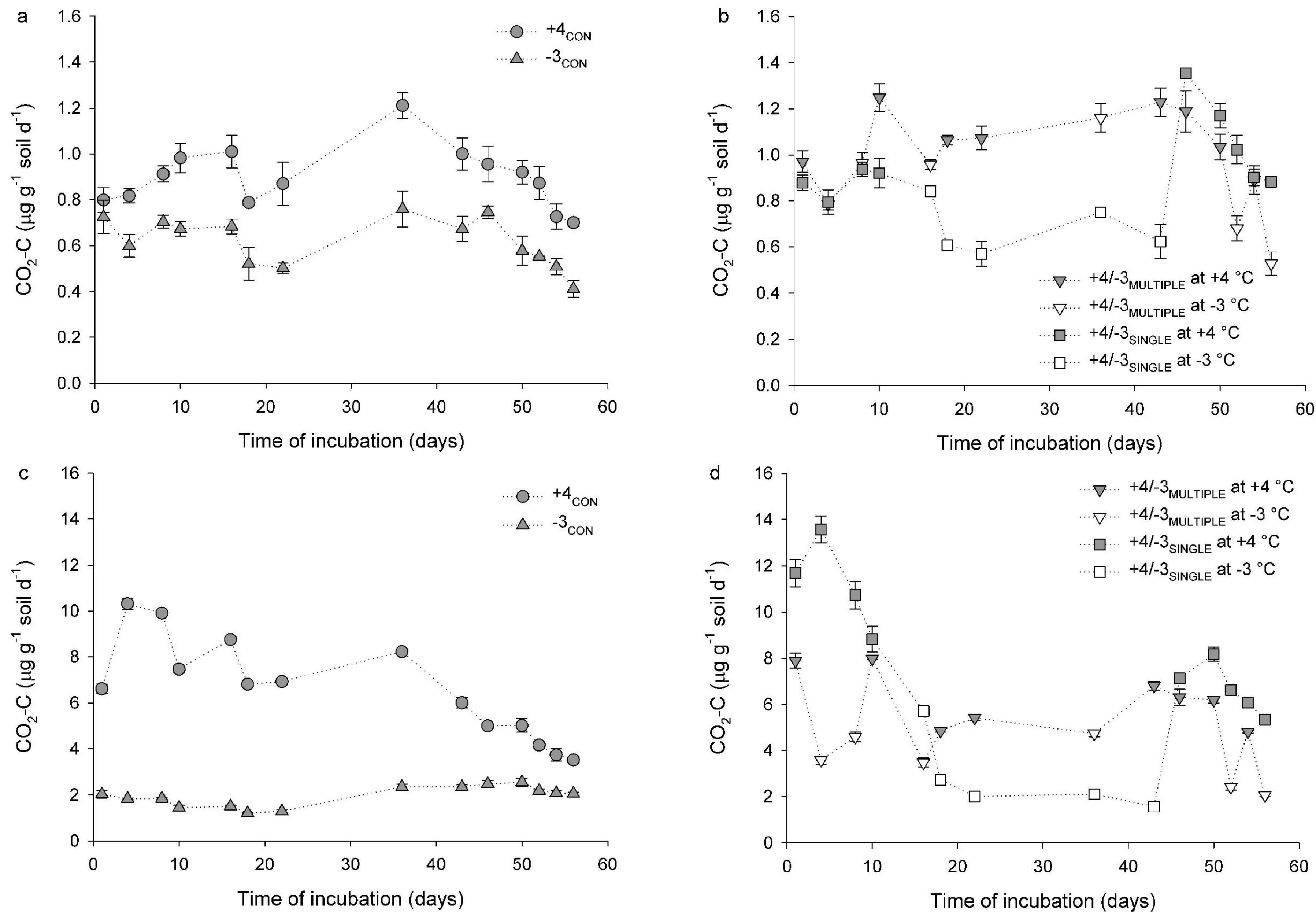

Fig. 3a-d. CO_2-C production rate of the control (a and b) and maize-amended (c and d) samples over a 56-day incubation period; error bars show ± one standard deviation (n = 5).

Cumulative CO_2 production (Fig. 4) in the control samples averaged 48 µg C g^{-1} soil and increased in the order $-3_{CON} < +4/-3_{SINGLE} < +4_{CON} < +4/-3_{MULTIPLE}$. Total CO_2 production after application of maize residues was highest in the $+4_{CON}$ treatment, with around 396 µg C g^{-1} soil, which was almost four times higher than in the -3_{CON} treatment. 21% of respired CO_2-C originated from the added maize C in the $+4_{CON}$ treatment, compared with only 3% in the -3_{CON} treatment. No significant difference in total C mineralization was found between the $+4/-3_{MULTIPLE}$ and the $+4/-3_{SINGLE}$ treatments, at roughly 300 µg C g^{-1} soil. In both treatments, 11.5% of the evolved CO_2-C originated from the added maize C.

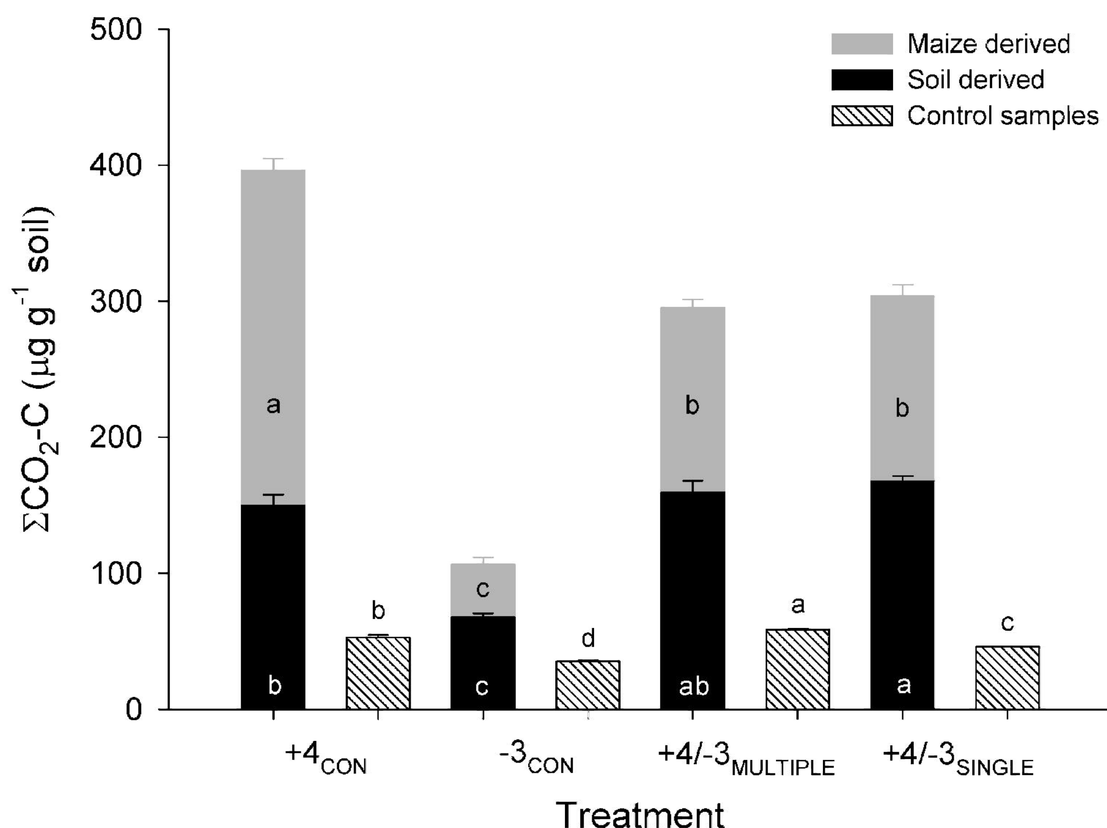

Fig. 4. Cumulative CO_2-C production of all temperature treatments at the end of a 56-day incubation period; error bars show ± one standard deviation (n = 5); different letters above the columns indicate a significant difference for the total CO_2-C production of the control samples ($P < 0.05$); different letters in the columns indicate significant differences for the maize- and the soil organic matter-derived CO_2-C production ($P < 0.05$).

The application of maize residues always significantly increased the mineralization of soil-derived C in comparison with the control samples (Fig. 4). The strongest increase in extra soil-derived CO_2-C production was found in the $+4_{CON}$ treatment as well as the freeze-thaw cycle treated samples. In the $+4_{CON}$ and -3_{CON} treatments, maize amendment led to an

additional cumulative mineralization of 96.6 and 31.2 µg SOC g^{-1} soil, according to an overall PE of 180% and 85% (equivalent to 0.7 and 0.2% of total SOC). Maize amended samples in the $+4/-3_{MULTIPLE}$ and $+4/-3_{SINGLE}$ treatments respired around 111 µg g^{-1} soil of additional soil C, equivalent to 0.8% of total SOC and an overall PE of 170% and 270%, respectively.

3.3.3 Soil microbial biomass and extractable C and N

The contents of microbial biomass C in the samples without application of maize residues at the end of the incubation period were very similar between the four treatments, at about 210 µg g^{-1} soil (Fig. 5a, Table 3). No significant effects of the temperature treatments were observed. Mixing of maize residues into the soil significantly increased microbial biomass C to a mean of 330 µg g^{-1} soil at the end of the incubation. While the significantly highest amount of microbial biomass C was in the $+4/-3_{SINGLE}$ treated samples, there was no significant difference for the +4- and -3_{CON} treatments or for the $+4_{CON}$ and $+4/-3_{MULTIPLE}$ treated samples (Fig. 5a, Table 3). Microbial biomass N in the control samples was not significantly affected by the temperature treatment and averaged 20 µg g^{-1} soil (Fig. 5b, Table 3). Except for the -3_{CON} treatment, application of maize residues significantly increased microbial biomass N to a mean of 27 µg g^{-1} soil. Samples of the $+4/-3_{SINGLE}$ treatment showed significantly highest amounts of microbial biomass N. In contrast to microbial biomass C, $+4_{CON}$ treated samples had significantly higher amounts of microbial biomass N than the -3_{CON} treatment. The microbial biomass C to N ratio significantly increased in the maize amended samples, except for the $+4_{CON}$ treatment, with highest values in the freeze-thaw cycle treatments (Table 3).

Without application of maize residues, the contents of fungal ergosterol were about 0.4 µg g^{-1} soil, samples of the -3_{CON} treatment showing highest amounts (Table 3). Application of maize residues significantly increased the ergosterol content, regardless of the temperature treatment, to an average of 0.6 µg g^{-1} soil. As for microbial biomass C and N, the highest contents of ergosterol were found in the $+4/-3_{SINGLE}$ treated samples. There was no significant difference between the other temperature scenarios. In comparison with the control samples, application of maize residues significantly increased the ergosterol to microbial biomass C ratio in the $+4/-3_{MULTIPLE}$ treatment (Table 3).

Table 3. K_2SO_4 extractable C and N, soil microbial biomass C, biomass N, ergosterol content, the microbial biomass C-to-biomass N ratio and the ergosterol-to-microbial biomass C ratio at the end of the 56-day incubation in control and maize amended samples of four temperature treatments.

Treatment	K_2SO_4 extractable C (µg g^{-1} soil)	K_2SO_4 extractable N	Microbial biomass C (µg g^{-1} soil)	Microbial biomass N	C/N	Ergosterol (µg g^{-1} soil)	Ergosterol / microbial biomass C (%)
Maize							
$+4_{CON}$	20.7 a	14.4 d	293 bc	27.2 b	10.8 cd	0.57 b	0.19 bcde
-3_{CON}	22.6 a	17.0 ab	277 c	23.4 cd	11.8 bc	0.58 b	0.21 bc
$+4/-3_{MULTIPLE}$	22.7 a	18.2 a	333 b	26.4 bc	12.9 ab	0.61 b	0.21 b
$+4/-3_{SINGLE}$	22.1 a	18.2 a	420 a	31.0 a	13.5 a	0.80 a	0.20 bcd
Control							
$+4_{CON}$	13.3 b	15.1 cd	201 d	20.2 e	10.3 d	0.35 d	0.18 e
-3_{CON}	16.2 b	15.1 cd	211 d	21.8 de	9.7 d	0.46 c	0.24 a
$+4/-3_{MULTIPLE}$	13.8 b	17.7 a	207 d	19.3 e	10.8 cd	0.38 d	0.18 de
$+4/-3_{SINGLE}$	14.4 b	16.0 bc	211 d	19.3 e	11.0 cd	0.40 cd	0.19 cde
CV (±%)	6.0	2.7	5.9	5.9	6.4	8.9	8.1

Different letters within a column indicate a significant difference ($P < 0.05$; Tukey HSD, n = 5); CV = pooled coefficient of variation between replicate incubations (n = 5).

Significant differences in the incorporation of maize-derived C into the microbial biomass were found between all temperature treatments (Fig. 5a, Table 2). From the added maize C, 6 and 4% were incorporated in the $+4_{CON}$ and -3_{CON} treatments, respectively. Multiple freeze-thaw cycles led to an incorporation of 7%, whereas the highest amount of maize-derived C was found in the $+4/-3_{SINGLE}$ treatment, at 10%. The highest amount of maize-derived N (12%) in the microbial biomass was also found in this treatment (Fig. 5b, Table 2). In the $+4_{CON}$ and the $+4/-3_{MULTIPLE}$ treatments, about 7% of the added maize N were incorporated, whereas only 2% of the added maize N were found in the -3_{CON} treatment.

After application of maize residues, soil-derived C and N were additionally incorporated into the microbial biomass in all temperature scenarios. This led to 20 to 40% increases in soil-derived microbial biomass C and N (corresponding to 0.3 to 0.7% of SOC and total soil N), being most significant for the $+4/-3_{SINGLE}$ treatments soil.

The contents of K_2SO_4 extractable C and N in the control samples were on average 14 and 4 µg g^{-1} soil, respectively (Table 3). Application of maize residues led to a general

increase in K_2SO_4 extractable C ranging from 39% (-3$_{CON}$) to 64% (+4/-3$_{MULTIPLE}$), and to a 15% increase in K_2SO_4 extractable N only in the -3$_{CON}$ and +4/-3$_{SINGLE}$ treatments.

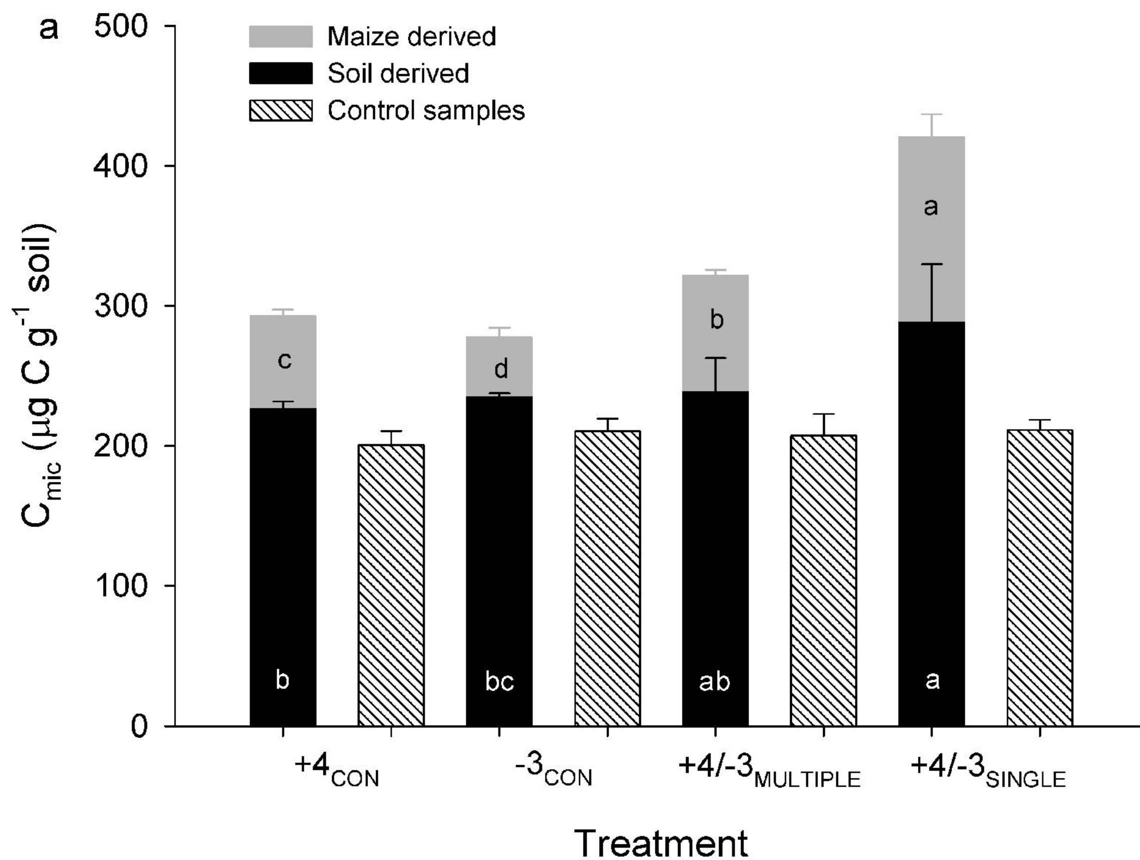

Fig. 5a. Maize and soil derived microbial biomass C as well as the respective control samples of all temperature treatments at the end of a 56-day incubation period; error bars show ± one standard deviation (n = 5); different letters in or above the columns indicate significant differences for the maize- and the soil organic matter-derived C ($P < 0.05$).

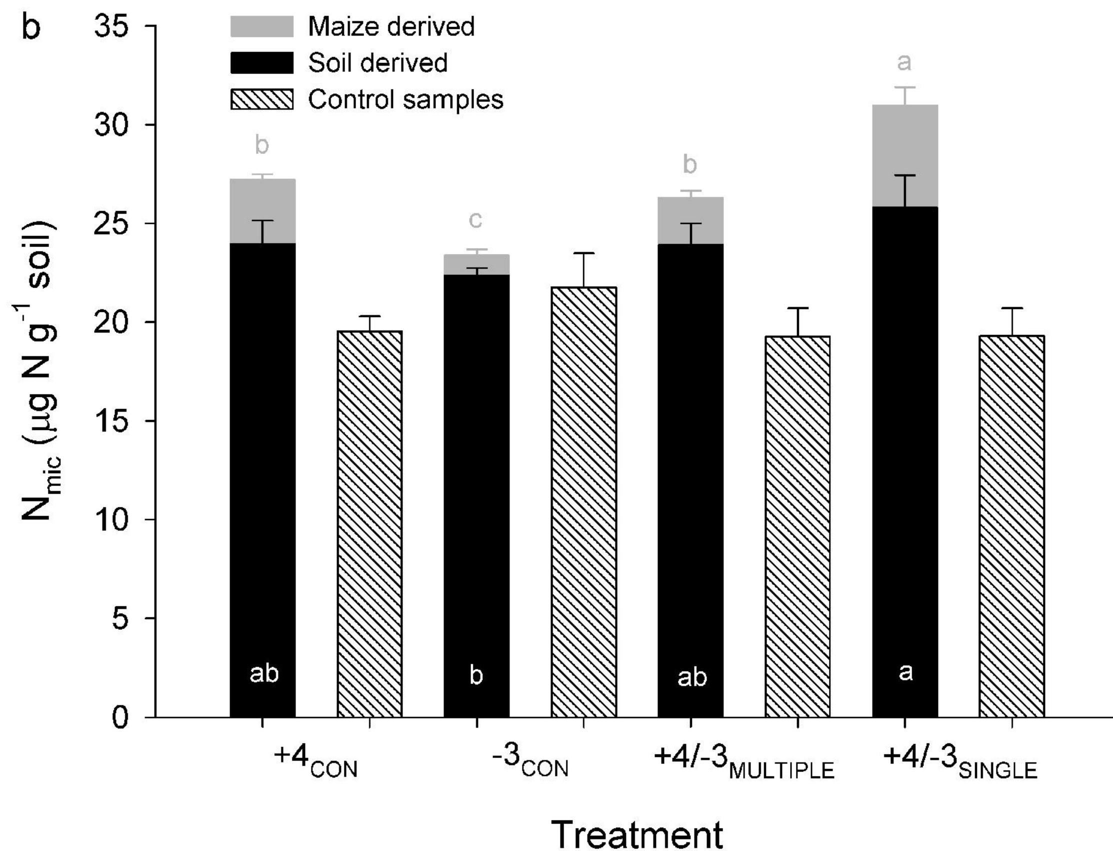

Fig. 5b. Maize and soil derived microbial biomass N as well as the respective control samples of all temperature treatments at the end of a 56-day incubation period; error bars show ± one standard deviation (n = 5); different letters in or above the columns indicate significant differences for the maize- and the soil organic matter-derived N ($P < 0.05$).

3.4 Discussion

3.4.1 Effects on C mineralization

Regardless of the temperature treatment, the C mineralization significantly increased in the residue-amended samples, indicating a clear turnover of the added maize residues at temperatures near and below 0 °C. However, as a consequence of the permanent frost, maize residue decomposition and thus mineralization rates in the -3_{CON} scenario were in general significantly decreased during the incubation and remained constantly low throughout the experiment. A similar pattern of microbial substrate use under constantly frozen conditions was found by Drotz et al. (2010). They observed a lag-phase in microbial CO_2 production of about 60 days in glucose amended soil samples frozen at -4 °C, after which the respiration rates strongly increased. This was accompanied by the production of glycerol, which they first detected after 34 days. Glycerol is known as a cryoprotective agent that limits intracellular

cell-damage and maintains microbial activity and CO_2 production (Beal et al., 2001; Fonseca et al., 2001; Drotz et al., 2010). The observed increase in C mineralization between day 22 and 36 (Fig. 3c) of roughly 55% might also indicate adaptation processes like the formation of glycerol or shifts in the membrane lipid composition.

A decreased substrate affinity (Nedwell, 1999) under constant frost may have hampered the respiration of the added maize residue C, so that only 3.3% were mineralized to CO_2. Drotz et al. (2010) reported a more then 4-fold higher mineralization of glucose after 99 days at -4 °C. This was most likely due to a direct microbial uptake of the soluble glucose C. In our study, the added maize residues contain complex components, e.g. cellulose, lignin and waxes (Incerti et al., 2011), which require the production of exo-enzymes for decomposition. This and the shorter incubation period explain the lower amount of C mineralised. In the $+4_{CON}$ treatment, microbial activity was not constrained by water availability (Brooks et al., 2011) or low substrate diffusion rates (Davidson and Janssens, 2006). Consequently, this temperature scenario was most favourable for microbial substrate use where the cumulative total C and maize-derived C mineralization rates were on average four- and six-fold higher, respectively, than under constant frost conditions. The mineralised maize C amounted to 36, 45 and 62% of the total respired CO_2-C in the -3_{CON}, the freeze-thaw cycle and the $+4_{CON}$ scenarios, respectively, indicating a clear shift in microbial substrate use from SOC to the added plant residue C with increasing temperature. This indicates that total and maize-derived C mineralization depended on the overall time of soil frost and not on the frequency of freeze-thaw events.

Although microbial activity under frozen conditions has been verified in numerous studies (Mikan et al., 2002; Öquist et al., 2004; Kurganova et al., 2007; Öquist et al., 2009a; Drotz et al., 2010), the limiting factor for decomposition and mineralization of SOM is the availability of unfrozen water (Romanovsky and Osterkamp, 2000; Öquist et al., 2009a). Even though the multiple freeze-thaw cycled samples were repeatedly exposed to short periods of frost (48 h at -3 °C), respiration rates in the frozen phases were significantly higher in comparison with the constant frost scenario. Here, isotopic analysis revealed an average 8-fold increase in mineralization of maize-derived C. Also Larsen et al. (2002) reported higher respiration rates in the frost phase of freeze-thaw treated mesocosms in comparison with constantly frozen controls. Freezing and thawing may strongly disrupt added plant material as well as soil macroaggregates, making additional nutrients available for microbial uptake (Harris and Safford, 1996; Schimel and Clein, 1996; Herrmann and Witter, 2002; Six et al., 2004).

Another indicator of an enhanced turnover of maize C induced by multiple freeze-thaw cycles when the soil is frozen is the observed mineralization of maize residue C in the single frost phase of the $+4/-3_{SINGLE}$ treatment, which was increased on average only 4-fold over the constant frost scenario and decreased with time. C mineralization in the +4 °C phases of the freeze-thaw cycled samples was also higher than in the $+4_{CON}$ scenario. This contrasts results of Larsen et al. (2002) and Sjursen et al. (2005), who reported lower or similar respiration rates, respectively, using simple organic substrates in their studies.

3.4.2 Effects on soil microbial biomass and extractable C and N

The application of constant frost and freeze-thaw cycles apparently had no significant negative effect on microbial biomass in the samples without application of maize residues (Table 3), which is in line with others (Grogan et al., 2004; Koponen et al., 2006, Sharma et al., 2006; Dam et al., 2012) but contrasts reported decreases of microorganisms after freezing and thawing (Winter et al., 1994; Schimel and Clein, 1996; Lipson et al., 1999; Lipson et al., 2000; Herrmann and Witter, 2002; Larsen et al., 2002; Pesaro et al., 2003; Dörsch et al., 2004; Feng et al., 2007). Although the different temperature scenarios affected neither the total amount of microbial biomass C nor the amount of microbial biomass N in the control samples, after 56 days the content of ergosterol as an indicator of fungal biomass was significantly increased under constant frost. Consequently, the ergosterol to microbial C ratio, as a relative indicator of the fungal contribution to the total microbial biomass (Djajakirana et al., 1996), was also significantly higher. As there was no input of fresh substrate, a reasonable explanation is that the turnover of ergosterol was reduced, leading to an accumulation of this cell-membrane component. On the other hand, one might suggest that a part of the frost-susceptible microorganisms, mainly bacteria as suggested by Sjursen et al. (2005), might be killed by freezing-induced drought stress (as found by Jensen et al., 2003) and subsequently used as an easily decomposable C and N source by more frost tolerant and slow growing fungi. Due to the low overall microbial metabolism at this temperature, the available nutrients were incorporated into microbial biomass rather than mineralized. This assumption is further supported by the cumulative CO_2 mineralization rate (Fig. 4), which was significantly lower in the constant frost treatment in comparison with the other scenarios. In both control and maize-amended samples, the ergosterol content was not negatively affected after prolonged frost and multiple freeze-thaw cycling. This indicates a frost tolerance of the fungal biomass, which contrasts findings of Feng et al. (2007) and Schmitt et al. (2008).

The temperature treatments had different effects on the microbial biomass after the application of maize residues. While both the total and maize-derived C mineralization were similar between the freeze-thaw cycle scenarios, markedly higher amounts of total and maize-derived microbial biomass C were found in the single freeze-thaw treatment in comparison with multiple freeze-thaw cycles. This means that the frequency of freeze-thaw cycles had no regulatory effect on substrate mineralization, but limited microbial assimilation of maize-derived C at the same time when the frequency of freeze-thaw events was high. In comparison with multiple freezing and thawing, samples of the constant 4 °C treatment also had similar amounts of total microbial biomass C and ergosterol but incorporated significantly less maize-derived C. This was accompanied by significantly higher mineralization rates, indicating a stronger turnover of the added substrate. Due to the faster turnover at constant 4 °C, a part of the freshly formed maize-derived microbial biomass might be mineralized explaining the lower amount of recovered maize C in the microbial biomass and a higher recovery in the mineralized CO_2-C. Microbial activity rather than microbial biomass was affected by temperature fluctuations around the freezing point in comparison with unfrozen conditions. In contrast to Larsen et al. (2002), who reported a decrease in microbial biomass C to N ratios between unfrozen and freeze-thaw cycled conditions (from 15 to 9), the microbial biomass C to N ratio in the present study was increased significantly from 11 to 13. This was due to a slight increase in microbial biomass C and a slight decrease in microbial biomass N caused by freeze-thaw cycling. The decreased amounts of total microbial biomass N under constant frost are in line with similar results of Sjursen et al. (2005).

The recovered extractable C in both maize-amended and control samples were not influenced by the temperature treatments. This is consistent with results of Sjursen et al. (2005) who also found no significant differences after 40 days of incubation. However, the application of maize residues significantly increased the extractable C content, implying that the additional amount must have come from soluble fractions of the crop residue C and/or residue-derived microbial C, because ^{13}C was slightly enriched in comparison with the control samples. In the constant frost scenario and the single freeze-thaw treatment with one prolonged frost period, differences in the ^{15}N enrichment of the extracts but similar amounts of increased extracted N suggest different sources of the additional N. In contrast, a high frequency of freezing and thawing generally increased the extractable N independently of maize application, whereas temperatures constantly above 0 °C had no effect. This is surprising, as the extracted N was similarly enriched in ^{15}N in both treatments, indicating similar amounts of N origin from the added maize residues or maize-derived microbial biomass.

3.4.3 Priming effect

In all temperature scenarios, the application of maize residues caused significant increases in soil organic matter-derived CO_2 evolution. An accelerated SOC mineralization after application of maize residues has been repeatedly observed (Vanlauwe et al., 1994; Ouedraogo et al., 2007 Rottmann et al., 2010; Zareitalabad et al., 2010). To our knowledge, this is the first time that priming effects have been experimentally verified on the basis of the microbial decomposition of a complex organic substrate at near and sub-zero temperatures. The additional CO_2 may originate from an increased turnover of microbial biomass C and/or from SOC (Kuzyakov, 2010) and is considered either as apparent or real priming, respectively (Blagodatskaya and Kuzyakov, 2008; Blagodatsky et al., 2010; Kuzyakov, 2010). According to Nottingham et al. (2009), a real priming effect can be assumed either when the excess of soil C mineralized in the substrate-amended soil exceeds the microbial biomass C or an increased incorporation in the same is detected. In our study, the cumulative additional soil C mineralized after maize straw application in the constant +4 °C and freeze-thaw cycle scenarios corresponded to only one third (32%) of the total microbial biomass C and to nearly half of the soil-derived microbial C (44%) after 56 days. In the constant frost treatment, the proportion of the extra soil-derived CO_2-C to the total and soil-derived microbial biomass C was even less (only 13%). This indicates that the release of additional soil-derived CO_2 in all temperature scenarios may be partly or solely driven by an increased turnover of microbial biomass, and thus at least an apparent PE occurred.

It is suggested that the "real" priming effect may be delayed for days or even weeks after substrate addition (Fontaine et al., 2003; Blagodatsky et al., 2010; Kuzyakov, 2010). In the first phase of substrate decomposition, r-strategists quickly metabolise the soluble C compounds (Dilly and Zyakun, 2008) and may contribute to priming due to their growth and endogenous metabolism shortly after substrate addition (Lundquist et al., 1999; Bell et al., 2003), whereas contributions of k-strategists increase in the later stage of decomposition, as they are more efficient in metabolizing more complex C compounds (Bottomley, 1999; Lundquist et al., 1999; Bell et al., 2003). Thus, "real" priming effects should occur when slow growing k-strategists dominate the microbial community (Blagodatskaya et al., 2009). In our study, the most pronounced acceleration of SOM-derived CO_2 evolution was found in the first three weeks of maize decomposition. Here, the C_3-CO_2 increased on average 2-, 5-, 6-, and 8-fold in the constant frost, constant +4 °C, multiple and single freeze-thaw scenarios, respectively. Thereafter, this amount decreased substantially and, in the last 20 days, was only 2- and 1-fold higher in the constant frost and constant +4 °C treatments, respectively.

Nevertheless, the amount of C_3-CO_2 of both freeze-thaw scenarios in the last 20 or 10 days was on average still 3 times higher than that of the control samples, indicating a significant effect of multiple freezing and thawing as well as a single thawing after prolonged frost. A possible explanation is that the extracellular enzymes generated by saprotrophic fungi to degrade the added maize residues (cellulases, lignin-modifying enzymes) at the later stage of decomposition are to some extent efficient in decomposing SOC (Fontaine et al., 2003; Kuzyakov, 2010).

The application of maize residues also increased the incorporation of soil-derived C into microbial biomass C in all temperature scenarios, which in turn is considered as real or true priming (Nottingham et al., 2009). Regardless of this, it remains unclear whether the enhanced incorporation of soil-derived C in the freeze-thaw cycle scenarios is due to maize residue decomposition or to disruption of soil aggregates (Oztas and Fayetorbay, 2003; Six et al., 2004), making occluded SOC available for microbial uptake (Soulides and Allison, 1961; Bullock et al., 1988; Christensen and Christensen, 1991).

3.4.4 Conclusions

Multiple freezing and thawing caused an intermediate substrate mineralization. However, cumulative substrate mineralization was not determined by the frequency of freeze-thaw events but regulated by the overall time of frost and thaw conditions. The opposite was found for the microbial biomass (including fungal biomass), where growth and assimilation of maize-derived C and N were lower at a high freeze-thaw frequency. A shift in microbial substrate use occurred from SOC to maize residue C with increasing soil temperature. A priming effect has been observed for the first time for temperatures around the freezing point, which might have implications for modelling SOC budgets at the annual time scale. Because decomposition of organic matter is not only affected by water availability but also by the soil texture, the use of soils with different contents of sand and clay would be interesting.

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4. Substrate use and survival of fungal plant pathogens on maize residues at winter temperatures around freezing point

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Abstract

Climate scenarios predict increasing temperatures and higher precipitation rates in late autumn to early spring, both of which holding the potential to change the dynamics of plant residue decomposition and overall microbial activity in soil. In company with consequences for nutrient release patterns influences on the survival of fungal plant pathogens and their phytopathogenicity can be expected. Both, litter decomposition and pathogen survival, was analyzed in a 70-day litterbag incubation experiment. Continuous +4 °C was compared to permanent frost (-3 °C) and different freeze-thaw cycles for the decomposition of maize residues and disease potential of soil-borne plant pathogens *Fusarium culmorum*, *Fusarium graminearum*, and *Rhizoctonia solani*. Frost generally reduced maize residue decomposition. On the lower levels of CO_2 production in the permanent or occasionally frost treatments pathogen inoculation had large effects on microbial maize use, indicating high saprotrophic activity of pathogens even in cold winter scenarios. Pathogen inoculation led to higher amino sugar contents of maize residue dwelling microbial organisms. At constant 4 °C remarkable high amounts of glucosamine were detected, indicating higher substrate use efficiency without frost. Both, temperature treatments as well as intra- and interspecific competition

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directed the development of pathogens after inoculation. *F. culmorum* took large advantage from the non-frost scenario, while no significant increase was found under continuous frost. *F. graminearum* was also able to increase its abundance at +4 °C. But this was strongly reduced when *F. graminearum* was in competition to the other two pathogens. In summary, *F. culmorum* was found to be highly frost tolerant and competitive against *F. graminearum*, particularly under conditions of freeze-thaw cycles since *F. culmorum* was able to take a large share of saprotrophic litter residue use under the cold conditions. Biomass of *R. solani* was strongly decomposed in all treatments. We conclude that constant mild conditions during winter can increase biomass of *F. culmorum* and *F. graminearum* in crop residues, causing increased infection pressure in the next season. In contrast to that, frost and freeze-thaw events can lower the build-up of *Fusarium* biomass and thus diminish the risk of crop infection.

Keywords: *Fusarium culmorum*; *Fusarium graminearum*; *Rhizoctonia solani*; Climate change; Freeze-thaw cycles; Decomposition; Amino sugars; CO_2

4.1 Introduction

Crop residues in the field such as maize debris are a major source of inoculum of necrotrophic plant pathogens (Buddemeyer at al., 2004; Maiorano et al., 2008). Infection of grain crops with *Fusarium culmorum* (W.G. Sm.) Sacc. and *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch) can cause high yield losses and a contamination of the grain by mycotoxins (e.g., deoxynivalenol and zearalenone) (Sutton, 1982; Parry et al., 1995; Mesterházy et al., 1999). Different *Fusarium* spp. can cause diseases individually or in mixed infections (Doohan et al., 1998). The soil-borne fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) with the anastomosis group (AG) *R. solani* AG-2-2-IIIB is pathogenic to a broad range of plant species including maize (Strausbaugh, et al., 2011) and is able to survive on debris of different host plants (Pfähler and Petersen, 2004). *R. solani* is responsible for diseases in sugar beet (*Beta vulgaris* L.), decreasing yields by up to 50% (Sneh et al., 1991; Rieckmann and Steck, 1995; Kiewnick et al., 2001, Kühn et al., 2009). Fungal plant pathogens produce a range of extracellular enzymes to facilitate plant colonization, for the decomposition of crop residues and degradation of components of the primary cell wall (Leplat et al., 2013). The survival of necrotrophic pathogens is related to the rate of residue decomposition, where residues at the soil surface provide nutrients and serve as a substrate allowing the pathogen to survive for long periods of

time, up to several years (Khonga and Sutton, 1988; Pereyra et al., 2004; Pfähler and Petersen, 2004).

Temperature is a key condition for growth of fungal pathogens and thus also controls competition advantages between *Fusarium* species and other fungi on plant debris (Doohan et al., 2003). The turnover of plant residues as a nutrient resource and inoculum media is also likely to be affected. Consequently, climate change is expected to have a strong direct impact on pathogen dynamics and also an indirect impact via decomposition processes of crop residues in temperate arable soils where temperatures remain close to the freezing point throughout the winter (Henry, 2008). In Germany, trend analysis has shown an increase in mean annual temperature by 0.8–1.1 °C from 1901 to 2000 with a marked increase in winter precipitation (Schönwiese and Janoschitz, 2008; Haberlandt et al., 2010).

Knowledge on the effects of changing winter climate temperatures on microbial colonization and decomposition of crop residues and the subsequent development of fungal pathogens is important for assessing their disease potential. Currently, there is little information available on the influence of winter temperatures around 0 °C or freezing and thawing events on pathogen survival because most experiments were carried out at too elevated temperatures or used artificial substrates (Beyer et al., 2004). We examined the effects of single and combined inoculation of three fungal plant pathogens on residue decomposition and the pathogen growth on maize residues at temperatures around the freezing point (Lukas et al., 2013). Also, the colonization with litter decomposing fungi and bacteria was monitored by the analysis of amino sugars, e.g. glucosamine and muramic acid. Those are highly specific indices for fungal and bacterial colonization, respectively, and do not occur in plants (Amelung 2001; Amelung et al. 2008). We addressed the following questions: (1) Do constant temperatures above 0 °C increase maize residue decomposition in comparison with permanent frost and different freeze-thaw cycles? (2) Do a high microbial activity and a considerable decomposition of plant debris during winter serve advantages for the survival of fungal plant pathogens? (3) Is frost and/or the frequency of frost events effective for the survival of fungal plant pathogens?

4.2 Material and methods

4.2.1 Soil and plant material

The arable soil used for the experiment was taken from the upper 10 cm of an experimental site in Neu-Eichenberg near Witzenhausen and is described in detail by Lukas et

al. (2013). Plant tissue, insects and stones were removed by hand; the soil was sieved wet (< 2 mm) and stored at 4 °C for 2 weeks before the experiment was started. Green maize leaves (*Zea mays* L.) were harvested from plants on a nearby field, dried (60 °C), chopped at < 2 cm and stored in a paper bag at 40 °C until the beginning of the experiment. The maize leaf residues contained 41.2% C with a $\delta^{13}C$ value of -13.4‰, 2.4% N and had a C/N ratio of 17.0.

4.2.2 Pathogen inoculum

For the inoculation with fungal plant pathogens macroconidia of *Fusarium culmorum* DSM 62184 (isolated from moldy maize grain in Germany by E. Seemüller) and *Fusarium graminearum* 210 (isolated from a wheat ear in Göttingen, Germany) and a mixture of mycelium and sclerotia of *Rhizoctonia solani* AG2-2 (isolated from sugar beet in Germany, provided by P. Kössler) were used. The macroconidia of *F. culmorum* and *Fusarium gramiearum* were obtained as described by Becher et al. (2010). Briefly, 50 ml of mung bean (*Vigna radiata* (L.) R. Wilczek) broth that was inoculated with colonized potato dextrose agar (PDA) plugs were filled into 300-ml flasks and incubated for 7 days at ambient light and temperature while shaking at 50 rpm. PDA medium in a single Petri dish was inoculated with an *R. solani* AG2-2 strain and incubated for 4 weeks at room temperature without light. Agar with *R. solani* mycelium was homogenized in a blender and the slurry was transferred into nutrient solution. To rule out possible effects of mung bean broth and sclerotia nutrient solution on the decomposition of maize leaf residues, the inoculum was centrifuged at 4,500 *g* for 10 min. The supernatant was discarded and the conidia/sclerotia in the pellets were suspended in autoclaved tap water. This step was repeated until colourlessness of the suspension indicated complete removal of the nutrient solution. The stock suspensions were kept at 4 °C.

4.2.3 Incubation procedure

The incubation experiment was carried out in 2 l glass jars, each containing moist soil equivalent to 350 g dry soil. Polyethylene litterbags (LB) (8 × 5 cm; 1 mm mesh) were filled with 3 g of oven-dried (40 °C) maize leaf residues, closed with staples and placed in a desiccator with moist paper towels for 24 h for remoistening and reducing the water repellency of the maize residues. After remoistening the inoculation of the maize residues was performed via pipette in Petri dishes (9 cm diameter) with 2 ml of each inoculum suspension containing 30,000 macroconidia of *F. culmorum* and *F. graminearum*, respectively, and 133.7 mg the mycelium/sclerotia mixture of *R. solani*. For the simultaneous investigation of maize

residue decomposition and C mineralization as well as the effects of the pathogen inoculation, three sample treatments were applied: (1) soil without litterbag application (control, n = 4), (2) soil with non-inoculated litterbags (non-inoculated, n = 3), (3) soil with litterbags inoculated with *F. culmorum* + *F. graminearum* + *R. solani* (inoculated, n = 4). To account for the different moisture levels, the non-inoculated litterbags were amended with 6 ml of autoclaved water. Additionally, a fourth sample treatment was applied only for temperature treatments one and two (see below) to test the viability of the macroconidia and mycelia/sclerotia mixture: (4) soil with litterbags inoculated with *F. culmorum*, *F. graminearum* or *R. solani* separately (n = 3). These samples were excluded from any measurements except for the analysis of the pathogen DNA at the end of the experiment to investigate possible pathogen interactions.

The litterbags were placed in the glass jars with contact to the soil. All samples were then incubated simultaneously by temperature treatment in two climate cabinets (MV 600, LinTek, Germany) for 70 days. To simulate winter climate regimes, four temperature treatments were applied (Lukas et al., 2013): (1) a constant +4 °C ($+4_{CON}$), (2) a constant -3 °C (-3_{CON}), (3) multiple freeze-thaw cycles of 48 hours at +4 and -3°C, respectively ($+4/-3_{MULTIPLE}$), and (4) a single freeze-thaw cycle of 34 days at -3 °C between two warm periods each of 18 days at +4 °C ($+4/-3_{SINGLE}$). Total number of days at +4 °C (36) and -3 °C (34) were equal for both freeze-thaw scenarios as well as the mean temperature (0.6 °C).

Air samples for CO_2 measurement were taken on days 2, 6, 8, 14, 20, 28, 30, 34, 36, 42, 44, 56, 58, 62, 64 and 70 after the incubation was started. For this purpose, a 35 ml syringe was connected to one of two ports on the PVC lid of each glass jar, which was attached with two rubber-bands to achieve an air-tight seal. The second port was used to connect a 50 L gas bottle with CO_2-free synthetic air (synthetic air 5.0, ≥ 99.999 vol% purity (20.5 ± 0.5% O_2 in 79.5 ± 0.5% N_2). Each glass jar as well as the connection of the attached syringe was flushed with synthetic air for about 2 min to remove the CO_2 before the first sampling. To adjust the temperature differences between the synthetic air and the climate cabinets, the synthetic air was first cooled in a styrofoam box by passing it through a 10 m flexible silicon tube covered with ice. After the flushing procedure, the first air sample (A_{T0}) was taken. The second air sample (A_{T1}) was taken after an accumulation period of 24 h. CO_2 concentrations were measured using an automated gas chromatograph with an electron capture detector according to Loftfield et al. (1997). The volume of CO_2 (ml) in the headspace volume of each jar under standard conditions for temperature and pressure was calculated as:

$$CO_{2_{Headspace}}\ (ml) = V_{Net} \times \Delta CO_2 \times \frac{p_n \times T_n}{p_n \times (T_n + IT)} \tag{1}$$

where V_{Net} is the headspace volume (ml), ΔCO_2 is the difference between the CO_2 concentrations A_{T1} and A_{T0} (expressed as %), p_n is the standard atmospheric pressure (hPa), T_n is the standard temperature (K) and IT is the temperature during the incubation (°C). Soil respiration expressed as CO_2-C was then calculated by the following equation:

$$CO_2\text{-C } (\mu g\ g^{-1}\ soil\ d^{-1}) = \frac{CO_{2_{Headspace}} \times M_{CO_2}}{V_{m0}} \times 0.2729 \times \frac{1000}{DW} \times \frac{24}{\Delta t} \quad (2)$$

where $CO_{2_{Headspace}}$ is the volume of CO_2 (ml) under normal conditions related to the respective incubation temperature, M_{CO_2} is the molar mass of CO_2 (mg), V_{m0} is the volume of one mol of a gas under normal conditions (ml), 0.2729 is the mass fraction of C in CO_2, DW is the dry weight of the incubated soil sample (g) and Δt is the time between A_{T0} and A_{T1} (h).

At the end of the incubation period, the litterbags were taken out of the glass jars. Soil sub-samples for analysis of microbial biomass C, biomass N and ergosterol were taken. Soil loosely adhering to the litterbags was removed carefully with a brush and a knife prior to opening the bag itself. The maize residues of each litterbag were dried at 40 °C for 48 hours, weighed and milled for further analysis (total C, total N, amino sugars, DNA extraction and subsequent quantitative polymerase chain reaction).

4.2.4 Analytical procedures

Microbial biomass C and biomass N were estimated by fumigation extraction (Brookes et al., 1985; Vance et al., 1987). A sub-sample of 20 g moist soil was separated into two portions of 10 g. One portion was fumigated at 25 °C with ethanol-free $CHCl_3$, which was removed after 24 h. Fumigated and non-fumigated samples were extracted for 30 min with 40 ml of 0.5 M K_2SO_4 by horizontal shaking at 200 rev min^{-1} and filtered (hw3, Sartorius Stedim Biotech, Göttingen, Germany). Organic C and total N in the extracts were measured via infrared and electrochemical detection, respectively, after combustion at 800 °C using a multi N/C® 2100S automatic analyser (Analytik Jena, Jena, Germany). Microbial biomass C was calculated as E_C/k_{EC}, where E_C = (organic C extracted from fumigated soil) – (organic C extracted from non-fumigated soil) and k_{EC} = 0.45 (Wu et al., 1990). Microbial biomass N was calculated as E_N/k_{EN}, where E_N = (total N extracted from fumigated soil) – (total N extracted from non-fumigated soil) and k_{EN} = 0.54 (Brookes et al., 1985).

The fungal cell-membrane component ergosterol was extracted from 2 g moist soil with 100 ml ethanol (96%) according to Djajakirana et al. (1996). Quantitative determination of ergosterol was then performed by reversed-phase HPLC analysis with 100% methanol as the

mobile phase and detection by light absorption at a wavelength of 282 nm (Dionex UVD 170 L).

The amino sugars glucosamine (GlcN), galactosamine (GalN) and muramic acid (MurN) were determined according to Appuhn et al. (2004) as described by Indorf et al. (2011) using OPA (o-phthalaldehyd) derivatisation. 800 mg of oven-dried (40° C) maize residue powder were hydrolysed with 10 ml of 6 M HCl for 3 h at 105 °C. Chromatographic separations were performed on a Hyperclone C_{18} column (125 mm length × 4 mm diameter) at 35 °C, using a Dionex (Germering, Germany) P 580 gradient pump, a Dionex Ultimate WPS – 3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelengths. Fungal C was calculated by subtracting bacterial glucosamine from total glucosamine as an index for fungal residues, assuming that muramic acid and glucosamine occur at a 1–2 molar ratio in bacterial cells (Engelking et al., 2007): mmol fungal C g^{-1} dry weight = (mmol glucosamine − 2 × mmol muramic acid) × 9. Bacterial C was calculated as an index for bacterial residues by multiplying the concentration of muramic acid by 45 (Appuhn and Joergensen, 2006). Although the conversion values may depend on variation of glucosamine and muramic acid concentration in fungi and bacteria, respectively, they have been repeatedly used by others (Potthoff et al., 2008; Murugan et al., 2013).

DNA extraction from the maize residues was conducted with some modifications as described by Brandfass and Karlovsky (2006) and Becher et al. (2010). Briefly, 50 mg of maize residue powder were blended in a 2-ml tube with 1 ml of cetyltrimethyl-ammonium bromide (CTAB) extraction buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-laurylsarcosi, 0.13 M sorbitol, 1% (w/v) polyvinyl-pyrolidone, pH set to 8.0 with NaOH), 2 µl mercaptoethanol and 1 µl proteinase K (from a stock solution 20 mg ml^{-1}). After an initial incubation period of 10 min at 42 °C and a second incubation for 10 min at 65 °C, during which the content of the tubes was mixed every 3 min, 0.8 ml of chloroform-isoamyl alcohol (24:1) were added. The samples were then thoroughly emulsified, incubated on ice for 15 min and centrifuged at 8,000 *g* for 10 min at room temperature. 600 µl of the upper phase were transferred to a 1.5-ml tube containing 200 µl of 30% (v/v) PEG 6000 and 100 µl of 5 M NaCl, mixed and centrifuged at 15,000 *g* for 15 min at room temperature. After carefully decanting the supernatant, the pellets were washed twice with 600 µl 70% (v/v) ethanol, dried, and dissolved in 50 µl of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl). Subsequent analysis of quality and concentration of total DNA was performed by agarose electrophoresis. A 1:20-dilution was used in the PCR. The extraction efficacy of DNA from this particular matrix was not tested because experience with different plant

matrices showed that when only 50 mg plant material is used per 1 ml CTAB buffer, negligible amount of DNA can be recovered from the residue after extraction (Rollwage and Karlovsky, unpublished results).

Quantification of the genomic DNA of *F. culmorum* and *F. graminearum* was carried out by real-time PCR using published protocols (Brandfass and Karlovsky, 2008); for *F. culmorum* the forward (GATGCCAGACCAAGACGAAG) and reverse (GATGCC AGACGCACTAAGAT) primer OPT18 (Schilling et al., 1996) and for *F. graminearum* the forward (ACAGATGACAAGATTCAGGCACA) and reverse (TTCTTTGACA TCTGTTCAACCCA) primer Fg16N (Nicholson et al., 1998) amplified anonymous, species-specific sequences from the genomes of both species. The amplification mix for *F. culmorum*-specific PCR consisted of NH_4-reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25°C; Bioline, Luckenwalde, Germany), 4 mM $MgCl_2$, 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of primer OPT18 F and OPT18 R, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM fluorescein (BioRad, Hercules, CA, USA; to facilitate the collection of well factors), 0.1x SYBR Green I solution (Invitrogen, Karlsruhe, Germany), template DNA (1 µL) and doubly distilled water (ddH_2O) filled to a total volume of 25 µL. The amplification mix for the *F. graminearum*-specific PCR consisted of 1x SYBR Premix Ex Taq (containing TaKaRa Ex Taq HS, dNTP Mixture, Mg^{2+}, and SYBR Green I, Takara Bio, Otsu, Japan), 0.3 µMof primer Fg16N F and Fg16N R, 10 nM fluorescein, template DNA (1 µL) and ddH_2O filled to 25 µL. The iCycler System (BioRad, Hercules, CA, USA) was used for amplification and melting curve analysis with the following temperature profile: initial denaturation for 1.5 min at 95 °C followed by 35 cycles with 30 s at 94 °C, 45 s at 64 °C and 45 s at 72 °C. The final elongation was performed for 5 min at 72 °C. An assay for *R. solani* AG2-2 DNA was used (Abbas et al., 2014), based on a forward primer AG22sp2 (TAGCTGGATCCATTAGTTTG) (Salazar et al., 2000) and reverse primer KhotR (GTTCAAAGAYTCGATGATTCAC) (Fredricks et al., 2010), which amplified part of the internal transcribed spacer between ribosomal RNA genes 18S and 5.8S. The qPCR system with SYBR Green detection was optimised for 25 µL qPCR reaction mixture containing PCR buffer (Bioline, Lückenwalde, Germany), 3 mM $MgCl_2$ (Bioline), 200 µM dNTPs (Bioline), 0.3 µM forward primer, 0.3 µM reverse primers each, 0.1 x of SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 0.25 u of Taq DNA polymerase (Bioline) and 1 µL *R. solani* DNA dilution series from 1.8 pg to 444.4 pg. The iCycler System (BioRad, Hercules, CA, USA) was used for amplification and melting curve analysis with the following temperature profile: initial denaturation for 3 min at 94 °C followed by 40 cycles with 30 s at 94 °C, 20 s at 59 °C and 30 s at 72 °C. The final

elongation was performed for 5 min at 72 °C. Standards were prepared by 3-fold serial dilution from pure genomic DNA of *F. graminearum*, *F. culmorum* and *R. solani*, which was quantified by densitometry as described before (Abbas et al., 2014; Brandfass and Karlovsky, 2008). Single technical replicates were used for sample analysis because biological variation was expected to exceed technical variation by an order of magnitude or more. The concentration of fungal DNA determined by qPCR was used to calculate the amount of DNA in maize residues, assuming that DNA extraction was quantitative.

4.2.5 Statistical analysis

The data presented in tables and figures are arithmetic means expressed on an oven-dry basis (about 24 h at 105 °C). The Kolmogorov-Smirnoff test was used to check for normal distribution. To test for treatment effects, a two-way analysis of variance was performed using a general linear model and inoculation (non-inoculated, inoculated) and temperature treatments as fixed factors. The significance of differences between the temperature treatments was tested by one-way analysis of variance (ANOVA) using post hoc Tukey HSD ($P \leq 0.05$). An independent-samples t-test was used to test for differences between inoculation treatments at the same temperature and the separately inoculated samples at +4 °C and -3 °C ($P \leq 0.05$). The relationships between substrate decomposition and total C mineralization as well as GlcN and both substrate decomposition and total C mineralization were tested by regression analysis. Additionally, Pearson product moment correlation analysis was performed to determine the relationship between fungal GlcN and the pathogen DNA. All statistical calculations were performed by SPSS Statistics 17.0 (SPSS Inc., Chicago, USA).

4.3 Results

4.3.1 Soil microbial biomass

Soil microbial biomass C in all samples (except non-inoculation at constant 4 °C) at the end of the incubation period were in a similar range with an average value of 287 µg g^{-1} soil (Table 4). Significant effect of temperature on microbial biomass C content was found in non-inoculated samples but not in inoculated samples and controls. Microbial biomass N averaged 54 µg g^{-1} soil, significant increases of 33% being found in samples with non-inoculated litterbags at constant 4 °C as compared to all other temperature regimes. Without litterbags, fungal ergosterol was present at about 0.5 µg g^{-1} soil (Table 4). A significant litterbag effect

was observed, leading to about 36% higher contents in comparison with the respective control samples.

Table 4. Amounts of soil microbial biomass C and N, ergosterol, the microbial biomass C to biomass N ratio as well as the proportion of ergosterol to microbial biomass C at the end of the 70-day incubation of the inoculated and not inoculated litterbag as well as the control samples of all temperature treatments.

Treatment	Microbial biomass			Ergosterol	Ergosterol / biomass C
	C	N	C/N		
	($\mu g\ g^{-1}$ soil)			($\mu g\ g^{-1}$ soil)	%
Inoculated					
$+4_{CON}$	312 a	64 a	4.9 a	0.67 ab	0.22 b
-3_{CON}	298 a	54 ab	5.5 a	0.87 a	0.29 a
$+4/-3_{MULTIPLE}$	305 a	53 b	5.7 a	0.63 b	0.21 b
$+4/-3_{SINGLE}$	288 a	55 ab	5.3 a	0.60 b	0.21 b
Not inoculated					
$+4_{CON}$	360 a	69 a	5.2 a	0.75 a	0.21 a
-3_{CON}	264 b	48 b	5.5 a	0.60 a	0.23 a
$+4/-3_{MULTIPLE}$	293 b	51 b	5.8 a	0.60 a	0.21 a
$+4/-3_{SINGLE}$	302 ab	55 b	5.5 a	0.56 a	0.19 a
Control					
$+4_{CON}$	271 a	48 a	6.1 a	0.48 a	0.17 a
-3_{CON}	271 a	50 a	5.4 a	0.46 a	0.17 a
$+4/-3_{MULTIPLE}$	291 a	50 a	5.9 a	0.51 a	0.18 a
$+4/-3_{SINGLE}$	260 a	49 a	5.3 a	0.49 a	0.19 a
Probability values					
Temperature	<0.05	<0.05	n.s.	n.s.	n.s.
Litterbag	<0.05	<0.01	n.s.	<0.01	<0.05
T × L	n.s.	<0.01	<0.05	n.s.	n.s.
CV (±%)	7.3	6.7	7.5	15	16

Different letters within a column indicate significant differences between temperatures within each treatment (inoculated, not inoculated, control) ($P < 0.05$; Tukey HSD); n.s. = not significant; CV = pooled coefficient of variation between replicates.

4.3.2 CO_2 production and maize residue decomposition

The soil microbial activity in samples without litterbag application was very low. Mean respiration measured at constant -3 °C was 0.54 µg CO_2-C g^{-1} soil d^{-1}, CO_2 evolution at constant 4 °C and in the freeze-thaw cycle treatments was about 26% higher. The highest mean C mineralization after application of maize residue litterbags was measured at constant 4 °C (17 0.54 µg CO_2-C g^{-1} soil d^{-1}, Fig. 6a). Here, the mineralization rates of both inoculated and not inoculated samples increased until day 28 and decreased continuously thereafter. Compared with not inoculated litterbag samples, the amount of CO_2-C evolved in the first four weeks was three times higher but on average 14% lower after the respiration peak. The average C mineralization after litterbag application at constant frost was also very low (1.2 µg CO_2-C g^{-1} soil d^{-1}) but increased by about 200% in the course of the experiment due to the inoculation (Fig. 6b). Respiration rates of the not inoculated litterbag samples were on average 2.6-fold lower and in the range of the control samples. Litterbag samples exposed to multiple freeze-thaw cycles showed a mean C mineralization of roughly 10 µg CO_2-C g^{-1} soil d^{-1} (Fig. 6c). In comparison with untreated litterbags, respiration rates of the inoculated samples in the first 36 days at both 4 and -3 °C were four times higher but about 12% decreased over the last five weeks. In the $+4/-3_{SINGLE}$ treatment both inoculated and not inoculated litterbag samples showed an average respiration rate of 13 µg CO_2-C g^{-1} soil d^{-1} (Fig. 6d). The inoculation lead to a 4-fold higher C mineralization during the first 4 °C period and to 38% higher respiration rates during the subsequent frost period. However, after the incubation temperature was set back to 4 °C the CO_2 evolution of the not inoculated litterbag samples was on average 16% higher.

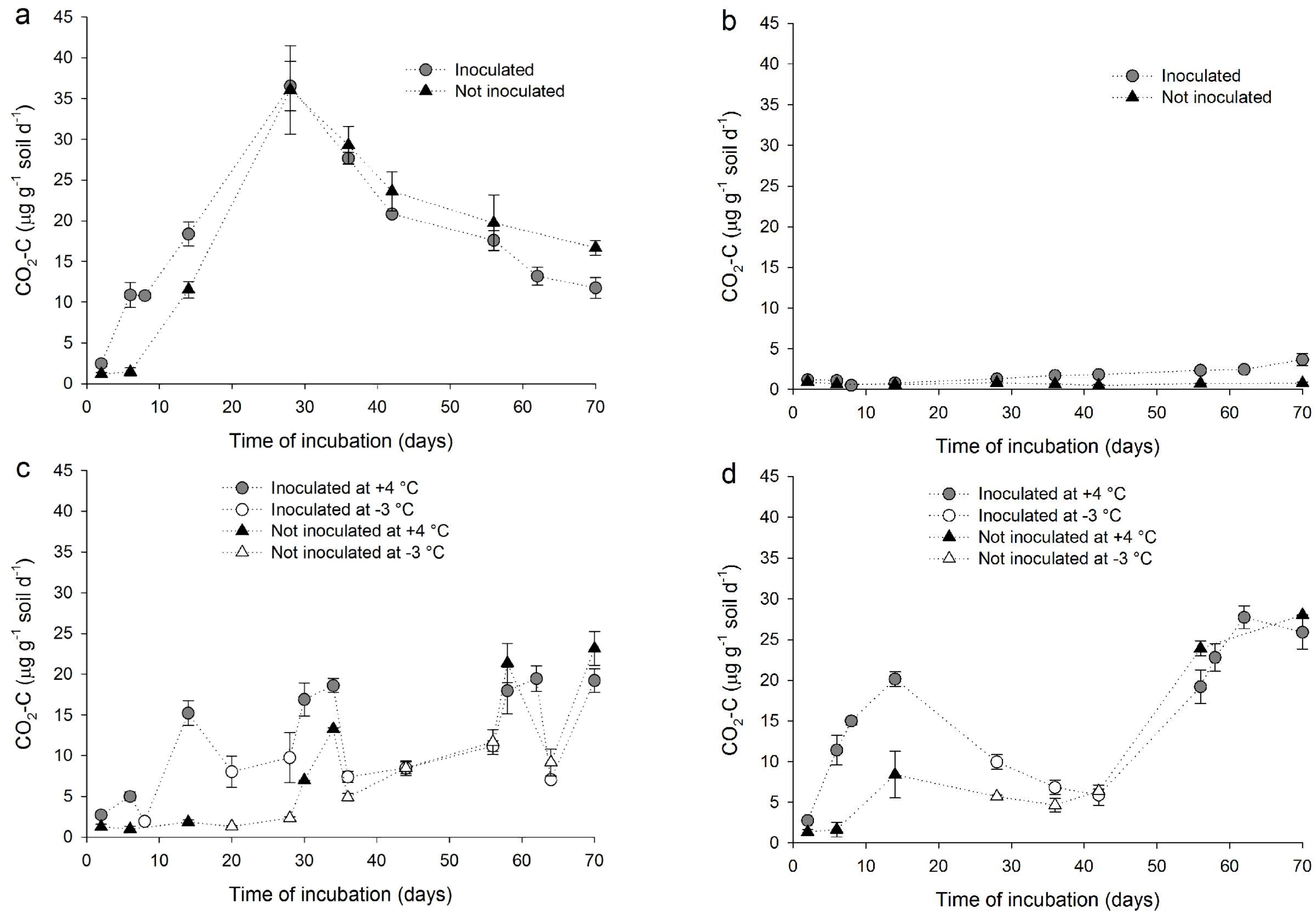

Fig. 6a-d. CO_2-C production of the inoculated (circles, n = 4) and not inoculated (triangles, n = 3) litterbag samples of the constant 4 °C (a), the constant -3 °C (b), the multiple (c) and single freeze-thaw cycle (d) scenario over a 70-day incubation period; error bars show ± one standard deviation.

Cumulative CO_2 production (Fig. 7) in the control samples averaged 44 µg C g^{-1} soil, no significant difference was found between both freeze-thaw scenarios. Total CO_2 evolution in samples with litterbags was highest at constant 4 °C and averaged 1,382 µg C g^{-1} soil, without further increase by pathogen inoculation. In contrast, 85 µg CO_2-C g^{-1} soil was evolved at constant frost, where the inoculation increased the C respired by about 135%. Both freeze-thaw scenarios showed an intermediate cumulative CO_2 production, which was significantly higher in the $+4/-3_{SINGLE}$ treatment (62%) for both inoculated and for the not-inoculated litterbag samples. In both temperature treatments, the pathogen inoculation significantly increased CO_2 production by on average 46% over the not-inoculated samples.

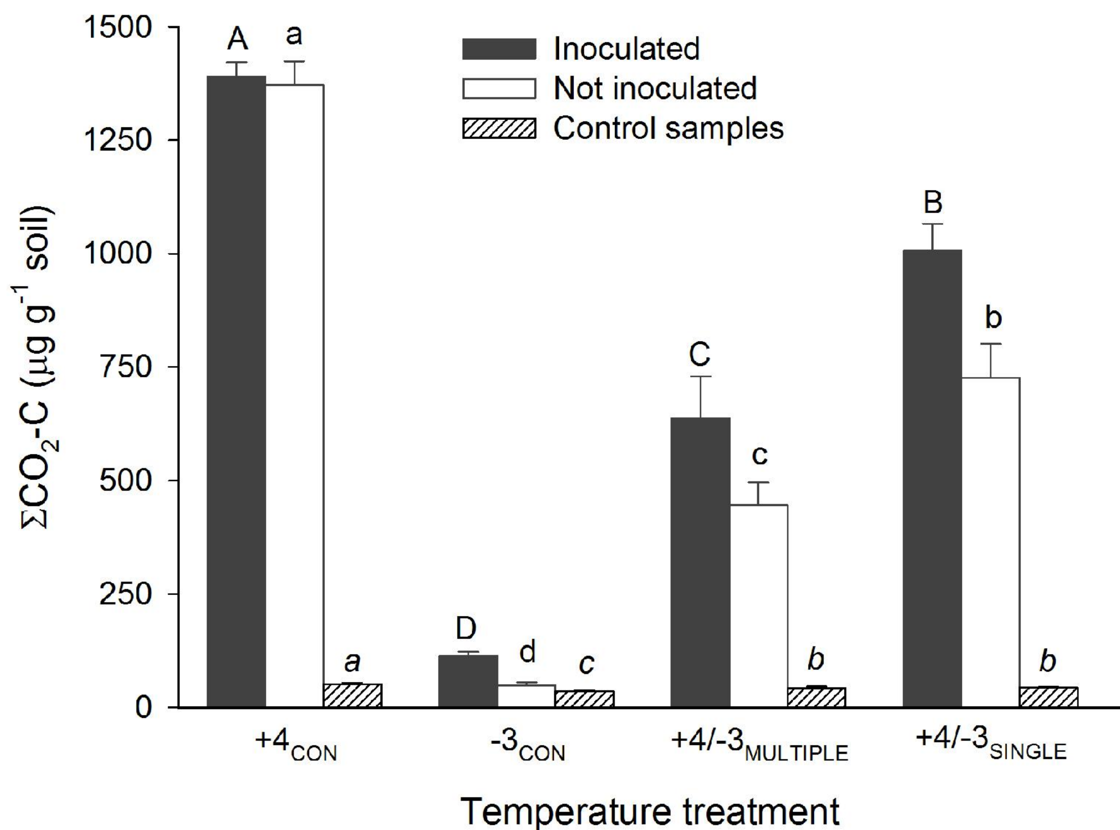

Fig. 7. Cumulative CO_2-C production of the inoculated (n = 4) and not inoculated (n = 3) litterbag as well as the control samples (n = 4) at the end of a 70-day incubation period; error bars show ± one standard deviation; different letters above the columns indicate significant differences ($P < 0.05$).

Maize residue decomposition followed similar pattern as CO_2 production (Fig. 8). Constant 4 °C strongly promoted the decomposition (on average 33%), the inoculation with pathogens had no increasing effect. In the other treatments, decomposition ranged between 1.2% (not-inoculated litterbags at constant frost) and 23% (inoculated litterbags in the +4/-

3_{SINGLE} scenario). Here, the pathogen inoculation significantly increased the maize residue decomposition by about 336% at constant frost und around 25% in the freeze-thaw scenarios over the respective not-inoculated litterbags.

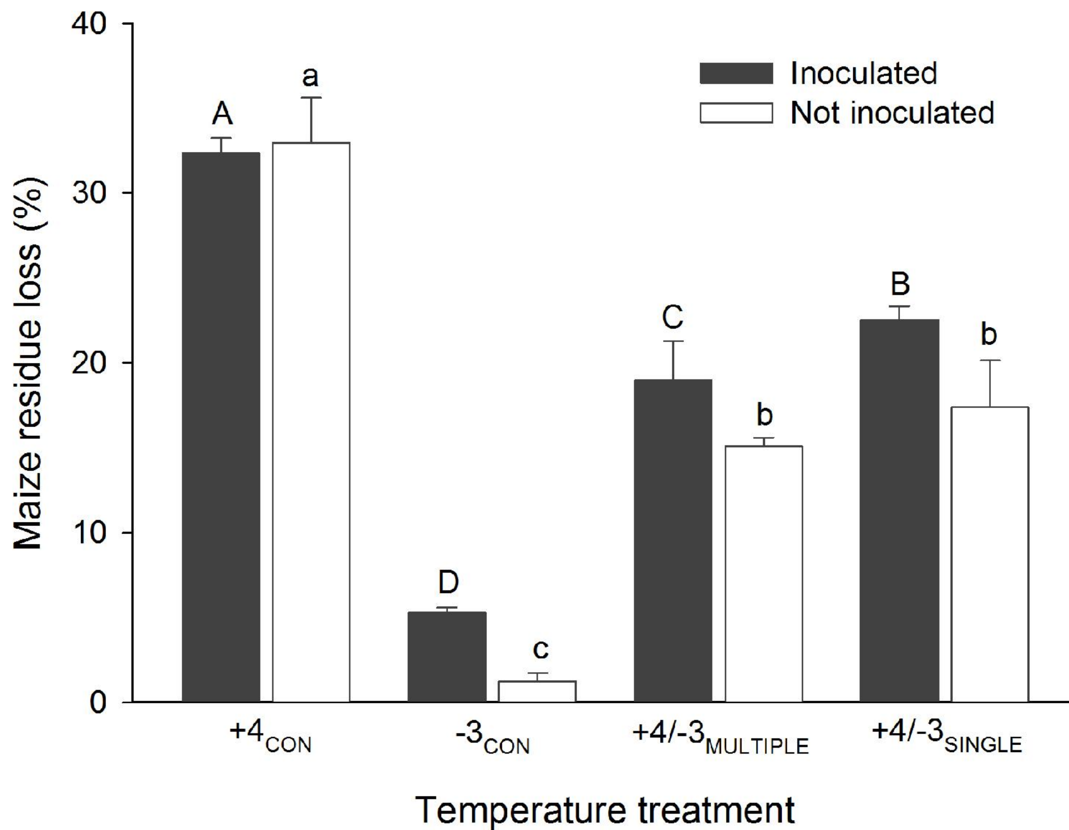

Fig. 8. Total weight loss of the inoculated (n = 4) and not inoculated (n = 3) maize leaf residues in percent of the initial amount at the end of a 70-day incubation period; error bars show ± one standard deviation; different letters above the columns indicate significant differences ($P < 0.05$).

4.3.3 Microbial colonization of maize leaf residues

Pathogen-inoculated litterbags at constant 4 °C revealed the significantly highest total amino sugars content (2.47 mg g^{-1} maize, Fig. 9), which was twice that of the respective non-inoculated litterbags. In comparison, constant frost as well as both freeze-thaw cycle scenarios decreased the total amino sugar content in the respective litterbag treatments by about 77% in the order constant frost > $+4/-3_{MULTIPLE}$ > $+4/-3_{SINGLE}$. Here, also twofold higher contents were observed on average in the inoculated litterbags.

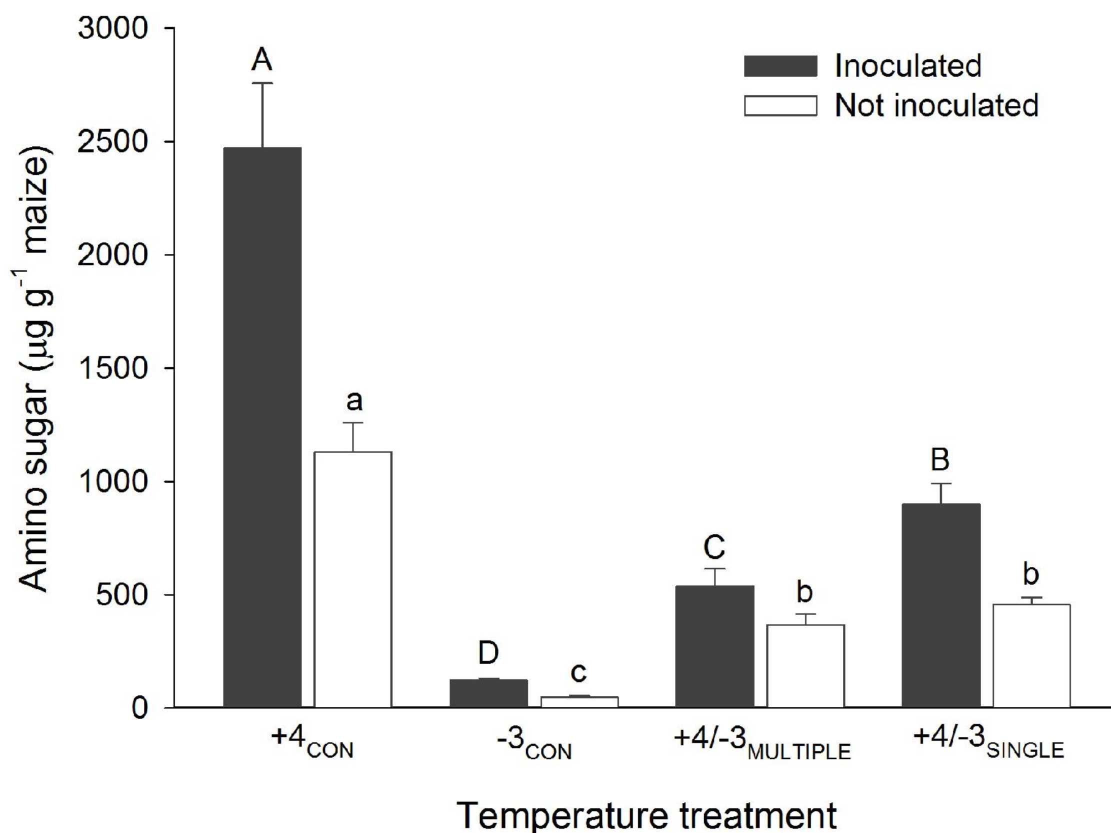

Fig. 9. Total amount of amino sugars of the inoculated (n =4) and not inoculated (n = 3) maize leaf residues of all temperature treatments at the end of the 70-day incubation; error bars show ± one standard deviation; different letters above the columns indicate significant differences ($P < 0.05$).

Fungal GlcN was the most abundant amino sugar, on average accounting for 92% on total amino sugars in the pathogen inoculated samples and 81% in the non-inoculated litterbags. The contribution of MurN to total amino sugars was very low, ranging from 0.3 to 4.8%. Samples at constant 4 °C showed the significantly highest content of GlcN and consequently the highest content of fungal C (Table 5), which was almost 3 times higher than in the respective litterbags without pathogen inoculation ($P < 0.05$). Consequently, the fungal C to bacterial C ratio, based on the amino sugars GlcN and MurN, was significantly increased over the non-inoculated litterbags. Similar increases of GlcN and fungal C contents by 2.5 times were found in the inoculated litterbags of the constant frost and $+4/-3_{SINGLE}$ treatments, leading to significant higher fungal C to bacterial C ratios. MurN and consequently bacterial C were lowest in the inoculated litterbags and not detectable without inoculation under constant frost (Table 5). A more than 3-fold increase due to the inoculation was found in the multiple freeze-thaw cycle treatment only, whereas the content of MurN in the constant 4 °C

and the +4/-3$_{SINGLE}$ treatment decreased by about 40% compared with non-inoculated litterbags.

Table 5. Concentrations of muramic acid (MurN), glucosamine (GlcN), galactosamine (GalN), fungal C, bacterial C and the fungal C to bacterial C ratio in the inoculated and not inoculated maize leaf residues of all temperature treatments at the end of the 70-day incubation.

Treatment	GalN	GlcN	MurN	Fungal C	Bacterial C	Fungal C/
	(μg g^{-1} DW)			(mg g^{-1} DW)		bacterial C
Inoculated						
+4$_{CON}$	73 a	2352 a	15 a	21 a	0.7 a	31 b
-3$_{CON}$	2.9 c	116 c	0.4 b	1.0 c	0.02 b	64 a
+4/-3$_{MULTIPLE}$	23 b	398 c	17 a	3.6 c	0.8 a	4.6 c
+4/-3$_{SINGLE}$	23 b	831 b	14 a	7.5 b	0.6 a	12 c
Not inoculated						
+4$_{CON}$	231 a	859 a	27 a	7.7 a	1.2 a	6.5 ab
-3$_{CON}$	n.d.	48 c	n.d.	0.4 c	n.d.	n.d.
+4/-3$_{MULTIPLE}$	81 b	288 b	5.0 b	2.6 b	0.2 b	12 a
+4/-3$_{SINGLE}$	46 b	317 b	22 a	2.9 b	1.0 a	5.6 b
Probability values						
Temperature	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Inoculation	<0.01	<0.01	<0.05	<0.01	<0.05	<0.01
T × I	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
CV (±%)	29	14	15	16	15	27

Different letters within a column indicate significant differences within litterbag treatments ($P < 0.05$, Tukey HSD); DW = dry weight, n.d. = not detectable; CV = mean coefficient of variation between replicates.

4.3.4 Fungal inoculum develpment

The initial amount of *F. culmorum* DNA applied with macroconidia to the litterbags was 0.49 ng mg^{-1} maize residues (Fig. 10a). At the end of the incubation period, similar values were found after constant frost in litterbags individually inoculated with *F. culmorum* and co-inoculated with the other pathogens (t-test, $P > 0.05$). In contrast, at constant 4 °C the content of *F. culmorum* DNA in both the individually and co-inoculated litterbags greatly increased to an average of 876 ng mg^{-1} maize residues. Differences between *F. culmorum* biomass in litterbags inoculated with *F. culmorum* only and those co-inoculated with the other pathogens

were insignificant at constant 4 °C (t-test, $P > 0.05$), indicating that interactions had no effect on development of *F. culmorum*. The average amount of DNA measured in the co-inoculated litterbags in the freeze-thaw cycle treatments was more than 3 times lower than at constant 4 °C but was still markedly increased over the constant frost samples and the initial value. No significant difference was found between multiple freeze-thaw cycles and the $+4/-3_{SINGLE}$ treatment.

The initial amount of *F. graminearum* DNA applied with the inoculation was 0.4 ng mg^{-1} maize residues (Fig. 10b). After 70 days of constant frost, *F. graminearum* DNA was not detectable in the separate inoculation as well as on maize residues co-inoculated with the other pathogens. The highest amount of *F. graminearum* DNA was found in the separately inoculated litterbag samples kept at constant 4 °C, where the DNA amount increased 55-fold over the initial value (t-test, $P \leq 0.05$). In contrast to the separate inoculation, at constant 4 °C DNA of *F. graminearum* co-inoculated with the other pathogens was significantly lower (t-test, $P \leq 0.05$) and only showed an average 4.6-fold increase over the initial amount (1.9 ng DNA mg^{-1} maize; t-test, $P \leq 0.05$). No significant difference was found between the freeze-thaw cycle treatments. Here, both treatments greatly decreased the amount of *F. graminearum* DNA in comparison with constant 4 °C and caused a roughly 62% reduction of the pathogen DNA compared with the initial amount.

For *R. solani*, biomass corresponding to 790 ng fungal DNA mg^{-1} maize residues was applied with the initial inoculation via mycelia/sclerotia (Fig. 10c). Compared to that, after 70 days of incubation the amount of DNA was significantly decreased in all treatments. At permanent frost, DNA of *R. solani* co-inoculated with the other pathogens was stronger decreased than in the separately inoculated litterbags (t-test, $P \leq 0.05$), where on average 55% of the initial amount could be detected. In contrast, at constant 4 °C the separately inoculated litterbags were found to be stronger reduced in DNA of *R. solani* compared to the co-inoculation (t-test, $P \leq 0.05$). The DNA of *R. solani* on maize residues co-inoculated with *F. graminearum* and *F. culmorum* showed a marked decline by on average 90% at all temperature regimes in comparison with the initial amount.

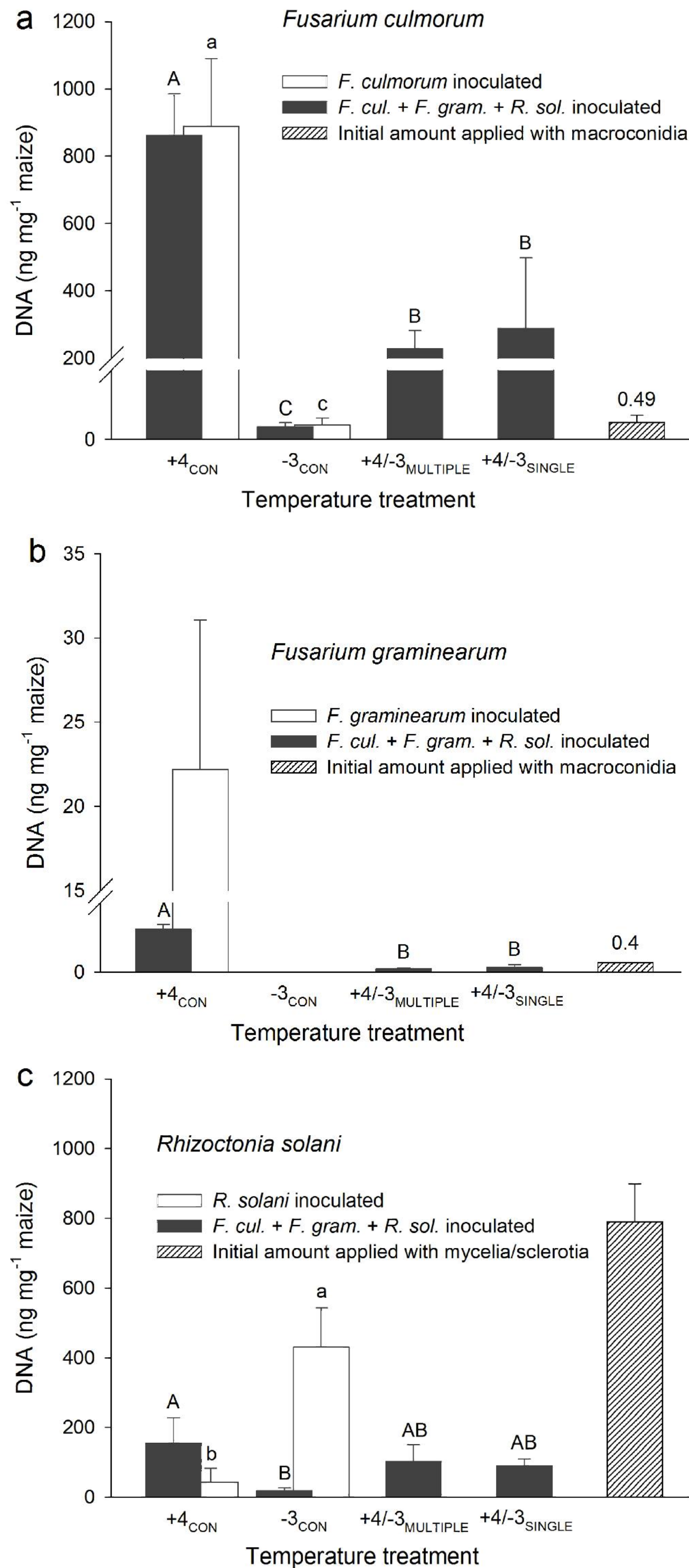

Fig. 10a-c. Amounts of DNA of *Fusarium culmorum* (a), *Fusarium graminearum* (b) and *Rhizoctonia solani* (c) of the inoculated litterbags (n = 4) and the separate inoculation (n = 3) of all temperature treatments at the end of the 70-day incubation as well as the initial amount (n = 3); error bars show ± one standard deviation; different letters above the columns indicate significant differences ($P < 0.05$).

4.4 Discussion

4.4.1 Effects on CO_2 production and substrate decomposition

In both litterbag treatments, the decomposition of the maize residues was strongly positively related to the CO_2 production (Fig. 11a), indicating that maize leaves were the main nutrient and energy source for the microbial decomposer community. Remarkably, constant 4 °C revealed no differences in CO_2 production and maize residue decomposition between both litterbag treatments while the accumulation of fungal GlcN indicated increased fungal colonization after pathogen inoculation. This discrepancy might be explained by higher substrate use efficiency of the pathogens at mild temperatures above 0 °C in comparison with the indigenous fungal population. Furthermore, for the inoculated residues the logarithmic relationships between substrate decomposition (CO_2-C production) and fungal GlcN (Fig. 11b+c) suggest a plateau phase at constant 4 °C, where substrate decomposition and C mineralization start to decline. Here, limited amounts of easily available nutrients or a limited surface area for further colonization may have constrained substrate decomposition, possibly leading to a shift in substrate utilization from maize to at least a part of the fungal and bacterial biomass present and growing on the maize residues during the experiment. Another possible explanation might be that many macroconidia did not germinate due to the low temperature or inhibitory substances produced by other microorganisms. This could not be detected by amino sugar analysis as macroconidia and mycelia have similar GlcN content (Gratzner, 1972; Schmit et al., 1975). In the constant frost and freeze-thaw treatments, the increase due to the inoculation can be directly related to stronger fungal colonization, indicating a higher frost resistance of at least part of the applied pathogens in comparison with the indigenous fungal population.

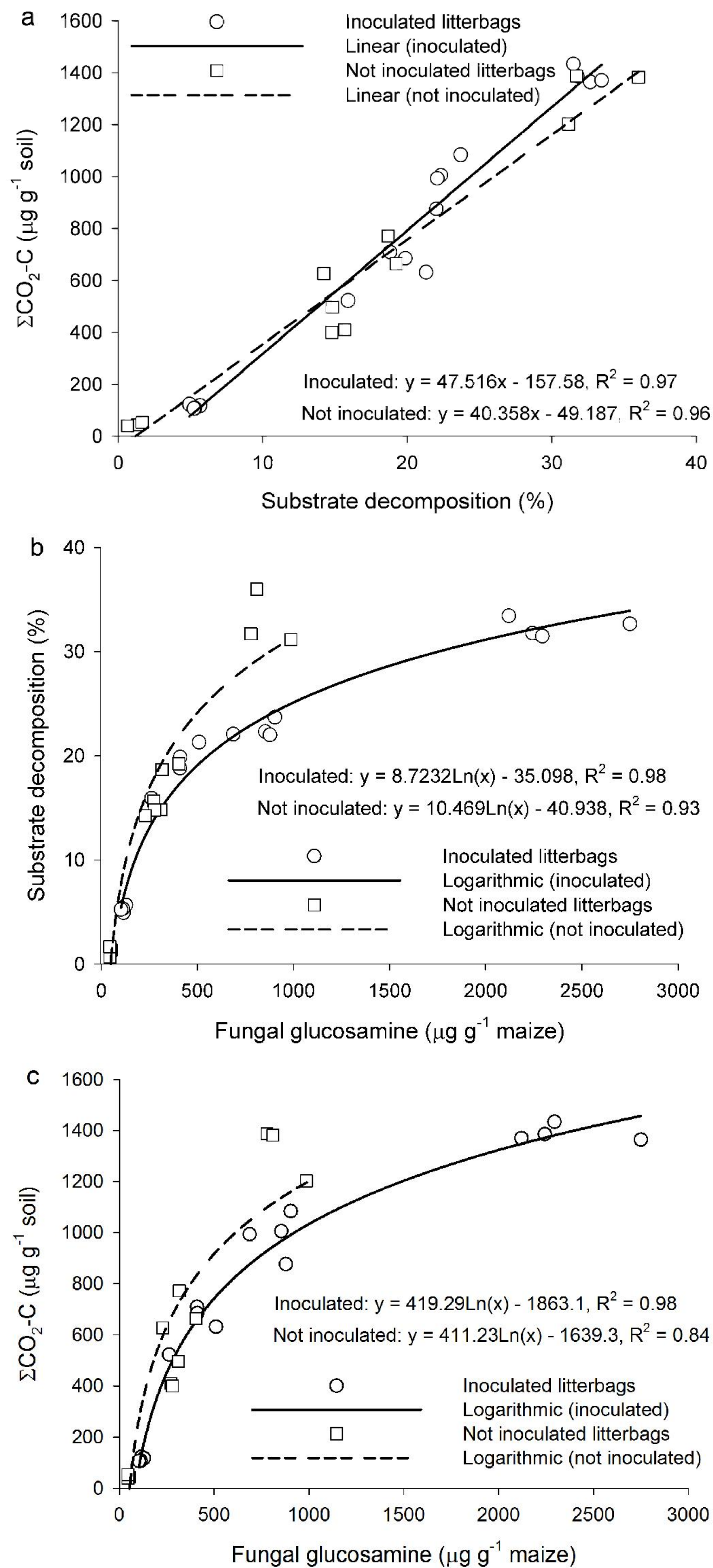

Fig. 11a-c. Regression analysis of substrate decomposition against cumulative CO_2-C production (a), fungal glucosamine against substrate decomposition (b) and fungal glucosamine against cumulative CO_2-C production (c) of the inoculated and not inoculated litterbag samples for all temperature treatments; data points represent values of each replicate.

4.4.2 Temperature and inoculation effects on bacterial and fungal colonization

At the end of the incubation period, amino sugars generally had very large proportions of fungal GlcN, which clearly demonstrates that fungi dominated the colonization and decomposition of maize residues at low temperatures. This is in line with reports by Lipson et al. (2002) and Schadt et al. (2003). The lowest content of fungal GlcN found on non-inoculated maize straw under constant frost indicates that the soil fungal community was not able to colonize the residues and the low temperature restricted the activity of the indigenous fungal population. In contrast, the results of both litterbag treatments at constant 4 °C and also the inoculated litterbags of the $+4/-3_{SINGLE}$ treatment point at conditions favourable for fungal colonization of maize residues.

Pathogen inoculation increased fungal GlcN in all temperature treatments even under constant frost on average by the factor 2.6 (± 0.2), except for the $+4/-3_{MULTIPLE}$ treatment. Here, repeated freezing and thawing allowed only a 1.4-fold higher content, indicating a reduced fungal colonization due to high freeze-thaw frequency. In all temperature treatments, the extra amounts of fungal GlcN showed a close positive relationship to the measured pathogen DNA for *F. culmorum* ($r = 0.91$, $P < 0.01$) and *F. graminearum* ($r = 0.96$, $P < 0.01$), suggesting a strong contribution of both pathogens, especially of *F. culmorum* to residue colonization and decomposition.

The generally low contents of MurN, a specific indicator for bacterial cell walls (Amelung et al., 2001; Glaser et al., 2004), indicate limited bacterial colonization under the current incubation conditions. The significant reduction of MurN in the pathogen inoculated litterbags in the constant 4 °C and $+4/-3_{SINGLE}$ treatments might be the result of an increased competition with fungi for water and nutrients. Multiple freezing and thawing is the only treatment in which MurN significantly increased to a similar level after the inoculation with plant pathogens, whilst fungal development was greatly reduced.

4.4.3 Development of fungal plant pathogens

F. culmorum prefers much higher temperatures (about 25 °C) for optimal growth *in vitro* (Brennan et al., 2003) than those used in the current study, optimal temperatures of *F. graminearum* are even higher. A positive relationship between temperature and growth rate for different isolates of *R. solani* was reported by Harikrishnan and Yang (2004) with an optimal temperature around 25 °C. Nevertheless, simulating a mild winter temperature scenario missing frost revealed an enormous growth potential of *F. culmorum* on infested maize residues. Here, co-inoculated *F. graminearum* and *R. solani* had no apparent negative

effect on the development of *F. culmorum*. This indicates higher competitiveness of *F. culmorum* at temperatures slightly above 0 °C, where the growth of *F. graminearum* was strongly reduced and the inoculum of *R. solani* was almost completely destroyed. The results of the constant frost and both freeze-thaw cycle treatments point to a considerable frost tolerance of *F. culmorum*, implying a high infection risk for succeeding crops after germination of the conidia at winter temperatures slightly above 0 °C.

The growth of *F. graminearum* and the germination of conidia are favoured by warm and humid conditions (Leplat et al., 2013). The optimum temperature for mycelial growth was found at 25 °C whereas at temperatures below 5 °C no growth was observed (Ramirez et al., 2006). In contrast, the results of our study clearly show growth of *F. graminearum* at constant 4 °C. At the presence of *F. culmorum* and *R. solani*, the increase of *F. graminearum* biomass at 4 °C was reduced, indicating a reduced ability of *F. graminearum* to compete for nutrients and/or growth substrate. This is consistent with findings showing *F. graminearum* to be a poor competitor for the colonization of crop residues (Pereyra et al., 2004; Leplat et al., 2013). Our results support the assumption that pathogen development can be limited by antagonistic fungi growing at the expense of *F. graminearum* on infested residues (Leplat et al., 2013). The results of the other temperature treatments clearly show that *F. graminearum* is not able to survive conditions of constant but mild frost but can maintain a small inoculum and thus disease potential after freezing and thawing events. Using conidial suspension as inoculum instead of a mixture of conidia and mycelia naturally present in the litter unlikely affected the relative survival and biomass accumulation of *Fusarium* spp. in our experiments.

The disease potential of *R. solani* depends on the germination of sclerotia that are present in the soil or on infested plant residues and the subsequent production of fungal hyphae (Coley-Smith and Cooke, 1971; Ritchie et al., 2009). On agar and in soil, Ritchie et al. (2009) found an optimal temperature range for sclerotial germination between 20 and 30 °C and 15 and 25 °C, respectively, whereas at 5 °C the germination was strongly restricted or not observed. At the end of our 70 d experiment, the amount of *R. solani* DNA was greatly decreased throughout all treatments compared with the initial value, except for the separate inoculation without *F. culmorum* and *F. graminearum* under permanent frost. This shows that the mixture of mycelia and sclerotia used for inoculation was to a great extent decomposed by other microorganisms. Also, sclerotia were probably unable to germinate and build up biomass under temperature regimes studied. Mechanical damage of sclerotia and mycelium of *R. solani* by homogenization of cultures used as inoculum might be responsible for the limited survival of the fungus in our experiments. Using maize litter with *in situ*-built sclerotia would help avoid mechanical damage but setting identical inoculum levels in different experiments

in this way would be difficult. Nevertheless, despite the strongly reduced inoculum of *R. solani*, the remaining biomass especially after prolonged frost in the absence of other pathogens might be capable of causing diseases on susceptible host plants when temperatures for germination and growth are more favourable in spring. Yet, it remains unclear whether the current sclerotia were still viable at the end of our experiment.

4.4.4 Conclusions

At mild winter conditions, pathogen colonization did not increase maize residue decomposition but significantly increased fungal GlcN in comparison with non-inoculated maize, indicating higher substrate use efficiency. Very large proportions of fungal GlcN detected on the maize residues demonstrated that fungi dominated the colonization and decomposition at temperatures around 0 °C. *F. culmorum* was found to be highly frost tolerant and revealed an increased competitive ability against *F. graminearum* and *R. solani* at mild conditions. Freeze-thaw events reduced the development of both *F. culmorum* and *F. graminearum* in comparison with constant mild conditions, whereas the freeze-thaw frequency had no effect. The results of the laboratory experiment suggest that mild winter conditions can increase the pathogen colonization of crop residues, whereas frost and freeze-thaw events can lower the build-up of *Fusarium* biomass and may diminish the risk of crop infection. Also, increased winter temperatures are likely to reduce infection pressure of *R. solani* due to degradation of pathogen inoculum.

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5. Substrate use, survival and development of fungal plant pathogens on infected maize residues under field conditions in winter – A soil warming experiment

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Abstract

Aims A soil warming experiment was performed to examine the effects of rising winter soil temperatures on the inoculum load and disease potential of fungal plant pathogens (*Fusarium culmorum*, *Fusarium graminearum*, *Rhizoctonia solani*) under field conditions.

Methods Using heating cables, arable soil was subjected to temperature treatments simulating medium (up to 2050) and long-term (up to 2100) climate warming scenarios. Mesh bags filled with pathogen-inoculated and non-inoculated maize residues were placed on top of the soil. After 152 days, changes in soil microbial biomass, maize residue decomposition as well as microbial amino sugars and pathogen DNA were measured.

Results Soil warming increased mean temperatures at 5 cm depth and decreased days of soil frost. Rising soil temperatures increased decomposition of pathogen-infested maize residues, without correlation to the degree of fungal colonisation. *F. culmorum* produced the largest amount of biomass, but showed no significant response to increased soil temperatures. In contrast, *F. graminearum* showed a considerably lower ability to colonize the maize residues,

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although it was able to increase the biomass with rising soil temperatures. Mycelia and sclerotia of *R. solani* were strongly decomposed and no growth was observed.

Conclusions An increased decomposition of maize residues does not significantly reduce the pathogen load. A high *F. culmorum* biomass and the increased inoculum of *F. graminearum* point to an increased infection risk with future climate warming.

Keywords: *Fusarium culmorum*; *Fusarium graminearum*; *Rhizoctonia solani*; Winter climate change; Soil warming; Decomposition; Amino sugars

5.1 Introduction

In wheat and other cereal crops, *Fusarium species* cause several diseases such as seedling blight, foot rot, Fusarium head blight, ear rot of maize and crown rot in wheat and barley (Sutton 1982; Burgess et al. 2001; Doohan et al. 2003; Xu and Nicholson 2009). *Fusarium culmorum* WG Smith and *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch) are considered to be the most important pathogens in crops worldwide, leading to high yield losses and to contamination of the grain with mycotoxins, such as deoxynivalenol and zearalenone (Parry et al. 1995; Mesterhazy et al. 1999; Popiel et al. 2008). The soil-borne fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) with the anastomosis group (AG) *R. solani* AG 2-2 IIIB is responsible for diseases in sugar beet (*Beta vulgaris* L.), decreasing yields by up to 50% in parts of Europe, Japan and the United States (Sneh et al. 1991; Rieckmann and Steck 1995; Kiewnick et al. 2001; Kühn et al. 2009).

Crop residues on the soil surface, in particular large amounts of maize debris, serve as substrate for pertotrophic pathogenic fungi; they represent a major source of inoculum for crop plant infection in the next season (Führer Ithurrart et al. 2004; Buddemeyer et al. 2004; Maiorano et al. 2008; Palumbo et al. 2008). Fungal survival and inoculum production in crop residues is limited by residue decomposition. In contrast to plant residues buried in soil after ploughing, residues on the soil surface provide nutrients and serve as a substrate for a longer period, allowing fungal plant pathogens to survive for several years (Khonga and Sutton 1988; Pereyra et al. 2004; Vogelgsang et al. 2011).

Climate change may have a strong impact on pathogen population dynamics and decomposition processes of crop residues on temperate arable soils, as they remain close to freezing point throughout the winter (Henry 2008). Here, temperature and water are key conditions influencing saprophytic growth and survival of fungal pathogens and the severity

of diseases they cause (Doohan et al. 2003). In Europe and especially in Germany, climate change has led to increased mean air temperatures over the last 50 years (Haberlandt et al. 2010) and trend analysis has shown an increase in the mean annual temperature of about 0.8 to 1.1 °C from 1901 to 2000, accompanied by a marked increase in winter precipitation (Schönwiese and Janoschitz 2008).

Knowledge on the effects of changing winter climate temperatures on infection and colonisation of crop residues and the subsequent development of fungal pathogens is important for assessing their disease potential. Litterbag experiments are commonly used to investigate decomposition of plant residues (Johansson et al. 1986; Knacker et al. 2003; Miura et al. 2008; Jacobs et al. 2011) and provide a useful tool to study the effects of rising soil temperatures on fungal pathogen dynamics on crop debris under field conditions. Colonisation with decomposing fungi and bacteria can be monitored by the analysis of amino sugars (Joergensen and Wichern 2008; Potthoff et al. 2008). Glucosamine and muramic acid are important and highly specific indices for fungal and bacterial biomass, respectively, as they do not occur in plants (Amelung 2001; Amelung et al. 2008).

A soil temperature manipulation experiment was carried out under field conditions to answer the following research questions: (1) To what extent are fungal pathogen dynamics on infected maize residues affected by increased winter soil temperatures? (2) Is the inoculum load regulated by the degree of substrate decomposition?

5.2 Material and methods

5.2.1 Soil warming facility

The experimental site is located in the northern part of Göttingen, Lower Saxony, Germany (51°33´29.28´´N, 9°55´59.46´´E) and was described in detail in a study of Siebold and von Tiedemann (2012). Briefly, the soil warming facility with a total size of 60 m^2 was designed in 2009, where the original stony and heterogeneous soil was replaced to a depth of 1 m by an arable top soil (high silty clay). The site consists of 12 plots (2 m × 2.5 m each) arranged in two rows. Heating cables were buried in each plot, also in the control plots, to ensure equal physical conditions, at a depth of 10 cm. The temperature offsets were set to +1.6 °C and +3.2 °C above the control plots, resulting in three soil temperature treatments: (ST1) ambient soil temperature, (ST2) ambient +1.6 °C and (ST3) ambient +3.2 °C. The soil warming treatments were applied to simulate warming scenarios for Lower Saxony by 2050 (medium-term) and 2100 (long-term), respectively (Jacob and Podzun 1997; Werner and

Gerstengarbe 2007). All temperature treatments were repeated four times in a randomised block design.

5.2.2 Experimental procedure

The soil used for the experiment was taken from the upper 10 cm of an arable field in Neu-Eichenberg near Witzenhausen (51°23´N, 9°55´E, Northern Hessia, Germany) in September 2011, sieved and stored at 4 °C for about two weeks. The soil was classified as a Haplic Luvisol (FAO-WRB 2006) with the following characteristics: 3.3% sand, 83.4% silt, 13.3% clay, a pH ($CaCl_2$) of 6.3, 1.4% total C, a soil organic $\delta^{13}C$ value of -26.4‰, 0.14% N and a C/N ratio of 10. Polypropylene cylinders (10 × 10 cm) closed at the bottom with polyethylene mesh (1 mm mesh size) were filled with the moist soil equivalent to 350 g dry soil and transferred to the experimental site at the beginning of October 2011. Per plot, two cylinders were inserted to a depth of 5 cm (24 cylinders in total).

Green maize leaves (*Zea mays* L.) were dried (60 °C), chopped at < 2 cm and stored in a paper bag at 40 °C until the beginning of the experiment. The maize residues contained 41.2% C and 2.4% N. For the inoculation with fungal pathogens, macroconidia of *Fusarium culmorum* and *Fusarium graminearum* and a mixture of mycelium and sclerotia of *Rhizoctonia solani* were used. The conidia were obtained as described by Becher et al. (2010). Briefly, 50 ml of mung bean (*Vigna radiata* (L.) R. Wilczek) broth that was inoculated with potato dextrose agar (PDA) plugs colonised with *F. graminearum* and *F. culmorum* were filled into 300-ml flasks and incubated for 7 days at ambient light and temperature while shaking at 50 rev min^{-1}. PDA medium in a single Petri dish was inoculated with an *R. solani* AG2-2 strain and incubated for 4 weeks at room temperature without light. Agar with *R. solani* mycelium was homogenised in a blender and the slurry was transferred into nutrient solution. To rule out possible effects of the mung bean broth and the sclerotia nutrient solution during the decomposition of maize residues, the inoculum solutions were centrifuged at 4500 *g* for 10 min in 120 ml polypropylene containers, the supernatant was discarded carefully and the conidia/sclerotia pellets were dissolved in autoclaved water. This step was repeated two to three times to reduce the remaining nutrients. The stock solutions were then kept at 4 °C before the experiment was started.

Polyethylene litterbags (LB) (8 × 5 cm; 1 mm mesh) were filled with 3 g of the oven-dried maize residues, closed with staples and placed in a desiccator with moist paper towels for 24 hours to reduce the water repellency of the maize residues. After remoistening, the maize residue of 12 litterbags was inoculated via pipette in Petri dishes (9 cm diameter) with

2 ml of each inoculum solution containing 30,000 macroconidia of *F. culmorum* and *F. graminearum*, respectively, and 133.7 mg mycelium/sclerotia of *R. solani*. Another 12 litterbags, each filled with 3 g of non-inoculated maize residues, served as controls. To account for the different moisture levels, the non-inoculated litterbags were amended with 6 ml of autoclaved water. At the end of October 2011 (two weeks after the cylinders had been inserted), all litterbags were transferred to the cylinders at the experimental site. Per plot, each of the two cylinders received one litterbag (either pathogen-inoculated or non-inoculated control), which was placed on top of the soil. The cylinders were then covered with polyethylene meshes (2 mm mesh size) to protect the litterbags from animals, but allowing any precipitation to pass.

At the end of the experimental period, the litterbags as well as the soil filled cylinders were recovered. Soil sub-samples for analysis of microbial biomass C, biomass N and ergosterol were taken. Soil loosely adhering to the litterbags was removed carefully with a brush and a knife prior to opening the bag itself. The maize residues of each litterbag were dried at 40 °C for 48 h, weighed and milled for further analysis (total C, total N, amino sugars, DNA extraction and subsequent quantitative polymerase chain reaction).

5.2.3 Analytical procedures

Microbial biomass C and biomass N were estimated by fumigation extraction (Brookes et al. 1985; Vance et al. 1987). A sub-sample of 20 g moist soil was separated into two portions of 10 g. One portion was fumigated at 25 °C with ethanol-free $CHCl_3$, which was removed after 24 h. Fumigated and non-fumigated samples were extracted for 30 min with 40 ml of 0.5 M K_2SO_4 by horizontal shaking at 200 rev min^{-1} and filtered (hw3, Sartorius Stedim Biotech, Göttingen, Germany). Organic C and total N in the extracts were measured using a multi N/C® 2100S automatic analyser (Analytik Jena, Jena, Germany). Microbial biomass C was calculated as E_C/k_{EC}, where E_C = (organic C extracted from fumigated soil) – (organic C extracted from non-fumigated soil) and k_{EC} = 0.45 (Wu et al. 1990). Microbial biomass N was calculated as E_N/k_{EN}, where E_N = (total N extracted from fumigated soil) – (total N extracted from non-fumigated soil) and k_{EN} = 0.54 (Brookes et al. 1985; Joergensen and Mueller 1996).

The fungal cell-membrane component ergosterol was extracted from 2 g moist soil with 100 ml ethanol (96%) according to Djajakirana et al. (1996). Quantitative determination of ergosterol was then performed by reversed-phase HPLC analysis with 100% methanol as the mobile phase and detected at a wavelength of 282 nm (Dionex UVD 170 L).

The amino sugars glucosamine (GlcN), galactosamine (GalN) and muramic acid (MurN) were determined according to Appuhn et al. (2004) as described by Indorf et al. (2011) using OPA (o-phthalaldehyd) derivatisation. 800 mg of oven-dried (40 °C) maize residue powder were hydrolysed with 10 ml of 6 M HCl for 3 h at 105 °C. Chromatographic separations were performed on a Hyperclone C_{18} column (125 mm length × 4 mm diameter) at 35 °C, using a Dionex (Germering, Germany) P 580 gradient pump, a Dionex Ultimate WPS – 3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelengths. Fungal C was calculated by subtracting bacterial GlcN from total GlcN as an index for fungal residues, assuming that MurN acid and GlcN occur at a 1 to 2 molar ratio in bacterial cells (Engelking et al., 2007): mmol fungal C g^{-1} dry weight = (mmol GlcN − 2 × mmol MurN) × 9. Bacterial C was calculated as an index for bacterial residues by multiplying the concentration of MurN by 45 (Appuhn and Joergensen, 2006). Microbial residue C was estimated as the sum of fungal C and bacterial C.

DNA extraction from the maize residues was conducted as described by Brandfass and Karlovsky (2006). The amount of genomic DNA of *F. culmorum, F. graminearum* and *R. solani* in plant residues was determined by real-time PCR (Brandfass and Karlovsky 2008; Abbas et al. 2014).

5.2.4 Statistical analysis

The data presented in tables and figures are arithmetic means expressed on an oven-dry basis (about 24 h at 105 °C). Normality of distribution of the data was tested using the Kolmogorov-Smirnoff and Shapiro-Wilk test and data were transformed when appropriate. To test for treatment effects, a two-way analysis of variance was performed using a general linear model and inoculation treatments (non-inoculated, inoculated) as well as mean soil temperatures of the ST1, ST2 and ST3 plots as fixed factors. The significance of differences between the soil temperature treatments was tested by one-way analysis of variance (ANOVA) using post hoc Tukey HSD ($P \leq 0.05$). All statistical calculations were performed by SPSS Statistics 17.0 (SPSS Inc., Chicago, USA).

5.3 Results

5.3.1 Effects of soil heating on soil temperature and water content

Soil heating significantly increased temperature at 5 cm depth in both warming treatments ($P < 0.001$). From the end of October 2011 until the end of March 2012, differences in mean daily temperatures at 5 cm soil depth were 1.3 °C (± 0.5) and 2.7 °C (±0.4) between the unheated ST1 plots and the ST2 and ST3 plots, respectively (Fig. 1). Over the 152 days, mean soil temperature was 5.0 °C in the ST1 plots, 6.4 °C in the ST2 plots and 7.7 °C in the ST3 plots. Over the course of the field trial, 15 days with soil temperatures below 0 °C were recorded in the ST1 plots at 5 cm depth. Both soil warming treatments reduced the number of days with soil frost to 11 and 6 in the ST2 and ST3 plots, respectively. Additionally, heating of the surrounding soil significantly decreased the soil water content in the collars from 22% in the ST1 plots to an average of 16% in the ST2 and ST3 plots ($P < 0.05$).

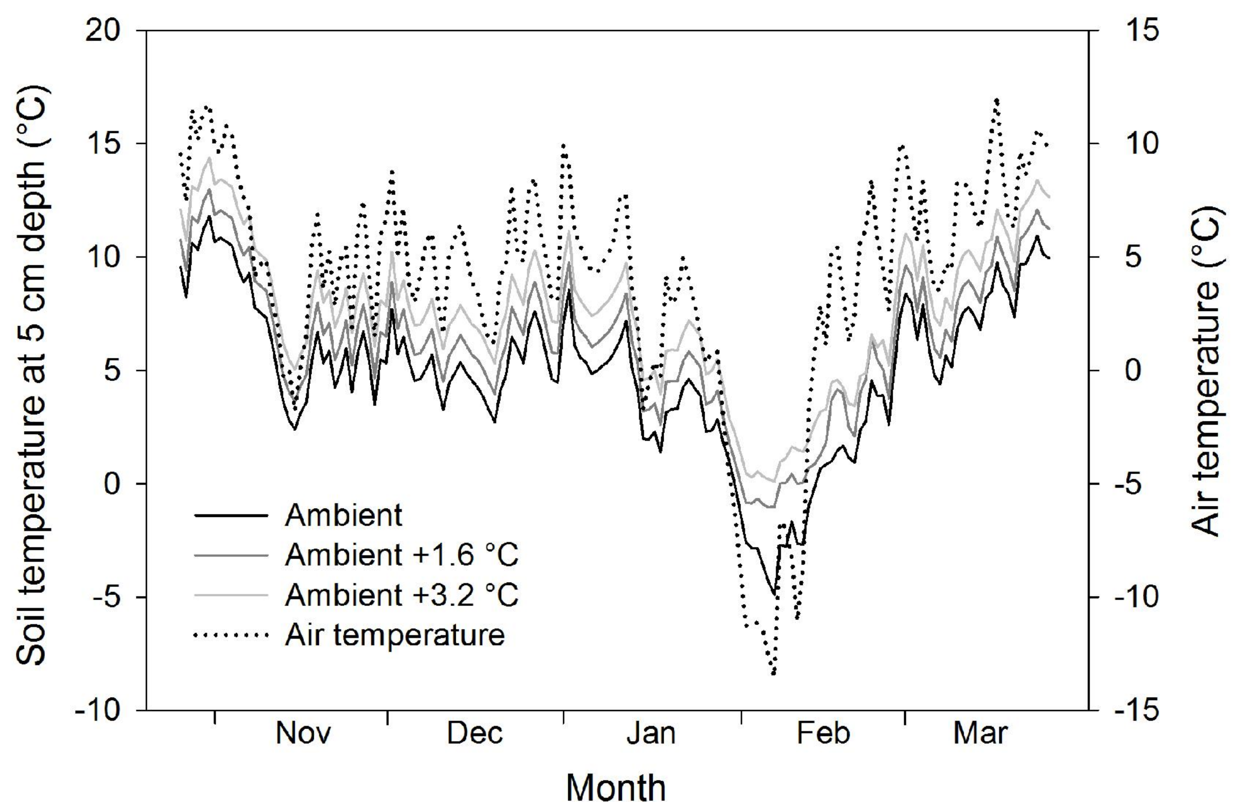

Fig. 12. Soil temperatures at 5 cm depth in the reference (ambient) and the two soil warming treatments (ambient+1.6 and ambient+3.2) in the field experiment compared with the air temperature based on daily mean temperatures.

5.3.2 Soil microbial biomass indices

The contents of soil microbial biomass C and biomass N at the end of the experimental period were very similar between all inoculation and soil temperature treatments and averaged 301 µg and 58.5 µg g^{-1} soil, respectively (Table 6). The average soil microbial biomass C/N ratio was 5.4. All samples contained around 0.33 µg ergosterol g^{-1} soil and had an average proportion of ergosterol to microbial biomass C of about 0.12%. For all soil microbial biomass indices, no significant effects of mean soil temperature, inoculation or temperature × inoculation interaction were found (two-way ANOVA).

Table 6. Contents of microbial biomass C and N, biomass C to biomass N ratio, ergosterol and ergosterol to biomass C at the end of the 152-day field experiment in the soil of the not-inoculated and inoculated litterbag samples of all temperature treatments.

Treatment	Microbial biomass			Ergosterol	Ergosterol / biomass C
	C	N	C/N		
	µg g^{-1} soil			µg g^{-1} soil	%
Not-inoculated					
+5.0°C	272	48	5.8	0.32	0.12
+6.4°C	293	82	4.6	0.35	0.14
+7.7°C	292	57	5.2	0.31	0.11
Inoculated					
+5.0°C	320	56	5.8	0.31	0.10
+6.4°C	314	54	5.7	0.36	0.12
+7.7°C	315	54	5.5	0.31	0.10
Probability values					
Temperature	n.s.	n.s.	n.s.	n.s.	n.s.
Inoculation	n.s.	n.s.	n.s.	n.s.	n.s.
T × I	n.s.	n.s.	n.s.	n.s.	n.s.
CV (±%)	14	21	13	20	19

N.S. = not significant (two-way ANOVA, $P > 0.05$, n = 24); CV = pooled coefficient of variation between replicates.

5.3.3 Decomposition of maize residues

In the non-inoculated and inoculated litterbags, around 52% and 57% of the maize residues were decomposed, respectively. A significantly stronger decomposition of about 9% was observed for pathogen-inoculated litterbags of the ST3 plots when compared with inoculated litterbags of the unheated ST1 plots. In addition, a significant 10% increase in decomposition over the respective non-inoculated maize residues was found for the pathogen-inoculated maize residues of the ST2 and ST3 plots ($P < 0.05$). Regardless of the soil heating treatments, this resulted in a maize decomposition rate of about 10.3 mg DW d^{-1} in non-inoculated and 11.3 mg DW d^{-1} in pathogen-inoculated litterbags. Only within the ST2 and ST3 plots, the decomposition rate of the pathogen-inoculated maize residues was significantly increased by about 1 mg d^{-1} over the non-inoculated litterbags ($P < 0.05$). Mean soil temperature ($P < 0.05$) and pathogen inoculation ($P < 0.001$) had significant effects on maize residue decomposition (two-way ANOVA).

5.3.4 Bacterial and fungal distribution

Total amino sugar contents increased from 1922 (ST1 plots) to 3031 (ST2 and ST3 plots) µg g^{-1} DW on non-inoculated maize residues and varied around 2112 µg g^{-1} DW in pathogen-inoculated litterbags (Table 7). Significant treatment effects were found for mean soil temperature and pathogen inoculation (two-way ANOVA, $P < 0.01$). The highest amino sugar contents found on the non-inoculated maize residues of the ST2 and ST3 plots were on average 36% higher than in the respective pathogen-inoculated litterbags.

GlcN was the most abundant amino sugar, on average accounting for 87% in all treatments. Highest GlcN and thus fungal C contents were found again in the non-inoculated litterbags of the ST2 and ST3 plots. Here, the values on maize residues of the ST3 plots were increased by about 47% over the respective pathogen-inoculated litterbags ($P < 0.05$). GlcN and fungal C were significantly affected by mean soil temperature, inoculation treatment as well as their interaction (two-way ANOVA, Table 7).

Bacterial MurN accounted for 1.5% of total amino sugars and varied around a mean of 36 µg g^{-1} DW, resulting in 1.64 mg bacterial C g^{-1} DW, without significant treatment effects (two-way ANOVA). However, one-way ANOVA within pathogen-inoculated maize residues revealed a significant increase of about 37% for the ST2 and ST3 plots over samples of the ST1 plots (Tukey HSD, $P < 0.05$). The fungal C to bacterial C ratio varied around 10.4 and significantly increased from 9.7 (ST1 plots) to 17.2 (ST3 plots) on maize residues without pathogen inoculation. Again, the fungal C to bacterial C ratio was significantly wider in the

non-inoculated litterbags of the ST3 plots than in the respective pathogen-inoculated litterbags ($P < 0.05$, Table 7).

On average, GalN contributed 10% to the total amino sugar content on non-inoculated and 6% on pathogen-inoculated maize residues. In both inoculation treatments, soil warming did not affect GalN. However, the GalN content in pathogen-inoculated maize residues was on average 47% lower than that in non-inoculated residues, leading to a significant inoculation effect (two-way ANOVA, $P < 0.001$).

5.3.5 Fungal plant pathogen inoculum

At the end of the experiment, inoculated maize residues were dominated by *Fusarium culmorum* in all temperature treatments (95% in ST1, 98% in ST2 and 91% in ST3). The amount applied with macroconidia was 0.49 ng mg^{-1} DW and averaged 2390 ng mg^{-1} DW after 152 days, without significant differences between soil heating levels (Fig. 13a). However, in both soil-warming treatments, *F. culmorum* DNA on maize residues decreased by about 13% in comparison with the non-heated control plots. The amount of *Fusarium graminearum* DNA applied with macroconidia was 0.4 ng mg^{-1} DW. After 152 days, *F. graminearum* showed the lowest distribution (0.7% in ST1, 1.2% in ST2 and ST3), and a significant increase with rising soil temperatures was found (Fig. 13b). In both soil warming treatments, the amount of *F. graminearum* DNA increased by 39 (ST2) and 111% (ST3) in comparison with the ST1 plots (15.0 ng mg^{-1} DW), leading to a strong positive correlation with mean soil temperature ($r = 0.91$, $P = < 0.0001$, Pearson correlation). About 427 ng of *Rhizoctonia solani* DNA mg^{-1} DW were applied as a mixture of hyphae and sclerotia. Of this, nearly 70% were lost throughout all temperature treatments (Fig. 13c). The recovery of *Rhizoctonia solani* DNA decreased in the order ST3 (54%) > ST1 (30%) > ST2 (7%).

Table 7. Concentrations of total amino sugars, muramic acid (MurN), glucosamine (GlcN), galactosamine (GalN), fungal C, bacterial C and the fungal C to bacterial C ratio of the inoculated and not-inoculated maize residues of all temperature treatments at the end of the 152-day field experiment.

Treatment	Total amino sugars	GalN	GlcN	MurN	Fungal C	Bacterial C	Fungal C/ bacterial C
	($\mu g\ g^{-1}$ DW)				($mg\ g^{-1}$ DW)		
Not-inoculated							
ST1	1922 b	259 a	1582 b	34.5 a	13.6 b	1.6 a	9.7 b
ST2	2953 a	246 a	2580 a	54.4 a	22.7 a	2.4 a	11.5 ab
ST3	3109 a	264 a	2715 a	31.7 a	23.8 a	1.4 a	17.2 a
Inoculated							
ST1	1854 a	120 a	1609 a	26.1 b	14.0 a	1.2 b	12.0 a
ST2	2378 a	134 a	2126 a	35.1 a	18.5 a	1.6 a	11.7 a
ST3	2103 a	151 a	1842 a	36.6 a	15.9 a	1.6 a	9.7 a
Probability values							
Temperature	0.001	n.s.	<0.001	n.s.	<0.001	n.s.	n.s.
Inoculation	0.002	<0.001	0.003	n.s.	0.003	n.s.	n.s.
T × I	n.s.	n.s.	0.03	n.s.	0.02	n.s.	0.03
CV (±%)	12	27	12	23	12	23	23

Different letters within a column indicate significant differences within inoculation treatments ($P < 0.05$, Tukey HSD); DW = dry weight; n.s. = not significant (two-way ANOVA, $P > 0.05$, n = 24); CV = pooled coefficient of variation between replicates.

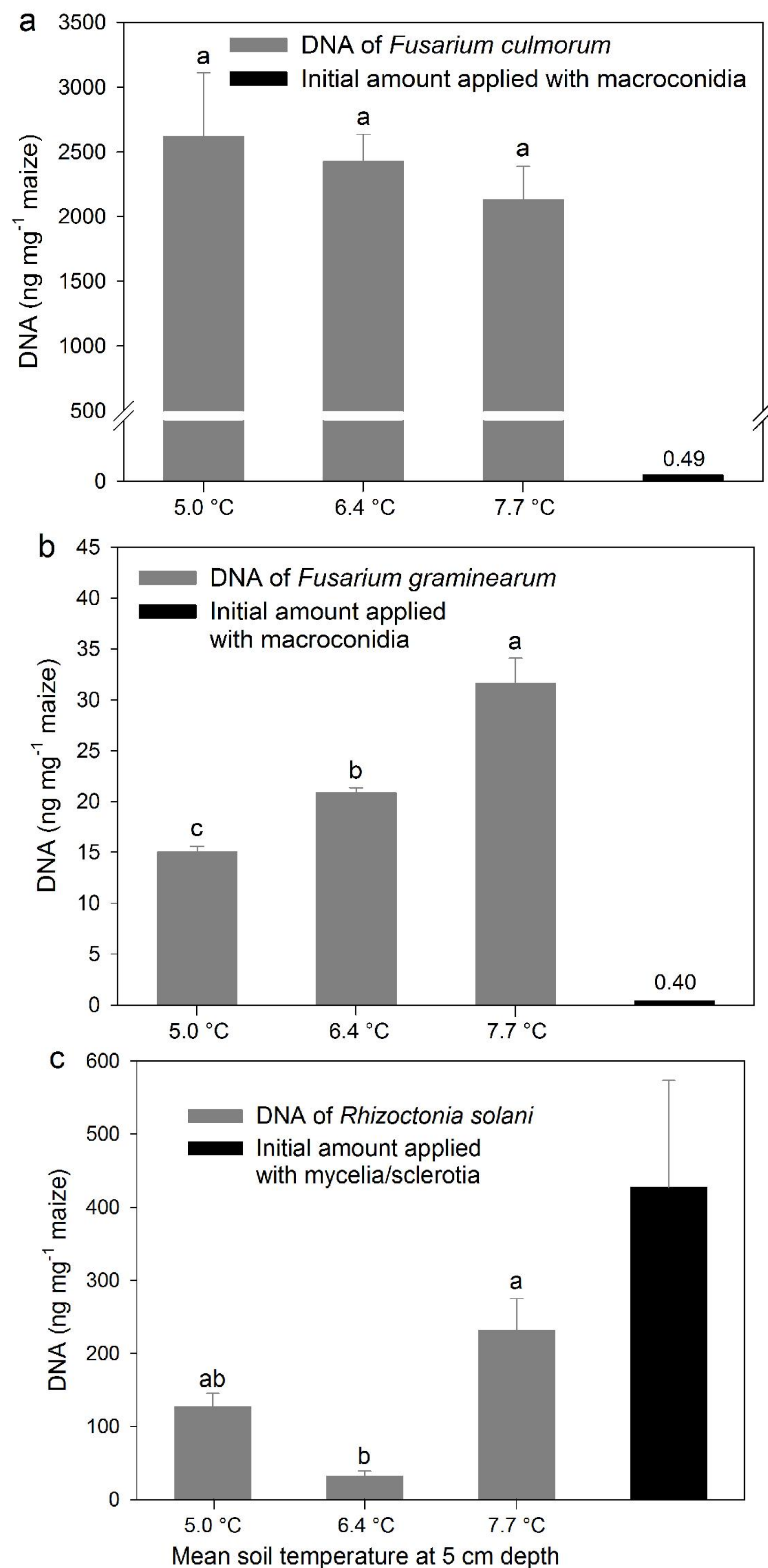

Fig. 13a-c. Amounts of DNA of *F. culmorum* (a), *F. graminearum* (b) and *R. solani* (c) of the inoculated maize leaf residues at the end of the 152-day field experiment; error bars show standard error of the mean; different letters above the columns indicate significant differences ($P < 0.05$, Tukey HSD).

5.4 Discussion

5.4.1 Temperature effects on decomposition and bacterial and fungal colonisation

More than 50% of the maize residues was decomposed after 152 days under field conditions. The resulting average decomposition rate of about 11 mg DW d^{-1} is similar to decomposition rates of wheat straw reported by others (Bowen 1990; Pereyra et al. 2004), ranging between 2 and 38 mg DW d^{-1}. In non-inoculated litterbags, increasing soil temperatures did not accelerate the decomposition but significantly increased the degree of fungal colonisation. In contrast, decomposition significantly increased in the ST3 plots by pathogen inoculation in comparison with the ST1 plots and with the non-inoculated litterbags of both warming treatments. There was no relationship between pathogen inoculation and fungal colonisation at the end of the experiment.

Non-inoculated green maize leaves showed a low microbial colonisation. The initial amount of fungal GlcN as a biomarker for fungal tissue was 49.4 µg g^{-1}, which is similar to the value found by Indorf et al. (2011) but considerably lower than calculated amounts of fungal C reported by Rottmann et al. (2011) on maize residues. In contrast, MurN was below the limit of quantification and not detectable on the original material. At the end of the field experiment, total amino sugars as an indicator for microbial residues on both pathogen-inoculated and non-inoculated maize residues generally had very large proportions of fungal GlcN (on average 84%), which were very similar between all treatments. The low overall amounts of muramic acid found after 152 days indicate reduced bacterial growth. This demonstrates the fungal dominated colonisation and decomposition of plant residues. Especially in winter, fungal dominated microbial communities have been reported by Lipson et al. (2002) and Schadt et al. (2003).

In the present experiment, the increase in total amino sugars in non-inoculated maize residues was significantly correlated with increasing mean soil temperature ($r = 0.70$, $P < 0.05$). The same was true for GlcN as an indicator for fungal tissue and fungal growth ($r = 0.74$, $P < 0.01$) but not for MurN, which is of bacterial origin. In contrast, in the pathogen inoculated maize residues a significant correlation with mean soil temperature was found for MurN ($r = 0.84$, $P < 0.01$) but not for fungal GlcN. Thus, these results suggest that either growth of the indigenous litter fungi or the colonisation by soil-derived fungi was promoted by increasing soil temperatures. Generally, the contents of GlcN and MurN were far more variable at each temperature on the non-inoculated than on the pathogen-inoculated maize residues, with coefficients of variation ranging from 4% to 60% (mean CV of 29%) and 2% to 10% (mean CV of 7%), respectively. This indicates that a large variation of natural microflora

populating plant residues may mask temperature effects and make comparisons with the decomposer community on pathogen-inoculated residues more difficult.

5.4.2 Development of fungal plant pathogens

Among the inoculated plant pathogens, *Fusarium culmorum* was most dominant and showed the greatest growth potential on aboveground maize residues during winter. This is similar to previous results of Lukas et al. (2014), investigating the survival of different plant pathogens on maize residues at temperatures around freezing point in a laboratory experiment. In the current study, the differences between the ST1 plots and the soil heating treatments were not significant, which might be due to the limited number of replicates per warming treatment and the resulting high variation between plots. Although there was no significant relationship between residue decomposition and pathogen distribution at the end of the experiment, the amount of DNA was decreased by 19% in ST3 plots, while the decomposition of maize residues in ST3 was significantly increased over ST1 plots. This indicates a possible link between decomposition of pathogen-infested crop residues and the amount of primary inoculum due to future soil warming in winter. Furthermore, Bowen (1990) reported a rapid decomposition of wheat residues inoculated with *F. culmorum* during the first three weeks, which gradually declined thereafter. It is known that *F. culmorum* can degrade cellulose and hemicelluloses (Harper and Lynch 1985) but is not able to degrade lignin (Bowen and Harper 1988). After the depletion of cellulose and hemicelluloses, *Fusarium* species seem to be replaced by other microorganisms, further degrading the residues (Hudson 1968; Bowen 1990). This would also explain the lower colonisation in the later stage of substrate decomposition at the end of the experiment, when major amounts of easily available nutrients were exhausted. Based on our results, neither an increase nor a significant decrease of *F. culmorum* primary inoculum on crop residues is to be expected due to future soil warming in winter for Lower Saxony. However, both the large amounts of pathogen DNA as well as the temperature-independent extended survival still point to an elevated disease potential for agricultural crops susceptible to *F. culmorum*.

In contrast to *F. culmorum*, *F. graminearum* is most common in warm and continental climates (Parikka et al., 2012) and the germination of conidia as well as the fungal growth is favoured by warm and humid conditions (Leplat et al. 2013). The optimum temperature for mycelial growth was found to be 25 °C, whereas no growth was observed at temperatures below 5 °C (Ramirez et al. 2006). In comparison with *F. culmorum*, the results of our study revealed a considerably lower ability of *F. graminearum* to colonize maize residues under

field conditions in winter, underlining its poor adaptation to low temperatures. It is also known that *F. graminearum* is a poor competitor for the colonisation and decomposition of crop residues over time, compared with other *Fusarium* species (Pereyra et al. 2004; Leplat et al. 2013). Pereyra et al. (2004) reported a low saprophytic fitness of *F. graminearum*, which is rapidly replaced from colonised residues by other competitors including other *Fusarium* species. However, in our experiment no correlation between the biomass of the two pathogens was found. Nevertheless, in comparison with the initial amount of DNA, pathogen growth was found in all temperature treatments, which strongly correlated with the mean soil temperature. Here, the colonisation significantly increased with soil warming and more than doubled in the ST3 plots (simulating a future soil warming of 2.7 °C), whereas the amount of *F. culmorum* DNA tended to decline. This might indicate that, as a result of future warming, increased soil temperatures and accelerated substrate decomposition may reduce the dominance of one pathogen and simultaneously improve conditions for development of another. Our results are also similar to findings of other authors reporting a decline of *F. culmorum* and an increased prevalence of *F. graminearum* in the Netherlands (Waalwijk et al. 2003) and in Denmark (Nielsen et al. 2011). Furthermore, in a simulation study of Volk et al. (2010) up to 2050, the projected disease infection risk increased for *Fusarium* head blight caused by *F. graminearum* in North Rhine-Westphalia (Germany). Although their study focused on simulated infection risk in summer, the increased primary inoculum found at the end of our winter field experiment also confirms the increased infection risk due to future climate warming.

Because sclerotia of *R. solani* only undergo myceliogenic germination (Coley-Smith and Cooke 1971), the disease potential depends on the germination of sclerotia in the soil or on plant residues and their ability to initiate the growth of hyphae (Coley-Smith and Cooke 1971; Ritchie et al. 2009). Environmental factors influence the persistence of *R. solani* sclerotia (Coley-Smith and Cook 1971; Dorrance et al. 2003; Grosch and Kofoet 2003; Chang et al., 2004; Harikrishnan and Yang 2004); soil temperatures between 15 °C and 18 °C are known to favour survival of sclerotia (Benson and Baker 1974). In soil, Ritchie et al. (2009) found an optimal temperature range for sclerotial germination between 15 and 25 °C, whereas germination was completely inhibited at 5 °C.

The amount of *R. solani* DNA found in inoculated maize residues after 152 days was greatly reduced compared with the initial inoculation, suggesting that the mixture of mycelia and sclerotia applied as inoculum was decomposed and used as a nutrient source by other microorganisms, particularly fungi. This assumption is supported by the fact that the highest amount of GlcN was observed in the soil warming treatment with the lowest amount of *R.*

solani DNA. It is also possible that fungi other than the *Fusarium* spp. used in this study grew at the expense of *R. solani*. Mycoparasites (e.g. *Verticillium biguttatum* and *Trichoderma virens*) are known to destroy sclerotia of *R. solani* (Jager et al. 1991; Demirci et al. 2009; Liu et al. 2010). *Trichoderma* species are able to hydrolyse fungal structures (e.g. conidia and mycelia) via the production of various enzymes (Cooney et al. 2001) and utilize fungal mycelium as a nutrient source (Popiel et al. 2008). Ritchie et al. (2013) reported that only 60% of buried sclerotia of *R. solani* could be retrieved after 18 months. They also observed a strong decrease in sclerotia viability (between 10% and 35% remained viable). In our study, based on quantification of DNA, between 7% and 54% of the applied sclerotia were found at the soil surface after five months. This indicates an increased degradation when sclerotia are directly exposed to environmental conditions.

5.4.3 Conclusions

Soil warming increased the degree of fungal colonisation on non-inoculated maize residues but did not accelerate decomposition. In contrast, substrate decomposition after pathogen inoculation increased with rising soil temperatures, which was not reflected by higher amounts of fungal residues. An increased decomposition of maize residues does not significantly reduce the pathogen load. Among the inoculated plant pathogens, *Fusarium culmorum* was most dominant and showed the greatest growth potential on maize residues. Neither an increase nor a significant decrease of *F. culmorum* primary inoculum on crop residues is to be expected due to future soil warming in winter for Lower Saxony. However, the temperature-independent extended survival of *F. culmorum* points to an elevated disease potential for susceptible agricultural crops. *Fusarium graminearum* revealed a considerably lower ability to colonize maize residues under field conditions in winter, but the strongly increased primary inoculum found at the end of our field experiment also indicates an increased infection risk due to future climate warming. Mycelia and sclerotia of *Rhizoctonia solani* were strongly decomposed and apparently used as a nutrient source by other microorganisms.

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6. Zusammenfassung

In dieser Arbeit wurde der mikrobielle Abbau von Ernterückständen (Maisresiduen) und damit verbundene Nährstoffflüsse unter dem Gesichtspunkt sich wandelnder Winterklimate bei Temperaturen um den Gefrierpunkt untersucht. Um diese nachzubilden, wurden in einem Kurzzeitexperiment 4 verschiedene Temperaturszenarien in Klimakammern simuliert. Die C- und N-Dynamiken wurden dabei durch Unterschiede im natürlichen ^{13}C-Isotopenverhältnis zwischen der SOM des verwendeten Bodens und der eingesetzten Maisstreu sowie einer künstlichen Anreicherung des Isotops ^{15}N im Mais bestimmt. Dabei wurde die Verlagerung des streubürtigen C und N in die Fraktionen CO_2-C, SOC, Gesamtstickstoff des Bodens, extrahierbarer C und N, C_{mic}, N_{mic}, sowie POM-C und POM-N betrachtet. Die eingesetzten Temperaturszenarien wurden ebenfalls verwendet, um den Einfluss von Wintertemperaturen um 0°C oder von Frost-Tau-Ereignissen auf die Überlebensrate und Entwicklung bodenbürtiger, pilzlicher Phytopathogene zu untersuchen. Dieser Versuchsansatz wurde anschließend auf einer Bodenerwärmungsanlage im Feldversuch wiederholt, um die Überlebensrate der verwendeten Pathogene bei steigenden Bodentemperaturen im Winter unter natürlichen Bedingungen zu bestimmen.

Im ersten Experiment (Kapitel 3) konnte ein signifikanter Abbau der Maisresiduen bei variierenden Temperaturen um den Gefrierpunkt nachgewiesen werden. Dabei begünstigten konstante Taubedingungen mit Temperaturen knapp über 0 °C den mikrobiellen Substratabbau und führten zu einer steigenden C-Mineralisation. Es zeigte sich, dass die Gesamtmenge des in Form von CO_2 mineralisierten Kohlenstoffes nicht durch die Häufigkeit der Frost- und Tauereignisse, sondern durch die Gesamtzahl der Frost- bzw. Tautage reguliert wird. Außerdem wurde durch den mikrobiellen Abbau der Maisresiduen in allen Temperaturszenarien zusätzlicher Kohlenstoff aus der organischen Bodenmaterie mineralisiert, d.h. in Form von CO_2 veratmet, sowie durch häufiges Gefrieren und Tauen des Bodens auch signifikant in die mikrobielle Biomasse eingelagert. Dieser allgemein als Priming-Effekt bezeichnete Prozess wurde in der vorliegenden Arbeit erstmals bei Temperaturen um den Gefrierpunkt festgestellt und hatte einen zusätzlichen Verlust des bodenbürtigen C und N durch den mikrobiellen Abbau eingearbeiteter Pflanzenreste zur Folge. Eine mögliche Ursache ist hierbei, dass die von den Mikroorganismen im fortgeschrittenen Stadium der Zersetzung des Maisstrohs gebildeten Exoenzyme auch teilweise am Abbau der organischen Bodenmaterie beteiligt sind. Die Bereitstellung zusätzlicher Nährstoffe aus Maisresiduen vergrößert die mikrobielle Biomasse des Bodens. Verglichen mit konstanten Taubedingungen zeigten dabei weder Dauerfrost, noch häufige

Frost- und Tauereignisse einen negativen, d.h. verringernden Effekt. In Boden ohne zusätzliche Maisresiduen wurde die höchste pilzliche Biomasse nach Dauerfrost festgestellt. Dies ist möglicherweise auf einen reduzierten Umsatz, d.h. einer Akkumulation des Zellmembranbestandteils Ergosterol bei Temperaturen unter 0 °C zurückzuführen. Nach der Einarbeitung der Pflanzenreste wurde der höchste Gehalt saprotropher Pilze nach einem einzelnen Frost-Tau-Ereigniss gemessen. Große Mengen des maisbürtigen N wurden sowohl durch konstante Taubedingungen, als auch durch Frost- und Tauereignissen in den Gesamtstickstoff des Bodens verlagert.

In einem zweiten Netzbeutel-Laborversuch (Kapitel 4) wurden die Auswirkungen variierender Wintertemperaturen um 0 °C auf den Abbau von Maisresiduen, welche mit pilzlichen Pflanzenpathogenen (*Fusarium culmorum*, *Fusarium graminearum* sowie *Rhizoctonia solani*) infiziert wurden, untersucht. Gleichzeitig wurde die Überlebensrate und das damit verbundene Schadpotential der Pathogene bestimmt. Es konnte gezeigt werden, dass bei konstant milder Temperatur (+4 °C) ein 6fach höherer Substratabbau sowie 12fach erhöhte C-Mineralisationsraten auftreten, als bei Klimaverläufen mit Dauerfrost (-3 °C). Die Pathogeninokulation hatte dabei, verglichen mit nicht-inokulierten Maisresiduen, keine Effekt auf die C-Mineralisation sowie den Streuabbau, führte aber zur höchsten gemessenen Menge an Glucosamin. Dies deutet auf eine höhere Substratnutzungseffizienz der eingesetzten pilzlichen Pathogene hin. Trotz stark limitierter mikrobieller Aktivität hatte der Pathogenbefall bei Dauerfrost signifikante Auswirkungen auf die Substratnutzung. Im Gegensatz zu unbehandelten Maisresiduen waren hier die C-Mineralisation um 135% und der Substratabbau um 336% erhöht. Frost-Tau Ereignisse begünstigen den mikrobiellen Substratabbau sowie die C-Mineralisation ebenfalls deutlich, jedoch in weit geringerem Maße, als konstant milde Temperaturen. Häufige Frost-Tau Ereignisse verringern hierbei außerdem sowohl die CO_2 Freisetzung, als auch den Streuverlust im Vergleich zu einer andauernden, zeitlich aber begrenzten Frostperiode mit vorausgehender und anschließender Warmphase signifikant um 35 bzw. 16%. Sowohl der Substratabbau, als auch die C-Mineralisation waren in den Frost-Tau Szenarien durch den Pathogenbefall im Vergleich zu unbehandeltem Substrat signifikant um 25 bzw. 52% erhöht. Von den zur Inokulation verwendeten Phytopathogenen zeigte sich *F. culmorum* bei einer 3fach Inokulation (zusammen mit *F. graminearum* und *R. solani*) am konkurrenzstärksten. Bei konstant milden Temperaturen (+4 °C) konnte das größte Schadpotential von *F. culmorum* nachgewiesen werden. Die gemessene Menge an Pathogen-DNA lag 1776fach über dem Wert der Erstinokulation und sogar über 2000fach höher, als die gefundene DNA Menge bei

Dauerfrost. Die Ergebnisse der Frost-Tau Szenarien zeigen ebenfalls ein signifikantes Wachstum von *F. culmorum* im Vergleich zur Erstinokulation. Die gemessenen DNA Mengen waren jedoch durchschnittlich 70% niedriger als bei konstant milden Temperaturen. Dabei hatte scheinbar nicht die Häufigkeit der Frost-Tau Ereignisse, sondern die Gesamtfrostdauer einen Einfluss auf das Pathogeninokulum. Die höchsten Mengen an *F. graminearum* DNA wurden ebenfalls bei konstant milden Temperaturen gefunden. Diese lagen bei einer Einzelinokulation 55fach, bei einer 3fach Inokulation mit *F. culmorum* und *R. solani* jedoch nur 4,6fach höher als die Ausgangsmenge. Es ist daher anzunehmen, dass *F. graminearum* weniger konkurrenzstark ist und ein Teil seiner Biomasse von *F. culmorum* als C-Quelle genutzt wurde. Nach 70 Tagen Dauerfrost konnte kein Inokulum von *F. graminearum* auf dem Substrat nachgewiesen werden, was auf eine Degeneration der DNA schließen lässt. Frost-Tau Szenarien führten ebenfalls zu einem Rückgang der Pathogen DNA um 60% im Vergleich zur Erstinokulation. *R. solani* als Auslöser der "Späten Rübenfäule" konnte nach 70 Tagen ebenfalls in allen Temperaturszenarien auf dem Substrat wieder gefunden werden. Bei konstant milden Temperaturen lag die gemessene Menge an DNA bei einer 3fachen Inokulation mit *F. culmorum* und *F. graminearum* jedoch 80% unter dem Wert der Erstinokulation und war bei einer Einzelinokulation sogar um 95% reduziert. Die Anwendung von Dauerfrost sowie Frost-Tau-Ereignissen verringerte die Pathogen DNA ebenfalls um 97 bzw. 88%. Da der Schaderreger in Form von Dauerstadien, sog. Sklerotien, auf das Substrat aufgebracht wurde, ist davon auszugehen, dass die Biomasse des Erstinokulates sowohl von *F. culmorum* / *F. graminearum*, bei Einzelinokulation aber auch von anderen Mikroorganismen abgebaut wurde. Es ist davon auszugehen, dass konstante Temperaturen über 0 °C die Pathogenbelastung von Ernterückständen erhöhen, während Frost und Frost-Tau-Ereignisse die Kontamination limitieren und den Infektionsdruck verringern.

Im dritten Versuch (Kapitel 5) wurden von Oktober 2011 bis März 2012 Netzbeutel mit Maisresiduen auf einer Bodenerwärmungsanlage ausgebracht und mit den oben bereits erwähnten Schaderregerpilzen beimpft. Netzbeutel mit nicht beimpfter Maisstreu wurden ebenso ausgelegt. Teilparzellen der Anlage wurden durch Heizkabel im Boden auf +1,6 °C bzw. +3,2 °C über der Temperatur in unbeheizten Referenzparzellen erwärmt. Dies verringerte die Anzahl der Tage mit Bodenfrost von 15 auf 11 bzw. 6. Im Durchschnitt wurde in allen Varianten mehr als 50% des eingesetzten Substrates abgebaut. Nur bei einer Erwärmungsrate von +3,2 °C wurde in den pathogen-beimpften Netzbeuteln ein höherer Substratabbau von ca. 9% im Vergleich zu Netzbeuteln der nicht erwärmten Parzellen festgestellt. Dies war jedoch nicht mit dem Grad der pilzlichen Besiedelung korreliert. Es

wurde eine signifikant negative Korrelation zwischen der Pathogeninokulation und Glucosamin festgestellt, was in den erwärmten Parzellen gegenüber nicht-inokulierten Maisresiduen zu 25% geringeren Mengen an pilzlichem C führte. Die Entwicklung von *F. graminearum* wurde in beiden Erwärmungsszenarien gegenüber den Referenzparzellen signifikant positiv beeinflusst. Ein Temperaturanstieg um 1,3 °C führte zu einem Anstieg der Pathogen DNA um ca. 39%, eine Erwärmung des Bodens um 3,2°C sogar um 111%. Die Ergebnisse zeigen jedoch eine nur sehr geringe Fähigkeit von *F. graminearum*, Maisresiduen unter Feldbedingungen im Winter zu besiedeln. Im Gegensatz dazu wurde das Wachstum von *F. culmorum* durch die Bodenerwärmung leicht, jedoch nicht signifikant gehemmt. Gegenüber den unbeheizten Referenzparzellen war die gefundene Menge an Pathogen DNA in beiden Erwärmungsvarianten um durchschnittlich 13% reduziert. Dabei war die DNA Menge signifikant negativ mit dem Substratabbau korreliert, was auf eine Nährstofflimitierung hindeutet. Unabhängig vom Temperaturszenarium zeigt der Anstieg der DNA Menge am Ende des Versuches gegenüber der Erstinokulation um den Faktor $4,8 \times 10^3$ jedoch deutlich das enorme Potential von *F. culmorum*, Maisresiduen über die Wintermonate zu besiedeln. Von der aufgebrachten Menge an Sklerotien von *R. solani* konnten nach 152 Tagen unabhängig von der Temperaturbehandlung nur noch zwischen 7 und 54% als DNA nachgewiesen werden. Parallel zum Laborgefäßversuch ist auch hier anzunehmen, dass ein Großteil der Biomasse von den anderen Pathogenen abgebaut wurde. Es kann somit davon ausgegangen werden, dass speziell mit einem erhöhten Auftreten von Getreidekrankheiten zu rechnen, welche durch *Fusarium culmorum* hervorgerufen werden. Eine durch den Klimawandel verursachte Erwärmung des Bodens kann zu einer stärkeren Verbreitung von *Fusarium graminearum* führen.

7. Summary

In the present thesis, microbial decomposition of maize residues as well as associated nutrient fluxes under changing winter climate temperatures around the freezing point were investigated. For that, four different winter temperature scenarios were simulated in climate cabinets. In order to follow C and N dynamics, the natural $\delta^{13}C$ value of maize straw, which is usually different from that of soil organic matter, was combined with an artificial enrichment in ^{15}N. The fate of straw-derived C and N was studied by measuring CO_2-C, SOC, total soil N, extractable C and N, microbial C and N as well as POM-C and POM-N. The same temperature scenarios were used to investigate the effects of constant winter temperatures around 0 °C and freeze-thaw cycles on survival and development of soil borne fungal plant pathogens. In a subsequent soil temperature manipulation study, the survival and development of fungal plant pathogens at elevated soil temperatures was examined under field conditions.

In the first experiment (chapter 3), a significant decomposition of maize residues at temperatures around the freezing point was observed. Thereby, a constant +4 °C was most favourable for microbial substrate decomposition leading to an increased C mineralization. The cumulative maize mineralization was not determined by the frequency of freeze-thaw events, but regulated by the overall time of frost and thaw conditions. Additionally, the decomposition of maize straw significantly increased soil organic C mineralization (in all scenarios) and incorporation into microbial biomass (in the freeze-thaw scenarios only). So far, this so called priming effect has not been reported at temperatures around the freezing point and lead to an additional loss of total soil organic C and N as a result of microbial decomposition of a complex organic substrate. A possible explanation is that the extracellular enzymes generated by saprotrophic fungi to degrade the added maize residues at the later stage of decomposition are to some extent efficient in decomposing SOM. Microbial biomass was significantly increased after maize straw amendment, with constant frost and freeze-thaw scenarios not having any negative effect compared with constant +4 °C. Highest fungal biomass was found after constant frost without fresh substrates and also after extended frost followed by a warm period when fresh plant residues were present. A reasonable explanation is that the turnover of ergosterol was reduced at constant frost, leading to an accumulation of this cell-membrane component. Large amounts of the added maize N were recovered in the soil total N at constant 4 °C and in the freeze-thaw scenarios, with the strongest effect after single freezing and thawing.

In a second experiment (chapter 4) using litterbags, the impact of changing winter temperature regimes around 0 °C on decomposition of pathogen-infected (*Fusarium culmorum*, *Fusarium graminearum*, *Rhizoctonia solani*) maize residues was investigated. Additionally, the survival and disease potential of the inoculated fungal plant pathogens was determined. Results show an increased decomposition of the maize residues and a higher C mineralization at constant temperatures above 0 °C in comparison with constant frost. Fungal infection at constant 4 °C did not accelerate substrate decomposition or C mineralization compared with non-inoculated maize residues, but led to the highest amount of glucosamine, indicating higher substrate use efficiency. Although microbial activity was strongly limited at constant frost, the pathogen inoculation significantly increased C mineralization and maize residue decomposition by about 135 and 336%, respectively, compared with non-inoculated samples. Freezing and thawing also promoted the microbial substrate decomposition and C mineralization, but to a lesser extend then constant 4 °C. Thereby, multiple freeze-thaw cycles decreased both CO_2 evolution and substrate loss by 35 and 16%, respectively, compared with a single prolonged frost event between two warm periods. The pathogen inoculation significantly increased C mineralization as well as decomposition of the maize residues in the freeze-thaw cycle treatments compared with non-inoculated litterbags. Among the inoculated pathogens, *F. culmorum* was most competitive. Continuous +4 °C strongly increased the pathogen load of *F. culmorum*, while no significant increase was found under continuous frost. Increased amounts of the pathogen DNA were also found in both freeze-thaw cycle treatments. However, those were on average 70% lower in comparison with constant 4 °C. The results suggest that the pathogen growth was not regulated by the freeze-thaw frequency but the overall time of frost and thaw conditions. The highest amount of *F. graminearum* DNA was also observed at constant 4 °C, which was only 4.6 times higher than the initial amount when inoculated with the other pathogens, but on average 55-fold increased when inoculated individually. This suggests that *F. graminearum* is less competitive and part of its biomass was used as a nutrient source by *F. culmorum*. No DNA of *F. graminearum* was found at the end of the continuous frost scenario. Freezing and thawing events reduced the amount of DNA by 60% compared with the initial inoculation. *R. solani*, the causal agent of root rot in sugar beet was also recovered on the maize residues at the end of the experiment. However, the pathogen DNA was strongly reduced in all treatments indicating that the sclerotia were decomposed by other microorganisms. Therefore, constant warm conditions can increase the pathogen load of crop residues, whereas frost and freeze-thaw events reduce the contamination and diminish the risk of crop infection.

In the third experiment (chapter 5) using litterbags, maize residues inoculated with *F. culmorum*, *F. graminearum* and *R. solani* were placed on a soil temperature manipulation facility form October 2011 to March 2012. Non-inoculated maize residues served as controls. Soil warming increased mean temperatures at 5 cm depth by 1.3 and 2.7 °C and decreased days of soil frost from 15 to 11 and 6, respectively. In general, more than 50% of the initial maize straw was decomposed in all treatments. Rising soil temperatures increased the decomposition of the pathogen infected maize straw by about 9% in the +3.2 °C plots only, which was not correlated to the degree of fungal colonization. A significant negative correlation between the inoculation and glucosamine was observed, resulting in about 25% lower amounts of fungal C after soil warming, compared with non-inoculated maize straw. The development of *F. graminearum* was significantly positive affected due to soil warming, with an increase of the pathogen DNA of about 39 (+1.6 °C) and 111% (+3.2 °C) being found. However, the results revealed a considerably lower ability of *F. graminearum* to colonize maize straw under field conditions in winter. In contrast, due to soil warming growth of *F. culmorum* was slightly but not significant decreased by about 13% in comparison with the control plots. Thereby, a significant negative correlation between the pathogen DNA and substrate decomposition was observed, indicating a nutrient limitation. Nevertheless, the highest overall amount of *F. culmorum* DNA found in all treatments clearly demonstrates the great growth potential on maize residues during winter. The amount of *R. solani* DNA found on the inoculated maize residues after 152 days was greatly reduced compared with the initial inoculation, suggesting that the mixture of mycelia and sclerotia applied was decomposed and used as a nutrient source by other microorganisms, particularly fungi. The temperature independent extended survival of *F. culmorum* can increase the disease potential for agricultural crops. Neither an increase nor a significant decrease of *F. culmorum* primary inoculum on crop residues is to be expected due to future soil warming in winter for Lower Saxony. The strongly increased primary inoculum of *Fusarium graminearum* found at the end of the field experiment also indicates an increased infection risk due to future climate warming.

8. Schlussfolgerungen und Ausblick

In der vorliegenden Arbeit konnte gezeigt werden, dass wechselnde Frost- und Tauereignisse infolge abnehmender Niederschläge im Vergleich zu konstant milden Bedingungen, die unter einer geschlossenen Schneedecke im Winter vorherrschen können, eine mittlere Mineralisation von Ernterückständen zur Folge haben. Dabei ist jedoch nicht die Frost-Tau-Häufigkeit von Bedeutung. Die mikrobielle Aktivität und der damit verbundene Abbau pflanzlicher Residuen werden durch die Gesamtzahl der Frost- bzw. Tautage reguliert. Im Gegensatz dazu werden sowohl das Wachstum der mikrobiellen (einschließlich pilzlicher) Biomasse, als auch die Assimilation des streubürtigen C und N durch häufiges Gefrieren und Tauen des Bodens verringert. Steigende Temperaturen sind mit einer Verschiebung des mikrobiellen Substratnutzungsverhaltens von organischem Bodenkohlenstoff zur Mineralisation des Kohlenstoffes pflanzlicher Rückstände verbunden. Dabei ist auch bei Temperaturen um den Gefrierpunkt mit einem zusätzlichen Abbau von organischer Bodenmaterie zu rechnen, was zu sogenannten Priming-Effekten und einem weiteren Verlust an bodenbürtigem C und N führt. Dies sollte bei der Modellierung organischer Kohlenstoffvorräte in Böden berücksichtigt werden. Weiterhin konnten große Mengen des streubürtigen Stickstoffes im Gesamtpool des Bodenstickstoffs wiedergefunden werden, wovon gerade nach Frost- und Tauereignissen ein nicht unerheblicher Teil als leicht extrahierbar einzustufen ist und durch Auswaschung verloren gehen kann. Fortführende Untersuchungen des mikrobiellen Abbaus von Ernterückständen bei niedrigen Temperaturen sollten die Analyse von spezifischen mikrobiellen Biomarkern wie Aminozucker oder Phospholipid-Fettsäuren einschließen, um Veränderungen in der Zusammensetzung der mikrobiellen Gemeinschaft bestimmen zu können. Dabei kann der Einsatz von Isotopen (^{13}C, ^{15}N) Aufschluss über die am Abbau beteiligten Organismengruppen geben. Hierbei wäre auch eine Messung von Enzymaktivitätsprofilen über multiple Substrat-Enzym-Assays als ein Indikator für die mikrobielle funktionale Diversität hilfreich. Da der Abbau von organischer Materie nicht nur durch Temperatur und Wasserverfügbarkeit, sondern auch durch die chemische Zusammensetzung pflanzlicher Rückstände (C-N Verhältnis) und die Bodentextur beeinflusst wird, sollten verschiedene Pflanzenresiduen und Böden mit unterschiedlichen Sand- und Tongehalten zum Einsatz kommen.

Die vorliegenden Ergebnisse zeigen einen durch Pilze dominierten Abbau von Maisrückständen bei Wintertemperaturen um den Gefrierpunkt. Es wurde eine hohe Frosttoleranz von *Fusarium culmorum* sowie eine hohe Konkurrenzfähigkeit gegenüber *Fusarium graminearum* festgestellt. Sowohl im Labor, als auch unter Freilandbedingungen

wurde eine starke Besiedelung von Maisrückständen durch *F. culmorum* im Winter nachgewiesen. Deshalb ist mit hohen Infektionsraten und einem verstärkten Auftreten von Pflanzenkrankheiten, die durch *F. culmorum* hervorgerufen werden, zu rechnen. Dem sollte durch die Wahl entsprechender Fruchtfolgen, z. B. einem mehrjährigen Anbau resistenter oder Nicht-Wirtspflanzen entgegengewirkt werden. Unter konstant milden Bedingungen mit Wintertemperaturen um 4 °C führte eine Pathogeninfektion zwar nicht zu einem erhöhten Abbau der Maisresiduen oder einer verstärkten C-Mineralisation, jedoch zur stärksten Inokulumbelastung und dem höchsten Gehalt pilzlicher Residuen. Dies ist ein Anzeichen für eine höhere Substratnutzungseffizienz der eingesetzten Pathogene. Im Vergleich zu konstanten 4 °C führten Frost- und Tauereignisse zu einem reduzierten Wachstum der Pathogene. Dabei wirkte eine einzelne, andauernde Frostperiode zwischen zwei Warmphasen weniger hemmend als häufiges Gefrieren und Tauen. Konstante Wintertemperaturen über 0 °C können die Pathogenbelastung von Ernterückständen erhöhen, während Frost- und Tauereignisse die Kontamination und damit den Infektionsdruck reduzieren. Unter Freiland-bedingungen führten steigende Bodentemperaturen im Winter zu einem Anstieg der pilzlichen Besiedelung auf nicht infizierten Maisrückständen, was deren Abbau jedoch nicht beschleunigte. Umgekehrt wurde der Abbau pathogen-infizierter Maisresiduen durch höhere Bodentemperaturen verstärkt, was jedoch nicht in einem höheren Pilzbesatz widergespiegelte. Von den zur Inokulation verwendeten Pathogenen zeigte *F. culmorum* die größte Dominanz und das stärkste Wachstumspotential auf Maisrückständen. *F. graminearum* zeigte ein deutlich geringeres Vermögen, Maisresiduen unter Freilandbedingungen im Winter zu besiedeln. Hierbei war das Wachstum signifikant mit ansteigenden Bodentemperaturen korrliert. Sklerotien von *Rhizoctonia solani* wurden in allen Behandlungen stark abgebaut und von anderen Mikroorganismen als Nährstoffquelle verwendet. Die Ergebnisse deuten darauf hin, dass veränderte Umwelt- und Habitatbedingungen die Dominanz eines Pathogens, hier speziell von *F. culmorum*, verringern und gleichzeitig verbesserte Bedingungen für die Entwicklung andere Pathogene schaffen können. Ein erhöhter Substratabbau kann jedoch nicht dazu beitragen, die Pathogenbelastung signifikant zu verringern. Die hohe Besiedlungsdichte sowie das Überdauern niedriger Temperaturen im Winter können eine Erhöhte Infektionsrate anfälliger Nutzpflanzen im Frühjahr zur Folge haben.

9. Literatur

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