# A secret world

## C and N rhizodeposition of pea, influenced by plant development and growing conditions

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*to Tino,*

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**Preface**

The thesis is submitted to the Faculty of Organic Agricultural Sciences to fulfill the requirements for the degree "Doktor der Agrarwissenschaften" (Dr. agr.) and was funded by the German Research Foundation (DFG). The dissertation is based on three papers as first author, which have been published in or submitted to international refereed journals. The manuscripts are included in chapters 2, 3 and 4. Chapter 1 comprises a general introduction to the research topic as well as the objectives of this thesis. Chapter 5 contains the overall conclusions and an outlook on future research needs. Supplementary materials are found in chapter 8.

The following papers are included in this thesis:

Chapter 2:

Hupe, A., Schulz, H., Bruns, C., Joergensen, R.G., Wichern, F., 2016a. Digging in the dirt – Inadequacy of belowground plant biomass quantification. Soil Biology and Biochemistry 96, 137-144.

Chapter 3:

Hupe, A., Schulz, H., Naether, F., Bruns, C., Haase, T., Heß, J., Joergensen R.G., Wichern, F., 2016b. Even flow? Changes of carbon and nitrogen release from pea roots over time. Soil Biology and Biochemistry (under revision).

Chapter 4:

Hupe, A., Schulz, H., Bruns, C., Haase, T., Heß, J., Joergensen R.G., Wichern, F., 2016c. Get on your boots: Estimating root biomass and rhizodeposition of peas under field conditions reveals the necessity of field experiments. Plant and Soil (submitted).

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**List of abbreviations**

| | |
|---|---|
| α | Significance level |
| AGP-C | Aboveground plant carbon |
| AGP-N | Aboveground plant nitrogen |
| ANOVA | Analysis of variance |
| AMF | Arbuscular mycorrhizal fungi |
| asl | Above sea level |
| BBCH | Uniform decimal code for growth stages of crops and weeds |
| BGP | Belowground plant |
| BGP-C | Belowground plant carbon |
| BGP-N | Belowground plant nitrogen |
| C | Carbon |
| Ca | Calcium |
| CdfR | Carbon derived from Rhizodeposition |
| $CHCl_3$ | Chloroform |
| $CO_2$ | Carbon dioxide |
| cf. | Compare |
| cv. | Cultivar |
| DAS | Days after sowing |
| DFG | Deutsche Forschungsgemeinschaft (German Research Foundation) |
| dfR | Derived from rhizodeposition |
| DM | Dry matter |
| e.g. | For example |
| FAO | Food and Agriculture Organization |
| Fig. | Figure |
| J&B | Janzen and Bruinsma |
| K | Potassium |
| $k_{EC}$ / $k_{EN}$ | Extractable part of the total amount of carbon ($k_{EC}$) and nitrogen ($k_{EN}$) bound in the microbial biomass |
| $K_2SO_4$ | Potassium sulfate |
| L. | Linné |
| M | mol |

| | |
|---|---|
| MB | Mass balance |
| MBC | Microbial biomass carbon |
| MBN | Microbial biomass nitrogen |
| Mg | Magnesium |
| $myc^-$ | Non-mycorrhizal |
| N | Nitrogen |
| n | Number of samples |
| NA | Not applicable |
| n.d. | Not determined |
| NdfR | Nitrogen derived from Rhizodeposition |
| $NH_3$ | Ammonia |
| $NH_4^+$ | Ammonium |
| $nod^-$ | Non-nodulating |
| $NO_3^-$ | Nitrate |
| P | Phosphorus |
| p | Probability value for significance |
| WRB | World Reference Base |

**Summary**

In many respects, an accurate knowledge of the carbon (C) and nitrogen (N) input through crops into soil is highly important. Estimation of the additional amount of nitrogen originating from rhizodeposition, helps to improve crop rotations, which are extremely important especially in organic farming. The preceding crop effects of cultures can be more accurately evaluated and ultimately the use of artificial nitrogen fertilizer in conventional farming or the use of organic fertilizer in organic farming can be reduced and costs can be saved. Moreover, a better knowledge of the amount of carbon emitted by plants and incorporated in the soil improves the estimation of the C sequestration potential of soils. With an increasing focus on global warming and the increase in greenhouse gases, the ability of soil to store $CO_2$ from the atmosphere has become increasingly important over the last few years.

Therefore, the importance of rhizodeposition is far-reaching and not only limited to farming. There are a vast number of studies dealing with the estimation of C and N rhizodeposition. It is remarkable that the definition of rhizodeposition often differs between these studies. An important aspect of the present thesis is to demonstrate that rhizodeposition consists of a wide range of compounds: root exudates, including ion and gaseous compounds, sloughed cells or tissue root fragments from root turnover. This broad definition of rhizodeposition allows a better assessment of the C and N input into soil through plants. To characterize the amount of C and N that is available for subsequent crops, the term of "belowground plant biomass" is more suitable.

To estimate rhizodeposition, stable ($^{13}C$, $^{15}N$) or in some cases also radioactive ($^{14}C$) isotopes were used. There are a lot of different methods for plant labelling, e.g. labelling roots or shoots of plants or labelling the atmosphere. Unlike the labelling method, the calculation of rhizodeposition is often similar between the studies. With few exceptions, rhizodeposition has been calculated with the same equation since 1989. This calculation approach requires a homogeneous and constant enrichment of roots and rhizodeposits. Therefore, the calculation is particularly vulnerable for tracer relocation within the plant.

In a first experiment, a mass balance approach was developed as an alternative way of calculating N rhizodeposition. A mass balance takes into account the complete tracer distribution and is not only focused on the enrichment of root and soil. Calculating the rhizodeposition with mass balance is less sensitive to tracer relocation processes. Nevertheless, a constant labelling is required to prevent an over- or underestimation of rhizodeposition. For calculating the N

rhizodeposition of pea (*Pisum sativum* L. cv. Frisson) with mass balance, data were used from a pot experiment, which was conducted in the greenhouse at the University of Kassel, situated at Witzenhausen. The peas were labelled with $^{13}C$ and $^{15}N$ using the cotton wick method. To achieve a continuous enrichment, labelling was started as early as possible. Plants were labelled 5 times over a period of 10 weeks. The N rhizodeposition, or belowground plant N (BGP-N), was calculated in two different ways, with a mass balance approach and the equation of Janzen and Bruinsma, which has been established for many years. In order to show the sensitivity of the two calculation methods to methodological differences, data from a former experiment were also used. In this pot experiment, peas were also labelled with $^{13}C$ and $^{15}N$ using the cotton wick method. However, the duration of labelling and the time between the last labelling date and harvest differs between the two studies. It was shown that the amount of BGP-N calculated with the mass balance approach was always lower. This demonstrates that the Janzen and Bruinsma equation, which focuses only on root and soil enrichment, can lead to an overestimation of rhizodeposition, especially when tracer relocation within the plant (due to plant growth) may have influenced root enrichment. With increasing duration of labelling, the results of both calculations became more similar.

The development of rhizodeposition depends on the development of plant root. Therefore, it can be assumed that the amount of rhizodeposition changes during plant development. Another study was carried out to estimate the C and N rhizodeposition over the course of time. In detail, it was to be examined whether the amount of rhizodeposition correlates with the root to shoot ratio of pea plants. Moreover, a possible positive influence of mycorrhiza on C and N rhizodeposition was examined. *Pisum sativum* L. cv. Frisson and the non-mycorrhizal ($myc^-$) and non-nodulating ($nod^-$) pea mutant of Frisson, P2, were cultivated under controlled conditions in the greenhouse. The soil was collected at 0-30 cm depth from the research station. For an approximating continuous labelling, the plants were labelled five times in ten weeks with $^{13}C$ and $^{15}N$ using the cotton wick method, beginning 14 days after sowing (DAS). All pots were inoculated with "rootgrowth$^{TM}$ professional", placed directly below the seeds to ensure arbuscular (AMF) symbiosis. Plants were harvested at four different growth stages, depending on plant development (begin of flowering, end of flowering, green ripe and maturity). At each sampling date, the enrichment of plant parts and soil, the microbial biomass, extractable C and the inorganic N pool of soil were determined. With this, the incorporation of rhizodeposition into the different soil pools could be estimated. The amount of C and N rhizodeposition was calculated with the mass

balance approach, developed in the first part of the thesis. No significant differences could be detected for the incorporation of C and N derived from rhizodeposition (CdfR and NdfR) into the microbial biomass or into the extractable soil pool over time, even though a steady increase was observed. Nevertheless, microbial incorporation of rhizodeposits was on a low level throughout, indicating that rhizodeposits are not an easily available source of C and N. At the time of highest mycorrhization, Frisson released significantly more NdfR than P2, indicating a stronger N transfer into the mycorrhizosphere. Despite a high N fertilization at the beginning of the experiment, Frisson formed nodels, which might be an additional reason for differences in NdfR between Frisson and P2. For CdfR, however, no significant AMF effects could be found. Our results indicate that CdfR and NdFR release from roots follow different patterns: an even flow of C, driven by exudation at an earlier growth stage and root senescence during maturation and a steady flow of N.

Quantification of the C and N rhizodeposition under field conditions is difficult, as labelling plants with stable or radioactive isotopes and separating labelled and unlabeled roots is difficult and often creates unnatural conditions. As a consequence, experiments are often done under controlled conditions. To estimate a realistic amount of C and N rhizodeposition, experiments have to be conducted under field conditions without influencing the root system or the water and nutrient budget. Therefore, an experiment was carried out with peas grown under field conditions. The spring pea (*Pisum sativum* L. cv. Santana) was manually sown in microplots and grew without restricted root growth. Similarly to the pot experiment, plants were labelled with $^{13}C$ and $^{15}N$ using the cotton wick method, beginning with 3 leaves unfolded.

It was crucially important to find a way of labelling peas without influencing plant development. Therefore, a multicarrier was built, which is able to drive over plant stock. The multicarrier is height-adjustable and allows easier labelling without damaging plants. To reach a homogeneous and constant enrichment of plant and rhizodeposits, labelling solution was refilled when plants had taken up the solution completely. Plant harvest and analysis of plant and soil was similar to the former pot experiment. Soil samples were taken in each microplot at 3 depths (0-30; 30-60; 60-90 cm depth). In order to calculate the complete root biomass of one pea plant, a second soil sampling always took place in three defined sectors in the microplot (directly on one plant; between two plants in the row; between four plants and two rows) at two depths (0-30 and 30-60 cm). Then, the soil was washed and the root dry matter of each soil sample was determined. With this the total amount of root could be calculated. The amount of C and N

rhizodeposition was estimated according to the mass balance approach. The careful labelling of plants resulted in homogeneous and high plant enrichment with $^{13}C$ and $^{15}N$. Therefore, despite the greater effort required, the cotton wick method is recommended for the labelling under field condition.

It was shown that the quantity of C and N rhizodeposition did not change between flowering and dry ripe of pea. Around one third of C and N rhizodeposition was integrated into microbial biomass and $K_2SO_4$ extractable C or the inorganic N pool of soil, indicating that rhizodeposits consist of substantial particular amounts, such as root residues or root fragments, which take longer to be mineralized. When comparing this field study with the former pot experiment (with the same harvest dates, the same labelling method and the same soil), a shift of the root-to-shoot ratio and the rhizodeposition-to-root ratio has to be mentioned. This was caused by a higher root development until flowering and a higher root turnover after flowering, under field conditions. Because of differences in plant development, caused by restricted root growth in pot experiments, the calculated amount of rhizodeposition from pot experiments cannot be transferred easily into the field. Therefore, if it was aimed to estimate the additionally amount of carbon and nitrogen, coming from root and rhizodeposition, the experiment has to be conducted under field conditions, without a restricted rootgrowth.

## Zusammenfassung

Eine genaue Kenntnis des C und N Eintrags, durch ackerbauliche Kulturen, in den Boden ist in vielerlei Hinsicht von großer Bedeutung. Das Schätzen der zusätzlichen Stickstoffmengen, welche durch Rhizodeposition der Folgefrucht zur Verfügung steht, hilft die, besonders für den Ökolandbau, so wichtigen Fruchtfolgen zu verbessern, Kulturen hinsichtlich ihrer Vorfruchtwirkung besser beurteilen zu können und schlussendlich Kosten zu sparen, da auf die zusätzliche Ausbringung von mineralischen Düngern im konventionellen bzw. organischen Düngern im Ökolandbau verzichtet werden kann. Ebenso verbessert eine genaue Kenntnis der Kohlenstoffmenge, welche von Pflanzen assimiliert und im Boden gebunden wird, die Einschätzung des C-Sequestrierungspotentials von Böden. Die Eigenschaft von Böden, $CO_2$ aus der Atmosphäre zu binden, hat im Zuge der Klimaerwärmung und des weltweiten Anstiegs der Treibhausgase, in den letzten Jahren immer mehr an Bedeutung gewonnen. Der Einfluss der Rhizodeposition ist somit vielfältig und betrifft nicht nur ackerbauliche Problemstellungen.

Es gibt zahlreiche Versuche, welche sich mit der Bestimmung der C und N Rhizodeposition befassen. Auffällig ist, dass sich in diesen Untersuchungen die Definition der Rhizodeposition oftmals unterscheidet. Daher ist ein Anliegen der vorliegenden Arbeit zu verdeutlichen, dass die Rhizodeposition neben Wurzelexudaten, Lysaten, gasförmigen Verbindungen und Ionen immer auch Wurzelhaare, Wurzelrandzellen und Wurzelfragmente, welche durch Wurzelabbau nicht mehr der lebenden Wurzel zugeordnet werden können, beinhaltet. Diese weitgefasste Definition von Rhizodeposition ermöglicht es, den Eintrag von C und N in den Boden besser bewerten zu können. Der Begriff der unterirdischen Pflanzenbiomasse, welcher sowohl die Wurzel, als auch die Rhizodeposition umfasst, ist oftmals am geeignetsten, um die Menge C und N zu beschreiben, welche Folgekulturen zur Verfügung steht.

Um Rhizodeposition bestimmen zu können, wird zumeist mit stabilen ($^{13}C$, $^{15}N$), in seltenen Fällen auch mit radioaktiven ($^{14}C$) Isotopen gearbeitet. Diese gelangen durch unterschiedliche Methoden in die Pflanze und können durch massenspektrometrische Messungen der Pflanzenproben bestimmt werden. Die bisherigen Untersuchungen unterscheiden sich zwar häufig in der Methode, welche zum Markieren der Pflanzen verwendet wird, jedoch kaum in der Berechnung der Rhizodeposition. Bis auf wenige Ausnahmen wird die Rhizodeposition seit 1989 mit der gleichen Formel berechnet. Diese Rechnung erfordert eine gleichmäßige und über die

Zeit konstante Anreicherung der Wurzel sowie der Rhizodeposite und ist daher stark anfällig gegenüber Verlagerungsprozessen innerhalb der Pflanze.

In der vorliegenden Arbeit wurde daher in einem ersten Versuch mit Hilfe eines Massenbilanzansatzes eine Alternative entwickelt, die N Rhizodeposition zu berechnen. Eine Massenbilanz betrachtet die gesamte Verteilung der aufgenommenen stabilen Isotope in der Pflanze und ist daher unempfindlich gegenüber Verlagerungsprozessen innerhalb der Pflanze. Zur Berechnung der N Rhizodeposition wurden Daten aus einem Gefäßversuch verwendet, welcher im Forschungsgewächshaus der Universität Kassel, am Standort Witzenhausen durchgeführt wurde. Es wurden Erbsen (*Pisum sativum* L. cv. Frisson) mit den stabilen Isotopen $^{13}C$ und $^{15}N$ mittels Dochtmethode markiert. Um eine kontinuierliche Anreicherung zu erreichen, wurde mit der Markierung im frühestmöglichen Stadium der Pflanzenentwicklung begonnen. Über einen Zeitraum von 10 Wochen erhielten die Pflanzen insgesamt fünfmal 1ml der Isotopenlösung. Die Rhizodeposition bzw. der Stickstoff der unterirdischen Pflanzenbiomasse (BGP-N) wurde mit einer Massenbilanz und mit der bereits etablierten Berechnung nach Janzen und Bruinsma berechnet und miteinander verglichen. Um die Empfindlichkeit der beiden Rechenwege gegenüber methodischen Unterschieden besser darstellen zu können, wurden Daten aus einem früheren Versuch hinzugezogen. Auch in diesem Gefäßversuch wurden Erbsen mit der Dochtmethode mit $^{13}C$ und $^{15}N$ markiert, allerdings waren der Zeitraum der Markierung sowie der Abstand der letzten Markierung zum Erntetermin verschieden. Es ließ sich feststellen, dass in beiden Versuchen die BGP-N Menge kleiner war, wenn sie mit dem Massenbilanzansatz berechnet wurde. Dies zeigt, dass es bei der ausschließlichen Betrachtung der angereicherten Pflanzenwurzel und des angereicherten Bodens (wie es bei der Berechnung nach Janzen und Bruinsma geschieht) zur Überschätzung der Rhizodeposition kommen kann, insbesondere wenn der Abstand der letzten Markierung zur Ernte sehr groß ist sowie Pflanzenwachstum und Verlagerungsprozess innerhalb der Pflanze, die Anreicherung der Wurzel beeinflussen. Je kontinuierlicher die Markierung einer Pflanze erfolgt, desto ähnlicher werden sich die Ergebnisse beider Rechenwege.

Da die Rhizodeposition stark von der Entwicklung der Wurzel abhängt, verändert sich auch die Menge und Zusammensetzung der Rhizodeposition im Laufe der Vegetation. Daher sollte ein weiterer Versuch zeigen, ob die Menge der C und N Rhizodeposition mit dem Spross-Wurzel-Verhältnis der Pflanze korreliert und sich entsprechend der Pflanzenentwicklung im Vegetationsverlauf verändert. Weiterhin sollte untersucht werden, ob die Mykorrhizierung der

Pflanzenwurzeln die C und N Rhizodeposition positiv beeinflusst. Hierfür wurde ein Gefäßversuch im Forschungsgewächshaus der Universität Kassel am Standort Witzenhausen angelegt. Die Wintererbse Frisson (*Pisum sativum* L. cv. Frisson) und deren nicht mykorrhizierende und nicht nodulierende Isolinie P2 wurden in homogenisiertem Oberboden des Versuchsfelds angezogen und sobald drei Laubblätter vollständig entfaltet waren (14 Tage nach der Aussaat) mit Hilfe der Dochtmethode und den stabilen Isotopen $^{13}C$ und $^{15}N$ markiert. Um die arbuskuläre Mykorrhizierung (AMF) zu fördern wurde zur Aussaat "rootgrowth™ professional" direkt unter den Erbsensamen platziert. Um die Entwicklung der Rhizodeposition im Vegetationsverlauf darstellen zu können, wurden die Pflanzen zu vier verschiedenen Zeitpunkten geerntet (zur beginnenden Blüte, zum Ende der Blüte, zur Grünreife und zur Trockenreife der Erbsen). Zu jedem Termin wurde die Isotopenanreicherung in der Pflanze und im Boden bestimmt. Außerdem wurde, um den Einbau der Rhizodeposition in die verschiedenen Bodenkompartimente bestimmen zu können, die mikrobielle Biomasse erfasst sowie der $N_{min}$ und der $C_{org}$ Gehalt des Bodens ermittelt. Die Menge der C und N Rhizodeposition wurde mit Hilfe, des im ersten Projektteil entwickelten Massenbilanzansatzes errechnet. Eine Unterprobe der Wurzeln wurde mit Trypanblau angefärbt, um dann, mit Hilfe der „gridline Intersection" Methode, den Anteil der mykorrhizierten Wurzeln zu bestimmen.

Es konnte festgestellt werden, dass die C und N Rhizodeposition nur zu einem geringen Teil in die verschiedenen Bodenkompartimente eingebaut wird. Dieser Anteil bleibt auch im Laufe der Vegetation stabil. Das lässt darauf schließen, dass der größte Teil der Rhizodeposition eine nicht leichtverfügbare C und N Quelle darstellt. Zur beginnenden Blüte, dem Zeitpunkt der stärksten Mykorrhizierung, war die Menge der N Rhizodeposition von Frisson signifikant höher, verglichen mit P2, welche nicht zur Mykorrhizierung fähig ist. Ein möglicher positiver Einfluss der Mykorrhiza ist somit denkbar. Jedoch hat Frisson trotz einer hohen mineralischen N Düngung zu Versuchsbeginn Knöllchen gebildet, was auch Einfluss auf die N Rhizodeposition gehabt haben kann. Bei der C Rhizodeposition ließen sich zwischen Frisson und P2 keine Unterschiede in der Menge der Rhizodeposition feststellen. Die Ergebnisse deuten darauf hin, dass sich die C und N Rhizodeposition unterschiedlich verhält. Die Menge der N Rhizodeposition blieb im Vegetationsverlauf gleich, wohingegen die C Rhizodeposition, aufgrund von verstärktem Wurzelabbau in der generativen Phase, stetig zugenommen hat.

Die publizierten Versuche zur Quantifizierung der Rhizodeposition wurden bisher ausschließlich im Gewächshaus oder unter Freilandbedingungen mit eingeschränktem

Wurzelraum durchgeführt. Da die Rhizodeposition stark von der Entwicklung des Wurzelsystems beeinflusst wird, kann eine wirklichkeitsgetreue Abbildung der Rhizodeposition nur erfolgen, wenn die Pflanzen unter natürlichen Bedingungen, ohne eine Beeinflussung des Wurzelwachstums oder der Nährstoff- und Wasserverfügbarkeit, kultiviert und markiert werden. Jedoch sind die Methoden zur Pflanzenmarkierung oftmals nicht für Freilandversuche geeignet, bzw. ist eine Durchführung zu arbeits- und kostenintensiv und daher nicht realisierbar. Daher war es das Ziel einen Versuch durchzuführen, in dem erstmals Pflanzen markiert werden, welche unter praxisüblichen Bedingungen im Freiland wachsen. Hierfür wurde die Sommererbse Santana (*Pisum sativum* L. cv. Santana) auf dem Versuchsfeld der Universität Kassel in praxisüblicher Aussaatstärke in Mikroplots ohne Einschränkung des Wurzelraums kultiviert. Die Markierung erfolgte, ähnlich wie im Gefäßversuch, durch die Dochtmethode, ab dem BBCH Stadium 13.

Entscheidend in diesem Versuch war die Entwicklung einer Möglichkeit die Erbsen zu markieren, ohne sie im Wachstum zu beeinträchtigen. Hierfür wurde ein Wagen gebaut, mit dem es möglich ist, über den Erbsenbestand zu fahren. Dieser ist in der Höhe variabel, um die Pflanzen aus dem Liegen heraus zu markieren, ohne sie dabei zu beschädigen. Um eine kontinuierliche Markierung zu erreichen, wurde den Pflanzen kontinuierlich neue Markierungslösung zur Verfügung gestellt. Die Probennahmetermine sowie die Analyse der Pflanzen orientierte sich am Gefäßversuch. Bodenproben wurden in drei Tiefen (bis 90 cm) entnommen. Die Beprobung des Bodens erfolgte stets in drei definierten Sektoren der Mikroplots (auf der Pflanze; zwischen zwei Pflanzen in der Reihe; zwischen zwei Reihen), um die Wurzelmasse einer Erbsenpflanze vollständig berechnen zu können und Überschätzungen zuvermeiden. Anschließend erfolgte eine gewichtete Verrechnung der Wurzeltrockenmassen der drei Sektoren (in 0-30 cm und 30-60 cm Tiefe). Die Menge der C und N Rhizodeposition wurde mit Hilfe der Massenbilanz berechnet.

Durch die $^{15}N$ und $^{13}C$ Doppelmarkierung konnte eine hohe und kontinuierliche Anreicherung der Erbsen im Feld erzielt werden. Somit ist die Dochtmethode (trotz großem Aufwand) für eine Markierung von Pflanzen mit stabilen Isotopen im Freiland zu empfehlen. Es zeigte sich, dass sich die Menge der C und N Rhizodeposition zwischen der Blüte und der Trockenreife der Erbse nicht verändert. Ungefähr ein Drittel der Rhizodeposition wird in die mikrobielle Biomasse und in den $K_2SO_4$ extrahierbaren C bzw. $N_{min}$ Pools des Bodens integriert. Dies ist identisch mit den Ergebnissen aus dem vorher durchgeführten Gefäßversuch. Der Gefäßversuch ist in der Art und Weise der Markierung sowie in den Ernteterminen und in der

Beprobung/Analyse der Pflanzen und des Bodens identisch und kann daher zu einem näheren Vergleich herangezogen werden. Durch ein stärkeres Wurzelwachstum zu Kulturbeginn und einem stärkeren Wurzelumsatz im generativen Stadium der Pflanzenentwicklung, ist das Spross-Wurzel-Verhältnis sowie das Rhizodeposition-Wurzel-Verhältnis zwischen Gefäß- und Freilandversuch unterschiedlich. Daher ist es nicht ohne weiteres möglich, Ergebnisse aus einem Gefäßversuch auf Pflanzen zu übertragen, welche unter Freilandbedingungen, ohne Begrenzung des Wurzelraumes, gewachsen sind. Ist das Vesuchsziel die genaue Abschätzung des zusätzlichen Kohlenstoff- und Stickstoffeintrags durch Pflanzen in den Boden, muss somit der Versuch im Freiland und ohne Einschränkung des Wurzelwachstums durchgeführt werden.

# 1. General introduction

## 1.1. The importance of rhizodeposition

The rhizodeposition of plants represents an essential C and N input into soil and has been repeatedly studied (Wichern et al., 2008; Pausch et al., 2013). The calculation of rhizodeposition helps to improve N balances (Mayer et al., 2003a,b) and to estimate the C sequestration potential of plants, as this input source of plant biomass is often neglected, causing wrong C and nutrient budgets (Wichern, 2007). Moreover, the quantification of the root derived N of legumes is increasingly important (Unkovich et al., 2010), because for N availability to subsequent crops and an intercropping partner, it is essential to consider the N-input through rhizodeposition into the soil (Mayer et al., 2003a). Uren (2007) defined rhizodeposition as the process of release of organic and inorganic compounds, including ion and gaseous compounds, from living roots. Rhizodeposition mainly consists of soluble root exudates, sloughed cells and tissue root fragments from root turnover (Uren, 2007; Nguyen, 2003; Jones et al., 2009). Rhizodeposition is said to be an easily available source of C and N for soil microorganism and rapidly decomposable (Mayer et al., 2004; Kindler et al., 2006; Wichern et al., 2008). The quantity and quality as indicated by the C/N ratio of rhizodeposition has a strong impact on the microbial turnover and the mineralization rate of nitrogen in soil. Therefore, the simultaneous estimation of C and N rhizodeposition is crucially important (Wichern et al., 2008; Jones et al., 2009; Yasmin et al., 2010). To improve cropping systems, especially in organic farming, a better knowledge of C and N rhizodeposition of legumes under field conditions, without influencing the root growth or water and nutrient budget, is essential.

## 1.2. Estimating rhizodeposition

### *1.2.1. Methods for plant labelling*

For estimating rhizodeposition, plants were labelled with stable ($^{13}C$, $^{15}N$) or radioactive ($^{14}C$) isotopes. There are many different approaches for labelling plants to quantify rhizodeposition, like root (Schmidtke, 2005), shoot (Gylfadóttir et al., 2007; Wichern et al., 2009) or atmosphere labelling (Janzen and Bruinsma, 1989; Schmitt et al., 2012; Soong et al., 2014). Substantial differences can be found in the possible duration of labelling (Arcand et al., 2013a). Simultaneous investigations of C and N rhizodeposition are rare (Merbach et al., 1999; Wichern

et al., 2007a, b; Schenck zu Schweinsberg-Mickan et al., 2010; Yasmin et al., 2010), due to the fact that for C often other labelling methods were used than for N labelling. To prevent an over- or underestimation of rhizodeposition, the labelling needs to be constant over the vegetation period/ the time of the experiment. A continuous labelling depends on the method used for labelling. Leaf feeding, for example, a method with a fast and effective solution uptake, represents a pulse labelling method and is not suitable for constant labelling. In contrast, the split root technique (the root system of the plant is divided into two parts, one part grows in soil, enriched with stable isotopes and the other part grows in unlabelled soil) and the application of enriched gases allow a continuous labelling. Due to multiple pulses, continuous labelling is also possible with the cotton wick method. Therefore, this type of stem feeding technique offers an alternative method to the split root technique or the labelling with enriched gases, especially since normally labelling under field conditions is difficult to perform and usually requires greater technical effort (Mahieu et al., 2007; Wichern et al., 2008). Moreover, the cotton wick method offers the possibility of labelling plants simultaneously with $^{13}C$ and $^{15}N$ (Wichern et al., 2007a, b; 2010).

When using the cotton wick method, the stem of each plant is drilled with a 0.5 mm diameter drill, approximately 3 cm above the ground. Then, a cotton wick is passed through the hole in the stem. The ends of the wick are passed through silicon tubes and through the lid of a vial, containing the labelling solution. To prevent evaporation losses, the connections between wick and plant or wick and lid are sealed with kneading mass. If plants take up the solution completely, the labelling solution can be refilled by pipette. After solution uptake, the empty vials are filled with deionized water to guarantee complete solution uptake. For measuring the natural abundance of $^{13}C$ and $^{15}N$ of the plants, as background values for calculating rhizodeposition, unlabelled control plants have to be cultivated.

### *1.2.2. Calculation of rhizodeposition*

Janzen and Bruinsma (1989) developed an approach for the calculation of N rhizodeposition after labelling plants with $^{15}N$. This approach has been used in many studies dealing with rhizodeposition, especially those estimating N rhizodeposition. The Janzen and Bruinsma calculation requires a homogeneous and constant tracer distribution in roots and rhizodeposits in time and space to prevent over- or underestimation, because of tracer dilution or translocation processes. Therefore, the Janzen and Bruinsma equation is most suitable when using a constant

labelling method, like the split root technique or working with enriched atmosphere. The percentage of C or N derived from rhizodeposition (%CdfR / %NdfR) is calculated as the ratio of atom%excess in soil and atom%excess in roots (Fig. 1.1). The total amount of C or N rhizodeposition is calculated by multiplying the total amount of C or N in soil and the percentage of CdfR or NdfR.

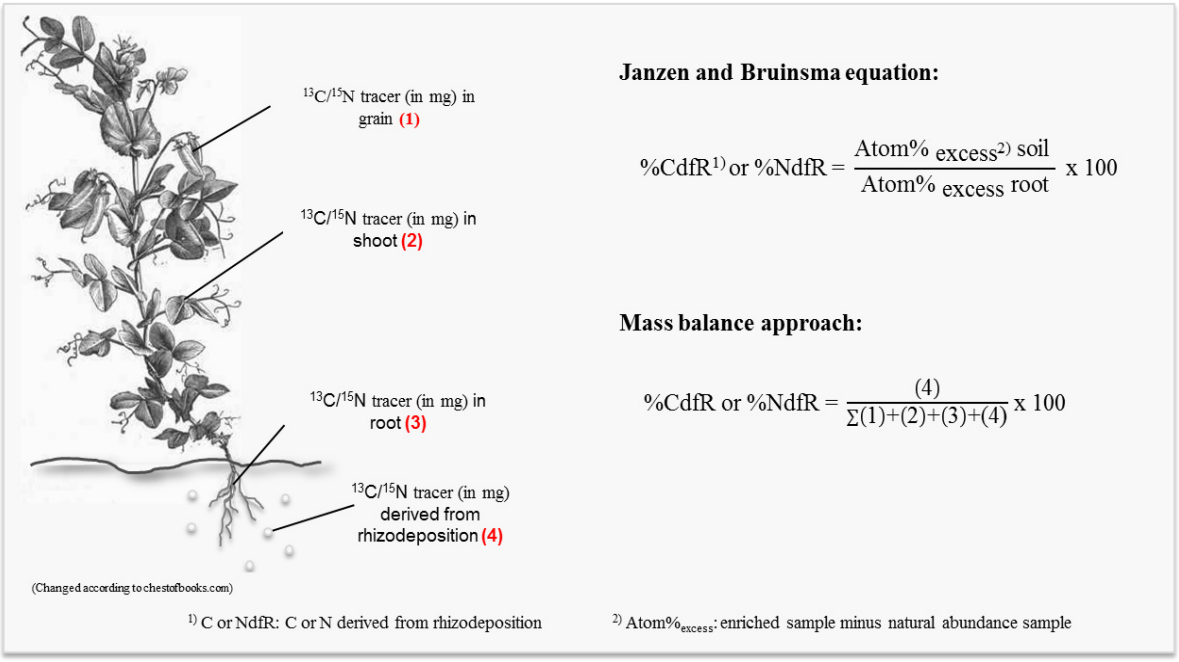


**Fig. 1.1**: The calculation of C and N rhizodeposition (%CdfR and %NdfR) with Janzen and Bruinsma equation and with mass balance approach.

Due to relocation processes during plant development, tracer relocation cannot be excluded. For example, N is highly relocated within plants during growth (Salon et al., 2001). Schiltz et al. (2005) found up to 11% of grain N in pea originating from roots. Tracer dilution in roots because of relocation can lead to an overestimation of rhizodeposition, because rhizodeposits are more highly enriched than roots. Otherwise, an accumulation of tracer in roots can cause an underestimation of rhizodeposition (Rasmussen, 2011). When estimating rhizodeposition you have to be aware that the method used for plant labelling and the duration of labelling influence the calculation. In particular, tracer dilution in roots because of plant growth or relocation processes influences the calculation of rhizodeposition when using the Janzen and Bruinsma equation. Therefore, a mass balance approach can be an alternative (Fig. 1.1).

A mass balance includes the tracer distribution in the entire plant and is not only focused on tracer in roots and rhizodeposits. Tracer relocation from root to grain, for example, cannot

influence the calculation of rhizodeposition. Khan et al. (2002) and Pausch et al. (2013) already used an isotope mass balance approach to determine belowground plant N (BGP-N) as a proportion of total N, and CdfR as a proportion of total C. With a further development of this mass balance approach, BGP-N and -C could be calculated in mg plant$^{-1}$.

## 1.3. Preliminary experiment to find a suitable labelling method under field conditions

Labelling plants under field conditions is a challenge. An important aim of the present study was to find a labelling method which allows a continuous labelling under field conditions without influencing plant development, especially without influencing root growth. Until now, studies have been conducted under controlled conditions in the greenhouse or under conditions similar to the field but with restricted root growth. This can lead to changes in plant development or in the root to shoot ratio. As rhizodeposition is closely related to root biomass, the amount and composition of rhizodeposition will be influenced too. The challenge is to find a labelling method that is suitable for labelling under field conditions without being work intensive or very expensive. Before starting the field experiment, a study was carried out to find the best labelling method. This method should have the following characteristics:

- to label plants continuously under field conditions
- the labelling has to start at an early stage of plant development and has to continue until maturity of pea plants
- the development of plants must not be influenced by the labelling method

The application of gases was not considered, because a continuous labelling with enriched gas is far too expensive and work intensive. The split root technique is not suitable for labelling under field conditions either. This method has a huge impact on the root system of plants and creates unnatural conditions. Therefore, we focused on the leaf feeding method and the cotton wick method, which we had already used in a pot experiment. We produced a 2% glucose and 0.5% urea solution, similar to the solution we would use in the field. However, we did not use stable isotopes, because we did not analyze the isotope ratio. We used *Pisum sativum* L. cv. Santana, a spring pea and *Pisum sativum* cv. L. E.F.B.33, a winter pea, the same varieties as those we later used in the field experiment. To begin with, we examined a possible influence of the C and N mixture in the labelling solution (Fig. 1.2 a) on development of algae or fungi in the

labelling system that can impede solution uptake. Therefore, we produced a further labelling solution in which C and N were separated (Fig. 1.2 b).

The cotton wick method requires very precise and careful work, which can be difficult under field conditions. Therefore, we examined whether the leaf feeding method can be an appropriate alternative (Fig. 1.2 c). For this, the leaves of *Pisum sativum* L.cv. E.F.B.33 (a regular leafed winter pea) were damaged through rolling between the fingers to improve the uptake of the labelling solution. *Pisum sativum* L.cv. Santana is a semi-leafless spring pea, making it difficult to label a single leaf. In addition, we examined whether the tendril (Fig. 1.2 d) or the tendril tip (Fig. 1.2 e) of Santana is also able to take up solution. To evaluate the continuity of the labelling method, the treatments differ in the frequency at which plants received a further amount of labelling solution: fortnightly, every week or as required.

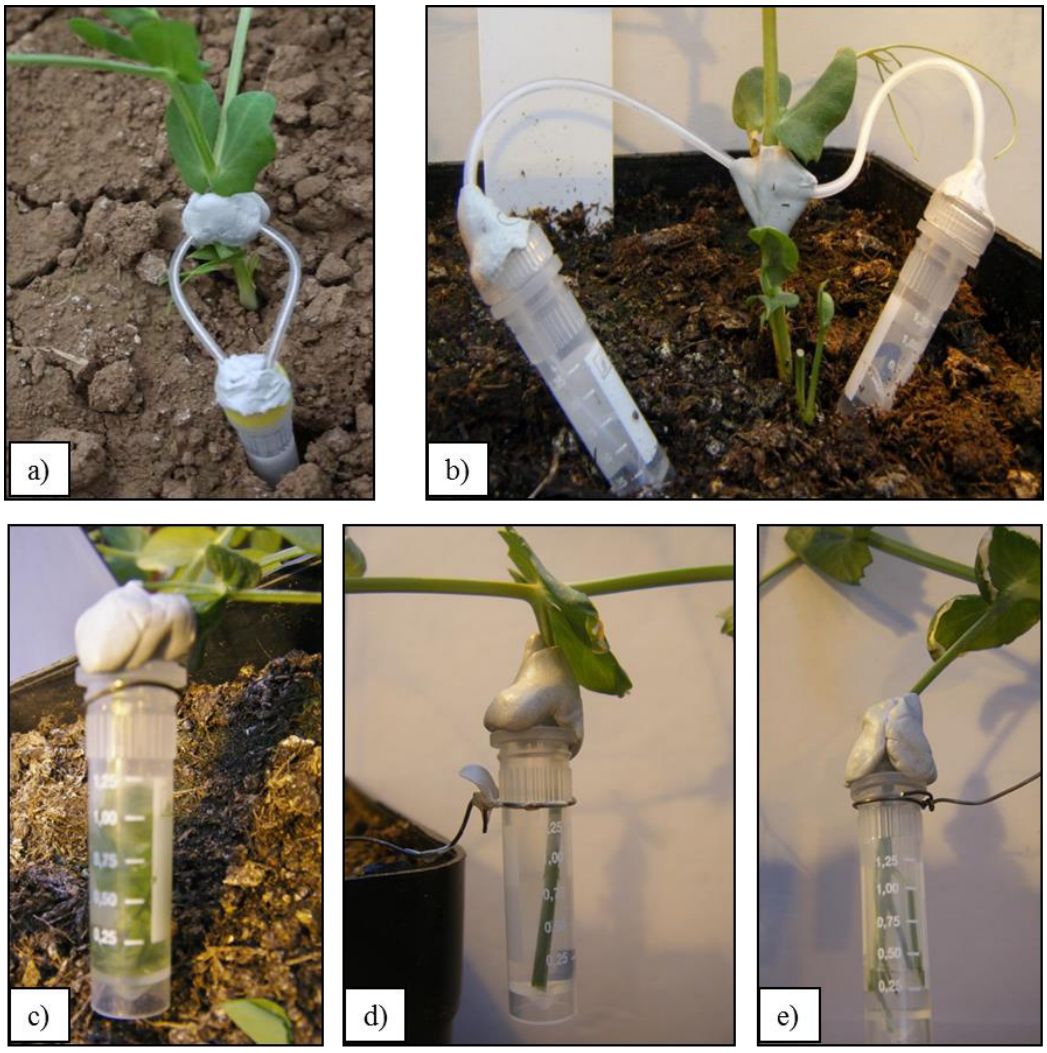


**Fig. 1.2**: Plant labelling methods: using a) cotton wick method with a mixture of C and N; b) cotton wick method with C and N separated; c) leaf feeding method; d) leaf feeding method using the tendril of a pea (*Pisum sativum* L.) and e) leaf feeding method using the tendril tip of a pea.

First, it could be shown that there was no difference between the treatments of C and N separated or in a mixture. The formation of algae or fungi probably only depends on clean working. It soon became clear that the leaf feeding method is not suitable for a continuous labelling under field conditions. The solution was taken up completely within the first 12 to 24 h. For a further solution uptake a new leaf must be taken, because the leaf or the tendril/tendril tip can be used only once. Therefore, a constant plant labelling during the whole vegetation period is not possible, because more leaves/tendrils are required than are produced. A crucial advantage of the cotton wick method is the possibility of reusing the labelling system, until the solution uptake decreases due to callus formation at the stem or the formation of algae/ fungi. A fixed interval for

solution refilling leads to a less continuous labelling. Therefore, the best way to labelling pea plants continuously under field conditions is the cotton wick method, where plants receive further solution immediately after complete uptake.

## 1.4. Objectives of this work

Rhizodeposition of different plant species has been investigated repeatedly. However, it is notable that the results presented differ considerably, depending on the definition of rhizodeposition, the labelling method and the experimental setup used. The main focus of the present thesis was to establish a method for estimating C and N rhizodeposition of legumes under field conditions and in particular to estimate the rhizodeposition of peas over the course of time and the incorporation of rhizodeposition in the different soil pools. In order to verify whether results of pot experiments can be transferred to the field, a second experiment was carried out, which was similar in labelling method, harvest dates, sample analysis and calculation. To evaluate potential errors caused by the method of calculating rhizodeposition, data from the pot experiment were used to develop an alternative calculation approach.

### *1.4.1. Objectives of the first publication: Developing a mass balance approach to calculate rhizodeposition and comparing this with the Janzen and Bruinsma equation*

When using the Janzen and Bruinsma equation for calculating rhizodeposition, a continuous plant labelling is required to guarantee a homogeneous enrichment of roots and rhizodeposits. Relocation processes within the plant can impede this. A mass balance was developed to estimate N rhizodeposition as an alternative approach to the Janzen and Bruinsma equation. Furthermore, these two approaches were compared and their potential error was assessed. For developing the mass balance approach, data from a greenhouse pot experiment were used, which is described in more detail below (1.4.2.). *Pisum sativum* L. was repeatedly pulse labelled with a $^{13}C$ glucose and $^{15}N$ urea solution using the cotton wick method. Additionally, data from a previous study using the same labelling approach were used for comparative BGP-N calculations. The two studies differed in the frequency and duration of labelling and the time of plant harvest. It was to be examined whether these differences in experimental setup can influence the calculated amount of rhizodeposition and whether the mass balance approach represents a less sensitive way of estimating rhizodeposition.

### *1.4.2. Objectives of the second publication: Quantification of C and N rhizodeposition of pea depending on plant development and mycorrhization*

The quantity and quality of rhizodeposition is expected to be affected by plant growth stage and soil microorganisms, especially arbuscular mycorrhizal fungi (AMF). A quantitative estimation of the C and N rhizodeposition allows the determination of the quality of rhizodeposition on the basis of the C / N ratio and the availability for microorganisms. A greenhouse pot experiment was carried out, which quantified the C and N rhizodeposition of peas over the course of time. In particular, the spatial and temporal distribution of rhizodeposition and their transfer into different soil compartments was tested. Furthermore, the percentage of the microbial biomass that originates from the rhizodeposition was quantified and compared between the non-mycorrhizal and non-nodulating pea (*Pisum sativum* L.) mutant P2 and the symbiotic isoline Frisson to estimate the influence of mycorrhiza on rhizodeposition. Therefore, all pots were inoculated with "rootgrowth™ professional", placed directly below the seed. For approximating continuous labelling, the plants were labelled fortnightly, five times during the experiment, beginning 14 days after sowing (DAS), with three leaves unfolded (Fig. 1.3 a). Peas were labelled with a solution of 2% $^{13}$C-glucose (99 atom%) and 0.5% $^{15}$N urea (95 atom%) using the cotton wick method (after Russell & Fillery, 1996 and Wichern et al., 2007). Sampling took place on four dates depending on plant development (beginning of flowering, end of flowering, green ripe [Fig. 1.3 b] and maturity). At every sampling date, plants were separated into root, shoot and grain. Plant and soil samples were dried for estimating dry matter, ground with a ball mill and weighed into tin cups for analyzing total C and N content and the isotope ratio of $^{13}$C/$^{12}$C and $^{15}$N/$^{14}$N. The isotope ratios were determined by isotope ratio mass spectrometry. For estimating microbial biomass, the chloroform fumigation method was used (Brookes et al., 1985; Vance et al., 1987), including a pre-extraction step to remove living roots (Mueller et al., 1992; Mayer et al., 2003a,b).

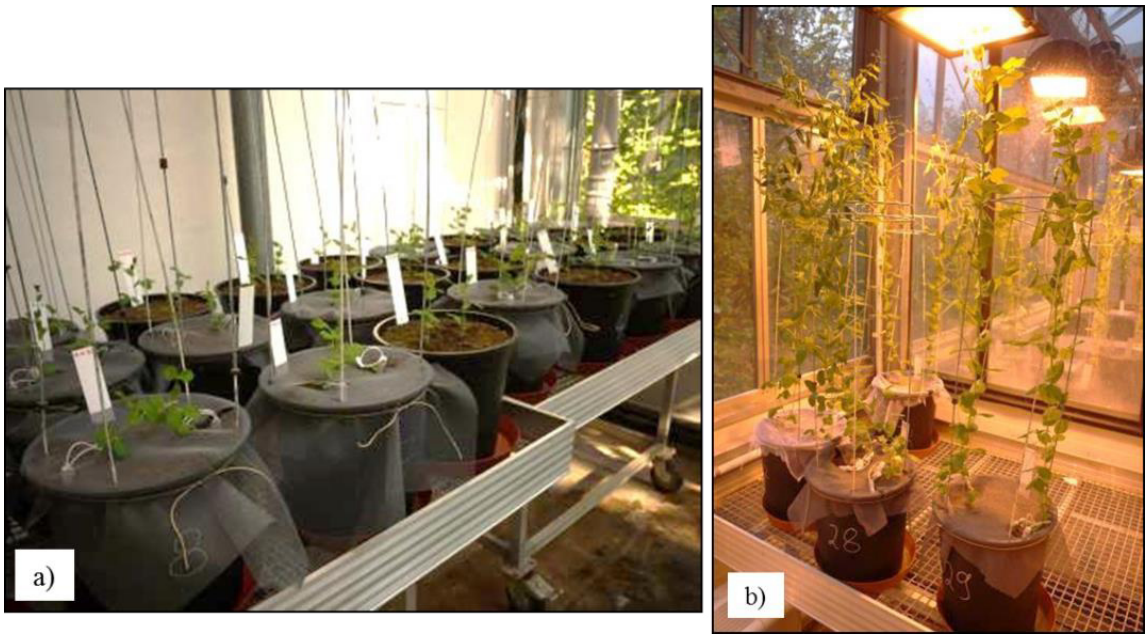


**Fig. 1.3**: Pea plants labelled with $^{13}C$ and $^{15}N$ using the cotton wick method
a) 14 days after sowing (DAS) and b) 71 DAS.

### *1.4.3. Objectives of the third publication: Estimating C and N rhizodeposition under field conditions without influencing root growth or the water and nutrient budget*

Quantification of the C and N rhizodeposition under field conditions is difficult, as labelling plants with stable or radioactive isotopes and separating labelled and unlabelled roots is difficult and often creates unnatural conditions. As a consequence, experiments are often done under controlled conditions. To estimate a realistic amount of C and N rhizodeposition, experiments have to be conducted under field conditions without influencing the root system or the water and nutrient budget. This is particularly true since the process of rhizodeposition is closely related to root biomass (Shamoot, 1968; de Graaf et al., 2007) and more root biomass will be developed under field conditions. Therefore, *Pisum sativum* L. cv. Santana was sown manually in microplots. In every microplot 12 pea plants were cultivated at a distance of 12.5 cm. Due to the fact that plant labelling had not been performed under field conditions until now, a first important step was to develop a method that allowed careful labelling, without influencing plant development (Fig. 1.4 a and b). With this, the peas were labelled with a labelling solution of 2% $^{13}C$-glucose (99 atom%) and 0.5% $^{15}N$ urea (95 atom%) using the cotton wick method (Fig. 1.4 c and d). To reach a homogeneous labelling, peas received labelling solution as required, until the day of harvest. The harvest dates, sampling and analysis of plant and soil were similar

to the pot experiment (a detailed description can be found under 1.4.2.). As estimating the total amount of root biomass under field conditions is difficult, a sampling scheme was developed which allowed the calculation of the complete root biomass. Therefore, soil samples were taken at 0 – 30 cm and 30 – 60 cm depth with a HUMAX, a special soil sampling drilling tool, in a sampling pattern based on the study of Anthes (2005). The soil was automatically filled in a plastic cylinder, which prevented a carry-over of the labelled soil from one microplot to the next. After soil washing, root dry matter of each sample was estimated and then total amount of root dry matter was calculated.

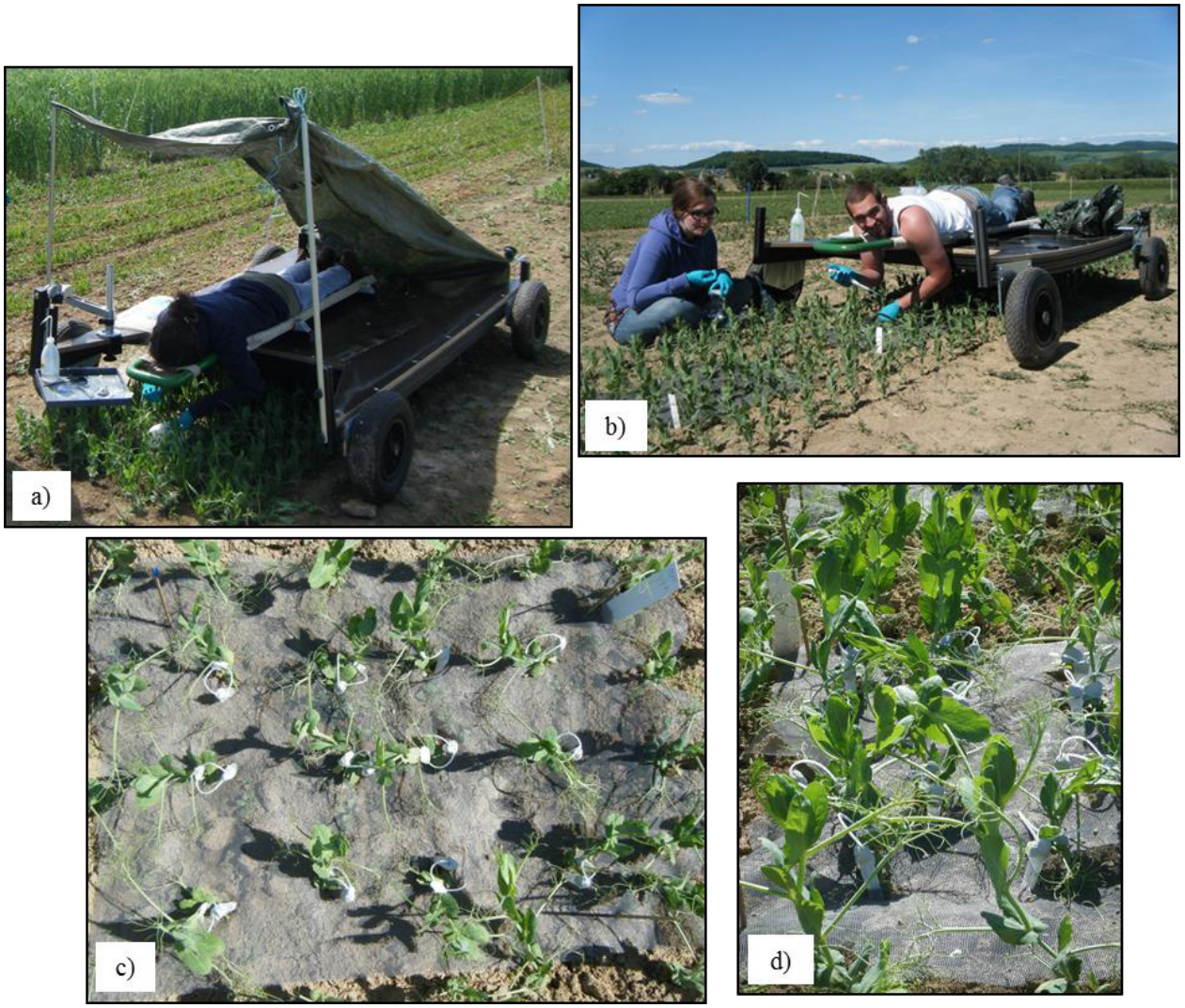


**Fig. 1.4**: a and b) Labelling pea plants (*Pisum sativum* L.) under field conditions with a multicarrier; c and d) peas labelled with $^{13}C$ and $^{15}N$ 41 days after sowing (DAS).

# 2. Digging in the dirt – Inadequacy of belowground plant biomass quantification

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**Abstract**

Accurate quantification of belowground plant biomass (BGP) is crucial to account for the carbon (C) and nitrogen (N) stored by plants. As soil sieving to recover roots leaves a large proportion of root borne compounds defined as rhizodeposits (fine roots, root fragments, exudates) unaccounted for, isotope-labelling approaches have frequently been used. The aim of the present study was to compare two approaches that estimate BGP-N from isotope labelling experiments and assess their potential error. *Pisum sativum* L. was grown in a pot experiment and repeatedly pulse labelled with a $^{13}C$ glucose and $^{15}N$ urea solution using a cotton wick method. Additionally, data from a previous study using the same labelling approach were used for comparative BGP-N calculations. In both experiments, the amount of BGP-N calculated with a mass balance approach was significantly lower compared with the classical calculation, indicating substantial overestimation of N rhizodeposition in previous studies. Multiple pulse labelling of plants with $^{15}N$ can result in homogeneous label distribution, which allows both calculation approaches to be used. However, when label distribution is heterogeneous, the classical approach overestimates N rhizodeposition and BGP-N.

Key words: belowground plant biomass; mass balance approach; $^{15}N$; pea; rhizodeposition

## 2.1. Introduction

Soils are one of the major terrestrial carbon (C) sinks and are highly relevant for future C sequestration. It`s important to reduce further greenhouse gas emissions from soils (Rees et al., 2005; Lal et al., 2007). Plants act as the major gateway of C input into soils. In particular the belowground plant biomass (BGP), often referred to as "the hidden half" (Den Herder et al., 2010; De Coninck et al., 2015), substantially contributes to C storage of plants and thus to sequestered C (Rees et al., 2005). When estimating nitrogen (N) fixation by legumes, it is important to consider the N stored belowground to obtain accurate values for the N input (Herridge et al., 2008; Peoples et al., 2009). Moreover, an increase in drought periods also in humid regions require adapted cropping systems with plants having extended root systems able to withdraw sufficient quantities of water and making more efficient use of plant nutrients (Franco et al., 2011).

When aiming at quantifying C or nutrients (e.g. N) stored in plants, an accurate estimation of BGP is therefore essential. However, often only parts of the BGP are measured. Most methods for the estimation of root dry matter, such as root sieving, underestimate the extent of the root system and its biomass (BGP), as fine roots are often not determined because of their small size and near transparency (Pierret et al., 2005). Moreover, root exudates and compounds released from dying roots are often not measured. This fraction often unaccounted for when considering visible roots only, can be summarized as rhizodeposits and is measured as C or N released from roots. Consequently, when assessing e.g. BGP-N, beside N stored in roots, all other N derived from roots is defined as N derived from rhizodeposition (NdfR), the process of N release from living plant roots (Uren, 2007; Wichern et al., 2008; Jones et al. 2009; Fustec et al., 2010). The same also holds true for C to some extent, even though other compounds, such as sugars for example, substantially contribute to C rhizodeposition (Nguyen, 2003; Wichern et al., 2008). Therefore, the question arises of how to calculate BGP, in particular rhizodeposition, correctly? Estimating the net rhizodeposition (release minus re-uptake), regardless of whether C or N rhizodeposition, is a complex task and results acquired are influenced by various aspects, such as plant species and age, soil type, nutrient availability in soil, water regime, temperature and pot size (Wichern et al., 2008; Jones et al., 2009; Kuzyakov, 2002; Poorter et al., 2012). However, apart from pot size, the most important factor affecting the prediction of rhizodeposition is the definition used. We therefore (Wichern et al., 2008) propose to use the definition of

rhizodeposition given by Uren (2007), who defined all compounds released from roots of living plants as rhizodeposition, including volatiles, gaseous compounds, root fragments and decaying roots.

Beside the definition of rhizodeposition, the method used for labelling plants with stable or radioactive isotopes also fundamentally influences the amount of rhizodeposition predicted, which was shown by Yasmin et al. (2006) and Mahieu et al. (2009). Yasmin et al. (2006) compared leaf, petiole and stem feeding methods for labelling the plants with $^{15}N$ and observed $^{15}N$ enrichment and $^{15}N$ recovery between the treatments, resulting in a high $^{15}N$ enrichment with leaf feeding and a very low (but more homogeneous) $^{15}N$ enrichment with petiole feeding. The methods of labelling differ also in the time of solution uptake (Yasmin et al., 2006) and influence the continuity of labelling. Apart from the split root technique, the labelling of the atmosphere with $^{15}N$ (as Janzen and Bruinsma [1989] did in their work) is a very effective method for a continuous labelling, which is an important assumption for the calculation of rhizodeposition. Due to a multiple pulse labelling, the cotton wick method approached an almost continuous labelling. However, leaf feeding, a method with a fast and effective solution uptake, is only suitable for pulse labelling. Due to this fact, leaf feeding cannot achieve a continuous labelling. Labelling the atmosphere and a multiple pulse labelling are the two most effective methods for estimating a realistic amount of BGP-N, as extensively discussed by Wichern et al. (2008).

Assessing belowground plant N (BGP-N), consisting of root N and rhizodeposition N, is particularly important for total N balances (Arcand et al., 2013a). Janzen and Bruinsma (1989) developed an approach for the calculation of N rhizodeposition after labelling plants with $^{15}N$. This approach has been used in most studies quantifying BGP-N inputs used until now. Their approach requires homogeneous distribution of $^{15}N$ in roots and rhizodeposits in time and space to prevent any dilution of isotopes. However, N is highly relocated within plants during growth (Salon et al., 2001); homogeneous enrichment with $^{15}N$ is difficult to reach. Tracer relocation may lead to an overestimation of N rhizodeposition because of tracer dilution in roots compared with rhizodeposits or to an underestimation when tracer is accumulated in roots and not released as rhizodeposits (Rasmussen, 2011). The influence of relocation can be limited by a continuous plant labelling until harvest, which is difficult to achieve with the available methods, especially under field conditions (Wichern et al., 2008). Khan et al. (2002) used an isotope mass balance approach to determine BGP-N ($^{15}N$ recovered in roots and soil) as proportion of total N ($^{15}N$ recovered in roots, soil and shoot). With a further development of this mass balance approach,

BGP-N and -C could be calculated in mg plant$^{-1}$. The calculation of BGP-N / BGP-C with an isotope mass balance approach may prevent over- or underestimations caused by tracer relocation processes (Rasmussen, 2011). Therefore, the objectives of the present study were:

- to compare a mass balance approach and the classical approach of Janzen and Bruinsma (1989) for quantification of BGP-N in peas,
- to assess the error associated with the classical calculation when plants are not continuously labelled, and
- to calculate the most realistic amount of BGP-N.

## 2.2. Materials and Methods

### *2.2.1. Soil*

For the pot experiment, the soil, collected at 0-30 cm depth from the research station of the University of Kassel in Neu-Eichenberg, Germany (51º 23´ N, 9º 55´ E, 220 m asl), was sieved (10 mm) and stored for six weeks before the experiment started. The silty loam (13 % clay; 83 % silt; 3 % sand) was classified as a Haplic Luvisol (FAO classification, 2014), with a pH of 6.0, 12 mg organic C g$^{-1}$ soil and 1.3 mg total N g$^{-1}$. At 0–30 cm soil depth, the soil contained 17 µg Ca-acetate lactate extractable P g$^{-1}$ soil, 66 µg K g$^{-1}$ soil, and 84 µg Mg g$^{-1}$ soil. One day before sowing, the soil was fertilized with 100 kg Ca ha$^{-1}$. Fertilizers (N, P, K, and Mg) were added at the day of sowing (150/17/43/9 kg ha$^{-1}$). The soil used in the column experiment was silty loam (16–23% clay, 75–82% silt, 2% sand) from the research station of the University of Kassel, which is located at Frankenhausen, Northern Hesse, Germany (51º 24´ N, 9º 25´ W, 230 m asl). For more information see Wichern et al. (2007a).

### *2.2.2. Experimental designs and labelling*

There were two experimental designs, one for *Pisum sativum* L. cv. Frisson (in 2012) and one for *Pisum sativum* L. cv. Santana (in 2005). Frisson was cultivated under controlled conditions in the greenhouse with a relative humidity of 60 % and 110 klxh d$^{-1}$. The mean temperature was 20 ºC during the day and 15 ºC during the night. The 8.5 l pots (28 cm diameter and 20 cm height) were filled with 11 kg soil and mechanically compressed to a wet density of 1.3 g cm$^{-3}$. Two plants per pot were cultivated with six replicates per treatment. For mycorrhization, all pots were inoculated with "rootgrowth™ professional", placed directly below the seed. Once a week, the pots were weighed and watered with deionized water, to keep a WHC between 60 and 80 %. For Santana,

the collected columns were placed in a box filled with soil and 4 plants per column were cultivated. For more details see Wichern et al., 2007a.

All plants (Santana and Frisson) were labelled with a $^{15}N$ urea (95 atom%) and $^{13}C$ glucose (99 atom%) solution, using a stem feeding technique (Russell and Fillery, 1996; Wichern et al., 2007b). The stem of each plant was drilled with a 0.5 mm drill, approximately 3 cm above ground. Then, a cotton wick was passed through the hole. The ends of the wick were passed through silicon tubes and through the lid of a two ml vial, which contained the labelling solution. To prevent evaporation losses, the connections between wick and plant and between wick and lid were sealed with Teroson (Henkel), a kneading mass. The feeding solution was produced with deionized water and then sterile filtrated (< 0.2 µm). All material used for labelling was steam sterilized for 20 min at 121 ºC. Frisson was labelled fortnightly with a 0.5 % urea and 2 % glucose solution. For labelling, the plants were multiple pulse labelled (5 times overall), beginning at BBCH 13 (3 leaves unfolded) 14 days after sowing (DAS) (Lancashire et al., 1991). The concentration of urea and glucose differed for Santana, depending on the estimated dry matter increase (between 0.6 % and 8.1 % for glucose and between 0.04 % and 0.89 % for urea). Plants were labelled twice with 1 ml solution, first in BBCH 15 (5 leaves unfolded; 33 DAS) and then in BBCH 18 (8 leaves unfolded; 45 DAS). After solution uptake, the empty vials of Frisson and Santana were filled with deionized water (between 0.5 ml and 1 ml) to secure a complete solution uptake. Pots and columns were covered with a 1 mm mesh to prevent soil contamination from falling leaves.

*2.2.3. Sampling and analyses*

At harvest, labelling systems were carefully removed from peas and stored for analyses. Frisson was harvested at 3 different dates, depending on plant development, beginning at BBCH 59 (first petals visible, flowers still closed; 45 DAS), BBCH 79 (pods have reached typical size; green ripe; 71 DAS) and at BBCH 89 (fully ripe: all pods are dry and brown; seeds are dry and hard; 95 DAS) (Lancashire et al., 1991). Santana was harvested at BBCH 85 (50% of pods are ripe, seeds have the final color, are dry and hard; 80 DAS). All pea plants were separated into roots, stem and leaves (incl. pods) and grain. After weighing, plant parts were dried for 48 h (Frisson) or 72 h (Santana) at 60 ºC. The dry plant parts were ground with a ball mill, weighed into tin cups and analyzed for total C, total N and the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios. At harvest, a soil subsample was sieved (< 2 mm), dried for 24 h at 105 ºC, ground and weighed into tin cups (as described above

for plant parts). The sample weight for analyzing the isotope ratio depends on the estimated amount of C and N of the subsamples. Total C and total N were analyzed with an elemental analyzer (Fisons, Milano, Italy). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios were determined with isotope ratio mass spectrometry (Finnigan MAT, Bremen, Germany). For a complete recovery of isotopes, the vials and labelling systems were extracted with 200 ml of 0.05 M $K_2SO_4$ according to Potthoff et al. (2003). The remaining amount of $^{13}C$ and $^{15}N$ in the extracts were calculated, assuming that this C and N were derived from the isotope solution.

*2.2.4. Calculation of N rhizodeposition*

For calculating NdfR, (I) the Janzen and Bruinsma equation (1989) and (II) a mass balance approach were used:

(I) Janzen & Bruinsma:

$$\text{NdfR}\ (\%) = \frac{\text{Atom\%}_{\text{excess}}\ \text{soil}}{\text{Atom\%}_{\text{excess}}\ \text{root}} \times 100 \qquad (1)$$

Values for $\text{atom\%}_{\text{excess}}$ were calculated by subtracting the $^{15}N$ atom% values of non-labelled plants from the $^{15}N$ atom% values of the labelled plants ($^{15}N$ abundance minus $^{15}N$ natural abundance). The total amount of N rhizodeposition was calculated by multiplying the total amount of N in soil by the percentage of NdfR. In order to apply this equation, the following assumptions have to be made to exclude dilution effects: (a) the enrichment of roots and rhizodeposits is equal, (b) the $^{15}N$ tracer is evenly distributed in the root system and, (c) the $^{15}N$ tracer content of roots is constant over the growth period of the plants (Janzen and Bruinsma, 1989; Mayer et al., 2003b; Rasmussen, 2011).

(II) Mass balance approach:

The mass balance approach defines the recovered amount of $^{15}N$ tracer in soil ($^{15}N_{tracer}$dfR) as a part of the total amount of $^{15}N$ tracer in all plant parts and in soil. This has to be equal for $^{14}N$ and $^{15}N$ from natural abundance. In this case, tracer dilution does not influence the calculation, because a mass balance approach focuses on the whole plant, not only on the plant roots. For estimating the total amount of NdfR, the amount of $^{15}N$ tracer in plant and in soil was calculated for each plant compartment (grain, shoot and leaves, root) and soil, considering the different

atomic weights of $^{15}N$ (15.0001) and $^{14}N$ (14.0031). The $^{15}N$ particle fraction was recalculated into a $^{15}N$ mass fraction (2). Then, the complete $^{15}N$ amount was the $^{15}N$ mass fraction multiplied by the measured amount of total N in the specific plant part (3). The total amount of $^{14}N$ has to be calculated as the difference between the total amount of N (of the specific plant part or the soil) minus the calculated amount of $^{15}N$ (4).

$$^{15}N \text{ mass fraction} = \frac{(\text{atom\% } ^{15}N \times \text{atomic weight } ^{15}N)}{[((100\text{-atom\% } ^{15}N) \times \text{atomic weight } ^{14}N)+(\text{atom\% } ^{15}N \times \text{atomic weight } ^{15}N)]} \quad (2)$$

$$^{15}N \text{ (mg)} = ^{15}N \text{ mass fraction} \times \text{total N (mg)} \quad (3)$$

$$^{14}N \text{ (mg)} = \text{total N (mg)} - ^{15}N \text{ (mg)} \quad (4)$$

In the next step, the $^{15}N_{tracer}$ was calculated as a proportion of the total amount of $^{15}N$ in a specific plant part or soil. For this, the enrichment of $^{15}N$ (atom%$^{15}N_{excess}$) was divided by the measured atom%$^{15}N$ (5). Now, the complete $^{15}N_{tracer}$ amount (in mg) was the total amount of $^{15}N$ multiplied by $^{15}N_{tracer}$ (% of $^{15}N$) (6).

$$^{15}N_{tracer}(\%\text{ of } ^{15}N) = \frac{\text{Atom\% } ^{15}N_{excess}}{\text{Atom\% } ^{15}N} \quad (5)$$

$$^{15}N_{tracer} \text{ (mg)} = ^{15}N_{tracer} (\%\text{ of } ^{15}N) \times ^{15}N \text{ (mg)} \quad (6)$$

As the enrichment of N depends on plant parts, the amount of $^{15}N_{tracer}$ was separately calculated for grain, roots, leaves and stem. Then, total $^{15}N_{tracer}$ was the sum of $^{15}N_{tracer}$ in grain, in roots, in stem and leaves and in soil (NdfR). Now, the $^{15}N_{tracer}$dfR in soil as a proportion of $^{15}N_{tracer}$ in total can be calculated (7).

$$^{15}N_{tracer}\text{dfR (in \%)} = \frac{^{15}N_{tracer}\text{ dfR}}{^{15}N_{tracer}\text{ in total}} \times 100 \quad (7)$$

The ratio of $^{15}N_{tracer}$ in rhizodeposition to $^{15}N_{tracer}$ in total (plant and rhizodeposition) corresponds to the ratio of N in rhizodeposition to total N (in plant and rhizodeposition) (8).

$$^{15}N_{tracer}\text{dfR (in \%)} = \text{NdfR (in \%)} \quad (8)$$

The total amount of rhizodeposition consists of $^{15}N$ from tracer plus $^{15}N$ from natural abundance plus $^{14}N$ from natural abundance. For estimating the total amount of N rhizodeposition, we used equation (8) and assumed that the ratio of $^{15}N_{tracer}$ derived from rhizodeposition in soil in relation to $^{15}N_{tracer}$ in total was the same as the ratio of $^{15}N$ derived from rhizodeposition in soil in relation to $^{15}N$ in total (in plant and rhizodeposition) (9). The same relationship was assumed valid for $^{14}N$ in soil and $^{14}N$ in total (as mentioned above) (10).

$$\frac{^{15}N_{tracer} \text{ in soil (mg)}}{^{15}N_{tracer} \text{ in total (mg)}} = \frac{^{15}NdfR \text{ (mg)}}{^{15}N \text{ in total (mg)}} \quad (9)$$

$$\frac{^{15}NdfR \text{ (mg)}}{^{15}N \text{ in total (mg)}} = \frac{^{14}NdfR \text{ (mg)}}{^{14}N \text{ in total (mg)}} \quad (10)$$

After rearranging the equation, $^{15}$NdfR and $^{14}$NdfR can be calculated (11 &12).

$$^{15}NdfR \text{ (mg)} = \frac{^{15}N_{tracer} \text{ in soil (mg)} \times ^{15}N \text{ in plant (mg)}}{^{15}N_{tracer} \text{ in plant (mg)}} \quad (11)$$

$$^{14}NdfR \text{ (mg)} = \frac{^{15}N \text{ in soil (mg)} \times ^{14}N \text{ in plant (mg)}}{^{15}N \text{ in plant (mg)}} \quad (12)$$

The total amount of N rhizodeposition is the sum of $^{15}$NdfR (including $^{15}N_{tracer}$dfR) and $^{14}$NdfR.

*2.2.5. Statistical analyses*

All statistical analyses were performed using IBM SPSS Statistics 22. Differences of the means between the dates of harvest (depending on plant development) were tested by a one-way analysis of variance (ANOVA), using the Scheffé post-hoc test. Student`s t-test was performed on pairs of means to determine differences between Santana and Frisson and between the calculation approaches (mass balance or Janzen & Bruinsma equation).

## 2.3. Results

*2.3.1. N derived from rhizodeposition*

Regardless of the calculation approach, the amount of N derived from rhizodeposition increased with time (Table 2.1). Calculated with mass balance, the amount of N derived from rhizodeposition was significantly lower 45 DAS compared with the amount of N rhizodeposition 71 and 95 DAS. In contrast, there were no differences for NdfR between the harvest dates, calculated with the Janzen and Bruinsma equation. At 45 DAS, the amount of NdfR was twice as high using the Janzen and Bruinsma equation compared with mass balance. At 71 DAS, the amount of NdfR was still over 50% higher using the Janzen and Bruinsma equation compared with mass balance. At 95 DAS, all significant differences disappeared between the two calculation approaches.

**Table 2.1**: $^{15}N$ enrichment of soil and root (Atom%$_{excess}$) and N derived from rhizodeposition (NdfR in mg plant $^{-1}$) of pea (Frisson) 45, 71 and 95 DAS. Values show means ± standard error of the mean (n = 6, except 45 DAS: n = 5), calculated with mass balance and Janzen & Bruinsma equation. Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$) of the calculation approaches. Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$).

| Frisson | DAS | NdfR (mg plant$^{-1}$) | | Atom%$_{excess}$ | |
|---|---|---|---|---|---|
| | | Mass balance | Janzen & Bruinsma | Soil | Root |
| BBCH 59 | 45 | 5.0 ± 0.7 a | 10.2 ± 1.3 a* | 0.0023 a | 1.76 a |
| BBCH 79 | 71 | 10.6 ± 1.1 b | 16.2 ± 1.5 a* | 0.0034 ab | 1.70 a |
| BBCH 89 | 95 | 12.5 ± 1.3 b | 16.2 ± 1.9 a | 0.0043 b | 2.15 a |

The results for NdfR, calculated as a proportion of total plant N were similar (Fig. 2.1). At 45 and 71 DAS, the two calculation approaches resulted in significant differences. Regardless of the calculation approach, the NdfR content of total plant N decreased over time. Furthermore, the NdfR content calculated with mass balance and the NdfR content calculated with the Janzen and Bruinsma equation converged in the course of plant development and with increasing intensity of labelling. Independently of the calculation approach, the NdfR content was significantly higher 45 DAS than 71 or 95 DAS.

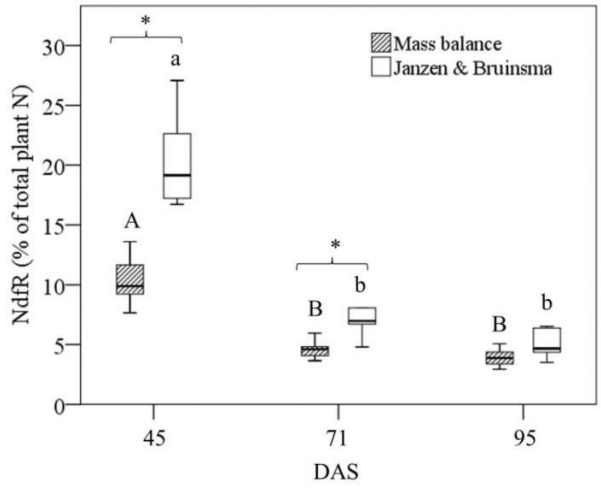


**Fig. 2.1**: N derived from rhizodeposition (NdfR in % of total plant N) of pea (Frisson) 45, 71 and 95 DAS, calculated with mass balance and Janzen & Bruinsma equation (n=6 except 45 DAS: n=5). Values with * indicate significant differences between the calculation approach (t-test, pairwise comparison, $p < 0.05$). Different letters (a, b) indicate significant differences between DAS, calculated with Janzen & Bruinsma equation, and different capital letters (A, B) indicate significant differences between DAS, calculated with mass balance (Scheffé, $p < 0.05$).

*2.3.2. Santana versus Frisson*

In the two experiments compared here, almost the same amount of roughly 5 kg soil was available for each pea plant. Because of a high N fertilization, Frisson had more than twice as much N available compared with Santana. On average, Santana reached 6.5 g and Frisson 10.0 g dry matter plant$^{-1}$ (Table 2.2). For Santana, the contribution of root to total plant dry matter was almost 5 times higher than for Frisson.

**Table 2.2**: Enrichment of $^{15}N$ (Atom%$_{excess}$), dry matter (DM in g plant$^{-1}$ and % of total DM) and total N (mg plant$^{-1}$) in various plant parts of pea (Santana and Frisson) and soil. Values show means ± standard error of the mean (n = 6 for Frisson and n = 7 for Santana).

| Plant parts and soil | | Santana (80 DAS) | Frisson (95 DAS) |
|---|---|---|---|
| Root | Atom%$^{15}N_{excess}$ | 0.6 ± 0.1 | 2.2 ± 0.1 |
| | DM (g plant$^{-1}$) | 0.5 ± 0.2 | 0.2 ± 0.01 |
| | DM (% of total) | 7.3 ± 1.9 | 1.5 ± 0.1 |
| | N (mg plant$^{-1}$) | 10.5 ± 2.5 | 3.9 ± 0.3 |
| Stem & leaf | Atom%$^{15}N_{excess}$ | 1.3 ± 0.1 | 4.1 ± 0.3 |
| | DM (g plant$^{-1}$) | 4.0 ± 0.2 | 4.3 ± 0.3 |
| | DM (% of total) | 62.8 ± 3.5 | 43.4 ± 3.1 |
| | N (mg plant$^{-1}$) | 65.5 ± 11.4 | 71.1 ± 11.3 |
| Grain | Atom%$^{15}N_{excess}$ | 1.2 ± 0.1 | 2.4 ± 0.2 |
| | DM (g plant$^{-1}$) | 2.0 ± 0.3 | 5.5 ± 0.5 |
| | DM (% of total) | 30.0 ± 2.7 | 55.1 ± 3.1 |
| | N (mg plant$^{-1}$) | 92.3 ± 17.8 | 232.0 ± 22.2 |
| Soil | Atom%$^{15}N_{excess}$ | 0.011 ± 0.003 | 0.004 ± 0.0004 |
| | DM (g plant$^{-1}$) | 5294 ± 281 | 5084 ± 56 |
| | N (mg plant$^{-1}$) | 3267 ± 73 | 7904 ± 76 |

On the other hand, the grain dry matter of Frisson was almost twice as high as that of Santana. The $^{15}N$ enrichment of Frisson was always higher than that of Santana, regardless of the plant part. These differences were mainly caused by the added amount of $^{15}N_{tracer}$. Santana was labelled twice and Frisson 5 times with 1 ml tracer solution. Differences in rhizodeposition were reflected by the $^{15}N$ enrichment in soil: Santana-cultivated soil was nearly three times more enriched than Frisson-cultivated soil.

At 95 DAS, the amount of NdfR and the proportion of NdfR as a percentage of total plant N did not differ for Frisson, independently of the calculation approach (Fig. 2.1, Table 2.1). In contrast, NdfR as % of total plant N differed significantly for Santana, depending on the calculation approach. The same was true for BGP-N as % of total plant N, calculated with the Janzen and Bruinsma equation and with mass balance (Fig. 2.2).

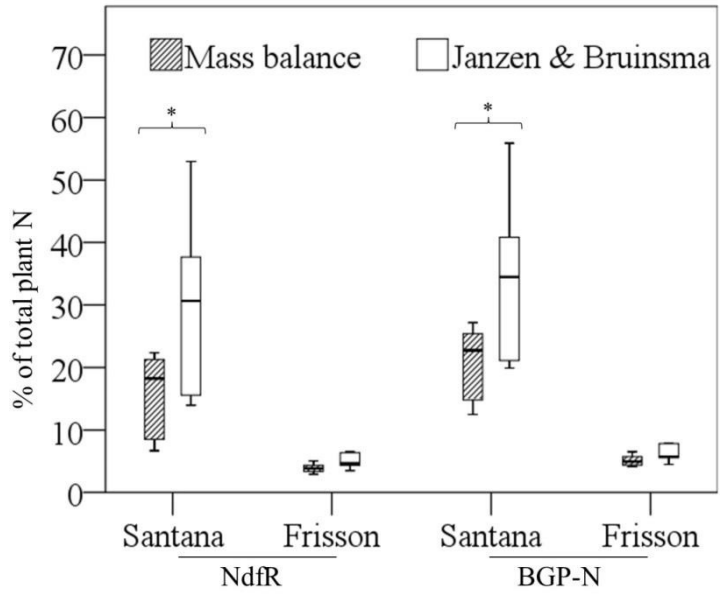


**Fig. 2.2**: N derived from rhizodeposition (NdfR in % of total plant N) and belowground plant N (BGP-N in % of total plant N) of pea (Santana and Frisson), calculated with mass balance and Janzen & Bruinsma equation. Values with * indicate significant differences between the means (n=6 for Frisson and n=7 for Santana; t-test, pairwise comparison, $p < 0.05$).

For Santana, the amount of NdfR was almost three times higher, calculated with the Janzen and Bruinsma equation, than that calculated with mass balance (Table 2.3). For Frisson, the amount of NdfR was only 30 % higher, calculated with the Janzen and Bruinsma equation, than that calculated with mass balance. In principle, amount and proportion of NdfR as % of total plant N was much larger for Santana compared with Frisson, regardless of the calculation approach. The same was true for amount and proportion of BGP-N.

**Table 2.3**: N derived from rhizodeposition (NdfR in % and mg plant$^{-1}$), belowground plant N (BGP-N in % and mg plant$^{-1}$) and recovery of $^{15}$N (%) in various plant parts, in soil, in wick system and in total of pea (Santana and Frisson). Values show means ± standard error of the mean (n = 6 for Frisson and n = 7 for Santana), calculated with mass balance and Janzen & Bruinsma equation. Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$) of the calculation approaches.

| | Santana (80 DAS) | | Frisson (95 DAS) | |
|---|---|---|---|---|
| | Mass balance | Janzen & Bruinsma | Mass balance | Janzen & Bruinsma |
| NdfR (% of total) | 15.3 ± 2.7 | 29.2 ± 5.7* | 3.9 ± 0.3 | 5.0 ± 0.5 |
| NdfR (mg plant$^{-1}$) | 32.7 ± 10.2 | 83.4 ± 31.3 | 12.5 ± 1.3 | 16.2 ± 1.9 |
| BGP-N (% of total) | 20.4 ± 2.3 | 33.5 ± 5.3* | 5.1 ± 0.4 | 6.2 ± 0.5 |
| BGP-N (mg plant$^{-1}$) | 43.2 ± 12.2 | 93.9 ± 33.3 | 16.3 ± 1.6 | 20.0 ± 2.2 |
| | Recovery $^{15}$N (%) | | | |
| Grain | 37.8 ± 4.0 | 35.3 ± 3.7 | 50.5 ± 0.5 | 47.3 ± 0.5 |
| Stem & leaf | 36.2 ± 4.0 | 33.8 ± 3.8 | 26.7 ± 4.4 | 25.0 ± 4.2 |
| Root | 2.0 ± 0.3 | 1.9 ± 0.3 | 0.8 ± 0.04 | 0.7 ± 0.03 |
| Soil | 14.2 ± 3.1 | 13.2 ± 2.9 | 3.2 ± 0.3 | 3.0 ± 0.3 |
| Wick system | 2.4 ± 0.5 | 2.4 ± 0.5 | 29.4 ± 2.5 | 29.4 ± 2.5 |
| Total | 92.5 ± 7.1 | 86.7 ± 6.6 | 110.6 ± 5.4 | 105.3 ± 5.2 |

*2.3.3. Recovery of $^{15}N$ in plant, soil and the labelling system*

During growth of both, Frisson and Santana, a large amount of $^{15}N_{tracer}$ was transferred into the pea plants. Some tracer solution remained in the labelling system. In general, the recovery of $^{15}N_{tracer}$ was higher in Frisson than in Santana (Table 2.3). Regardless of the calculation approach, most of the tracer recovered in plant and soil was accumulated in the grain (between 35 and 50 %, depending on the calculation approach and the variety). The absorption of the labelling solution was substantially reduced in the labelling system of Frisson due to formation of algae. Consequently, only 2.4 % of the tracer was found in the wick system of Santana, but almost 30 % in that of Frisson. NdfR as % of total N was much larger for Santana than for Frisson (Fig. 2.2), which was also reflected by the recovery of $^{15}N_{tracer}$ in soil (Table 2.3). Approximately 13 or 14 % of the tracer, depending on the calculation approach, was found in the Santana-cultivated soil, but only 3 % in the Frisson-cultivated soil.

## 2.4. Discussion

*2.4.1. Recovery*

The recovery of the tracer (without weighting of $^{15}N$ and $^{14}N$) is equal with the Janzen and Bruinsma equation and with mass balance. Due to the integration of the different atomic weights of $^{15}N$ and $^{14}N$, the recovery of the $^{15}N_{tracer}$ in plant and soil increases by 7 % (Table 2.3). The amount of N rhizodeposition increases only by 0.2–0.3 %, due to integrating the different atomic weights. However, for estimating real rhizodeposition different atomic weights should be included.

*2.4.2. Possible problems due to the calculation with enrichment values*

An important assumption for the Janzen and Bruinsma equation is an equal enrichment of roots over the growth period (Arcand et al., 2013a; Wichern et al., 2007b). As shown in Table 2.1, the $^{15}Natom\%_{excess}$ enrichment of soil increased between 71 DAS and 95 DAS from 0.0034 up to 0.0043 $^{15}Natom\%_{excess}$. Regardless of significance, different soil enrichment influences the calculated amount of rhizodeposition. In the present study, the enrichment of soil increases at the same time as the enrichment of roots. Consequently, the ratio of soil to root enrichment was equal during this period. With the Janzen and Bruinsma equation, an increasing amount of N rhizodeposition cannot be determined, assuming a constant N content in soil. The amount of N rhizodeposition was precisely 16.2 mg $plant^{-1}$ (Table 2.1) for both harvest dates. However, the

data clearly showed that the enrichment of soil and, thus, the amount of tracer-derived from N rhizodeposition increased from 0.0034 atom%$_{excess}$ to 0.0043 atom%$_{excess}$. This increase of N rhizodeposition can be detected with the mass balance approach. N rhizodeposition increased from 10.6 mg plant$^{-1}$ 71 DAS to 12.5 mg plant$^{-1}$ 95 DAS. As the mass balance uses real masses, small changes in enrichment can be detected as rhizodeposition. As Janzen and Bruinsma (1989) concentrated exclusively on the enrichment of soil and root, assuming that the ratio of soil and root enrichment is equal over time, their equation cannot calculate small differences in rhizodeposition and the calculated amount of rhizodeposition becomes inaccurate.

### *2.4.3. Leakage*

At 45 DAS, the amount of N rhizodeposition calculated with the Janzen and Bruinsma equation was twice as high as the amount of N rhizodeposition calculated with the mass balance (Table 2.1). The results of the two calculation approaches converge during plant growth. Gasser et al. (2015) observed a significant leakage of 0.5 % of the applied $^{15}$N within the first day after labelling. This leakage effect is a possible reason for an overestimation of N rhizodeposition using the Janzen and Bruinsma equation. Due to the assumption that the enrichment of roots is equal to that of rhizodeposits, the amount of $^{14}$N from soil pool assigned to N rhizodeposition will be overestimated.

The classical approach to calculate the N rhizodeposition refers to the soil N content. In contrast, the mass balance calculates N rhizodeposition as a proportion of the whole plant N, which increases with time. Due to the lower amount of plant N 45 DAS, the mass balance approach is less sensitive to a possible leakage than the classical calculation of Janzen and Bruinsma (1989). It is assumed that the proportion of tracer released into soil to the total amount of tracer is identical to the proportion of NdfR to the total amount of plant N. Because of the low total plant N 45 DAS (80 mg N plant$^{-1}$) in comparison with 95 DAS (500 mg plant$^{-1}$), the proportion of $^{14}$N, which is wrongly assigned by the leakage effect to the amount of N rhizodeposition, is also low. With mass balance, the amount of a possible overestimation of N rhizodeposition due to leakage of the applied $^{15}N_{tracer}$ depends on the total amount of plant N.

*2.4.4. Tracer distribution and dilution*

There are many different approaches for labelling plants to quantify rhizodeposition, like root (Schmidtke, 2005), shoot (Gylfadóttir et al., 2007; Wichern et al., 2009) or atmosphere labelling (Janzen and Bruinsma, 1989; Schmitt et al., 2012; Soong et al., 2014). Substantial differences can be found in the possible duration of labelling (Arcand et al., 2013a) and here in particular in the period of time from the last labelling to the harvest date. One single pulse of tracer or an increasing period between labelling date and harvest day may cause significant differences in root enrichment and tracer distribution (Fig. 2.3; Table 2.4).

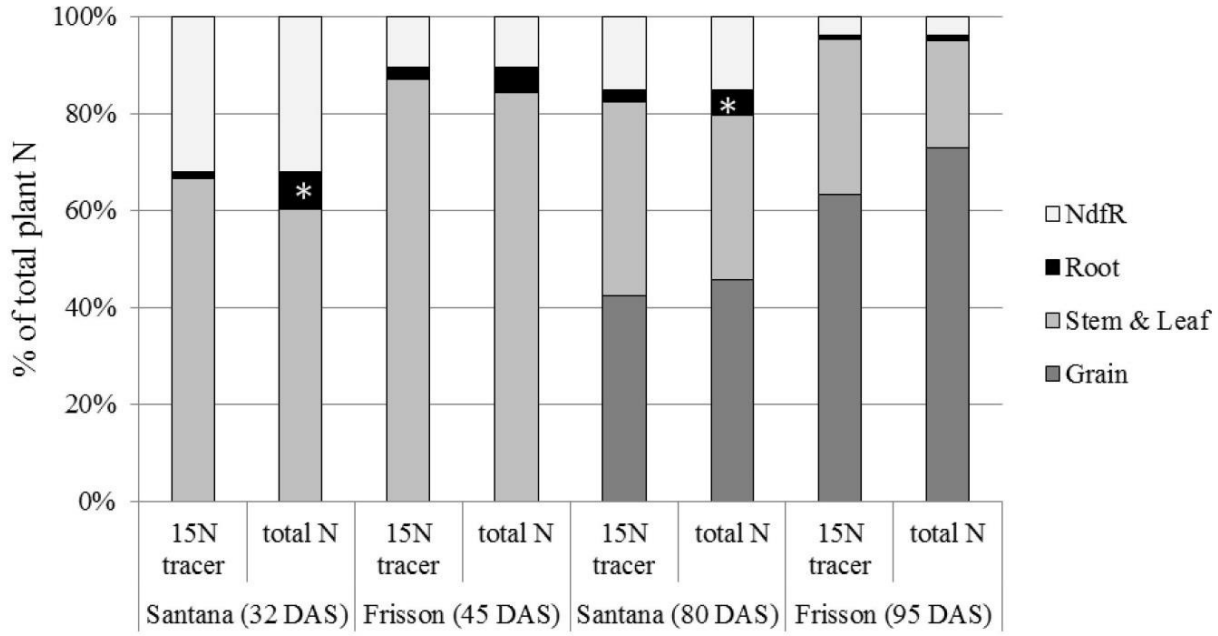


**Fig. 2.3**: Distribution of $^{15}N_{tracer}$ and total plant N in pea (Santana harvested 32 DAS and 80 DAS; Frisson harvested 45 DAS and 95 DAS). Values with * indicate significant differences between the means (n=5 for Frisson 45 DAS, n=6 for Frisson 95 DAS and n=7 for Santana; t-test, pairwise comparison, $p < 0.01$).

In the case of Santana, root growth was not completed after labelling, so the distribution of tracer did not correspond to the distribution of plant N. For Santana, root $^{15}N_{tracer}$ as % of total $^{15}N_{tracer}$ was significantly lower ($p < 0.001$) than the root N as % of total plant N. This indicates that pulse labelling (cf. Santana, harvested 32 DAS; Table 2.4) leads to an uneven distribution of the tracer and is a typical reason for overestimating N rhizodeposition, using the Janzen and Bruinsma equation. This is also obvious in the $^{15}N_{tracer}$ and N distribution in Frisson and Santana (Fig. 2.3). Wichern et al. (2009) already mentioned how important the label distribution is for calculating rhizodeposition. They point out the lack of documentation of tracer and plant N distribution in past studies and recommend that these details be included in future studies.

**Table 2.4**: Tracer uptake (mg plant$^{-1}$), number of pulses and NdfR (% of total) of pea (Frisson and Santana). Values show means ± standard error of the mean (n = 5 for Frisson harvested 45 DAS, n = 6 for Frisson harvested 95 DAS and n = 7 for Santana), calculated with mass balance and Janzen & Bruinsma equation. Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$) of the calculation approaches

| | Tracer uptake (mg plant$^{-1}$) | Number of pulses | NdfR (% of total) | |
|---|---|---|---|---|
| | | | MB | J & B |
| Santana (32 DAS) | 0.23 | 1 (24 DAS) | 32 ± 2.9 | 71 ± 3.0* |
| Frisson (45 DAS) | 1.89 | 2 (14, 28 DAS) | 10 ± 1.0 | 19 ± 2.2* |
| Santana (80 DAS) | 2.66 | 2 (33, 51 DAS) | 15 ± 2.7 | 29 ± 5.7* |
| Frisson (95 DAS) | 9.31 | 5 (14, 29, 42, 57, 70 DAS) | 4 ± 0.3 | 5 ± 0.5 |

Tracer dilution in roots also can be caused by plant growth, combined with N relocation (Hertenberger and Wanek, 2004). A previous experiment with peas has shown that up to 11 % grain N comes from the roots (Schiltz et al., 2005). Beginning 22 DAS, Janzen and Bruinsma labelled wheat plants with a $^{15}NH_3$ enriched atmosphere (22.193 atom%), over a period of 4 to 5 days. Plants were harvested 58 DAS, immediately after the last labelling. This labelling and sampling procedure guaranteed a homogeneous and continuous enrichment of roots.

In the present study, for Santana, significant differences between the amount of N rhizodeposition calculated with the Janzen and Bruinsma equation or calculated with the mass balance approach could be shown 80 DAS (Fig. 2.2; Table 2.3). In contrast, for Frisson, no differences in the calculated amount of N rhizodeposition could be determined 95 DAS. This may be due to the different labelling periods in both experiments or to the different times from the last labelling to harvest. Frisson was labelled over 10 weeks beginning 14 DAS. At this time, peas were strong enough to survive the installation of a labelling system. The peas were harvested almost 3 weeks after the last labelling. For these reasons, Frisson was almost completely homogeneously labelled as demonstrated by the equal enrichment of roots over time (Table 2.1) and by the homogenous distributions of $^{15}N_{tracer}$ and plant N (Fig. 2.3). Development of pea roots is mostly completed with flowering (Weaver and Bruner, 1927; Arcand et al., 2013b). So tracer dilution because of root growth may be excluded for the period of last labelling date and the date of harvest. Santana was labelled only twice, 33 and 45 DAS, when growth was not completed, so that root growth and processes of relocation may have caused tracer dilution.

The mass balance approach compensates possible tracer reallocation processes, because N rhizodeposition is calculated as a proportion of total plant N. For this reason, relocation processes in the plant do not influence the calculation of rhizodeposition. A high enrichment of the plant, in combination with multiple pulse labelling at an early stage of plant development, and finished shortly before harvest leads to a very similar amount of N rhizodeposition, when comparing the two different methods of calculation. If tracer dilution because of root growth or processes of relocation cannot be excluded, a mass balance approach can be the more precise way to calculate the rhizodeposition of plants. For a mass balance approach as well as for the Janzen and Bruinsma equation, the labelling needs to be constant over the vegetation period to prevent an over or underestimation of rhizodeposition. Regardless of the calculation approach for rhizodeposition, possible measurement errors need to be considered. By integrating the possible

error of mass spectrometry, the calculated amount of N rhizodeposition changes by up to 10%. The influence of the measurement error becomes smaller with increasing intensity of enrichment. Therefore, the aim should be the highest possible enrichment of plants. The current study was focused on N rhizodeposition of pea plants; however, the results can most likely also be transferred to C rhizodeposition.

## 2.5. Conclusions

For estimating rhizodeposition is important to accurately quantify the BGP for assessing C sequestration potential of plants and N storage of crops. As shown in this study using an isotopic labelling approach can result in substantial over- or underestimation using the Janzen and Bruinsma equation. Most studies estimating rhizodeposition, especially N-rhizodeposition, used pulse labelling approaches and consequently resulted in overestimation of rhizodeposition. Future studies thus have to guarantee continuous labelling of plants when aiming at quantifying BGP using isotope labelling methods. We thus urgently need alternative approaches to accurately quantify BGP-C and N in addition to the isotope labelling approach.

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# 3. Even flow? Changes of carbon and nitrogen release from pea roots over time

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**Abstract**

The C and N rhizodeposition represents an important part of belowground plant C and N (BGP-C; BGP-N). Therefore, a precise estimation of rhizodeposition can improve N balances or the estimation of the C sequestration potential of plants. The quality and quantity of rhizodeposition is expected to be affected by plant growth stage and soil microorganisms, especially arbuscular mycorrhizal fungi (AMF). To estimate the development of rhizodeposition, its microbial utilization over time and the influence of mycorrhiza, a 95-day pot experiment was carried out comparing a non-mycorrhizal and non-nodulating pea (*Pisum sativum* L. mutant P2) variety and its symbiotic isoline Frisson. The release of C and N from roots and the transfer into microbial biomass C and N, extractable organic C and inorganic N was investigated at four growth stages (beginning of flowering, end of flowering, green ripeness, and maturity). To approximate continuous and homogeneous labelling, the peas were labelled fortnightly with a solution of 2% $^{13}$C glucose (99 atom%) and 0.5% $^{15}$N urea (95 atom%), using the cotton wick method starting at three leaves unfolded. No significant differences could be detected for the incorporation of C and N derived from rhizodeposition (CdfR and NdfR) into the microbial biomass or into the extractable soil pool over time, even though a steady increase was observed. Nevertheless, microbial incorporation of rhizodeposits was on a low level throughout, indicating that rhizodeposits are not an easily available source of C and N. At the time of highest mycorrhization, Frisson released significantly more NdfR than P2, indicating a stronger N transfer into the mycorrhizosphere. For CdfR, however, no significant AMF effects could be found. Our results indicate that CdfR and NdFR release from roots follow different patterns: an even flow of C, driven by exudation at an earlier growth stage and root senescence during maturation and a steady flow of N, probably caused by N translocation within the plant or re-uptake into the plant during maturation. These changes in rhizodeposition quality have to be considered for C turnover studies and sequestration assessment.

*Key words*: $^{13}$C; $^{15}$N; belowground plant biomass; mycorrhiza; rhizodeposition; time course

## 3.1. Introduction

The rhizodeposition of plants represents an essential C and N input into soil and has been repeatedly studied (Wichern et al., 2008; Pausch et al., 2013). The calculation of rhizodeposition helps to improve N balances (Mayer et al., 2003a,b) and to estimate the C sequestration potential of plants, as this input source of plant biomass is often neglected, causing wrong C and nutrient budgets (Wichern, 2007). Uren (2007) defined rhizodeposition as the process of release of a mixture of root exudation, sloughed cells and tissue root fragments from root turnover, providing a rather broad definition, which is also used in the present study to consider the variety of C and N molecules released from roots. Moreover, rhizodeposition is a dynamic process, affected by biotic factors, such as plant species or variety and mycorrhiza (Jones et al., 2004; Rees et al., 2005) and abiotic factors, like soil properties (e.g. soil compaction, nutrient availability) (Rees et al., 2005) or climatic factors such as drought stress (Salon et al., 2007).

During the vegetation period, the N concentration in various plant parts can vary substantially (Salon and Munier-Jolain, 2010). The changes are caused by N availability and N translocation or remobilization processes, also affecting the quality and quantity of rhizodeposition (Arcand et al., 2013b). In pea plants, C is mainly transported as sucrose in the phloem (Kühn and Grof, 2010), whereas N is especially transported in the plant xylem, e.g. as urea (Witte, 2011) or as amino acids (Lalonde et al., 2003; Salon and Munier-Jolain, 2010). As a consequence, xylem sap of peas has a low C/N ratio of 1.5-6, whereas the C/N ratio of the phloem sap is often higher (Salon and Munier-Jolain, 2010). The phloem also delivers C compounds to the root system, where they are required for cell metabolism and division, leaving a large proportion of it respired and lost as $CO_2$. On the other hand, N is mainly taken up from the soil and transferred to the sinks, where it is required to build storage or structural proteins. As the dominating movement of C-assimilates is downward while that of N-compounds is upward, differences between C and N rhizodeposition patterns can be expected. To elucidate this, double labelling of plants with $^{13}C$ and $^{15}N$ isotopes in the form of a glucose-urea mixture is particularly suitable for measuring the time-course of C and N rhizodeposition of peas and oats (Wichern et al., 2007b) or chickpeas (Yasmin et al., 2010). In these experiments, C rhizodeposition ranged from 25 to 31% of total plant C and N rhizodeposition similarly from 28 to 36% of total plant N.

Quality and quantity of rhizodeposition are expected to change with time, depending on plant development as well as root growth and root turnover (Gavito et al., 2001). Arcand et al. (2013b)

studied the temporal dynamics of N rhizodeposition of peas. They found that the amount of N rhizodeposition increased over the course of time. They concluded that in the vegetative stage, N rhizodeposition mainly consisted of root exudates, whereas at maturity the amount of N rhizodeposition was dominated by N from root turnover (Arcand et al. 2013b).

Arbuscular mycorrhizal fungi (AMF), which form a symbiosis with over 80% of land plant species (Leifheit, 2014), provide additional phosphorus, water and micronutrients for the host plants in exchange for assimilates (Smith et al., 2010), resulting in a strong C flux into the mycorrhizosphere, amounting to as much as 20% of assimilated C (Parniske, 2008). AMF also affects root architecture, improve soil aggregation and influences the quality and quantity of rhizodeposition (Rillig and Mummey, 2006; Leifheit, 2014). A potentially useful, but not fully evaluated tool for investigating AMF effects on rhizodeposition is the use of AMF-resistant pea (*Pisum sativum* L.) mutants and their symbiotic isolines (Kleikamp and Joergensen, 2006; Jannoura et al., 2012).

The amount of roots and rhizodeposits and its microbial utilization shape the C sequestration potential of plants. Additionally, N is also stored in the belowground biomass and is partly immobilized and partly released by mineralization. To improve our understanding of these processes, a pot experiment was carried out, using a mycorrhizal and a non-mycorrhizal pea variety, labelled with $^{13}C$ and $^{15}N$, using a cotton wick stem feeding technique (Russell and Fillery, 1996; Wichern et al., 2007b). The underlying hypothesis is that AMF generally increase pea rhizodeposition by increasing C flow into the mycorrhizosphere. However, as mycorrhizal colonization depends on pea development, AMF effects on rhizodeposition may be lower during maturation and may differ for C and N. The differences in quantity and quality of rhizodeposition might also be reflected by differences in their incorporation into the microbial biomass C and N as well as in extractable organic C and inorganic N.

## 3.2. Materials and methods

### *3.2.1. Soil*

The soil, collected at 0-30 cm depth from the research station of the University of Kassel in Neu-Eichenberg, Germany (51º23´N, 9º 55´E 220 m asl), was sieved (10 mm) and stored for six weeks before the experiment started in boxes at 10 to 15 ºC in the dark. The silty loam (13% clay; 83% silt; 3% sand) was classified as a Haplic Luvisol according to the WRB-FAO classification system, with a pH of 6.0, an organic carbon content of 12 mg $g^{-1}$ soil and total nitrogen content of

1.3 mg $g^{-1}$ soil. One day before sowing, the soil was fertilized with N, P, K, Ca and Mg at a dose of 150, 17, 43, 100 and 9 kg $ha^{-1}$ to provide sufficient nutrients also for the non-mycorrhizal and non-nodulating pea mutant P2. According to Mahieu et al. (2009), with a high N fertilization, no differences in plant dry matter of Frisson and P2 are expected.

*3.2.2. Experimental design and labelling*

The experiment was carried out at the University of Kassel, Faculty of Organic Agricultural Sciences in Witzenhausen, Northern Hesse, Germany. *Pisum sativum* L. cv. Frisson and the non-mycorrhizal ($myc^-$) and non-nodulating ($nod^-$) pea mutant of Frisson, P2, were cultivated under controlled conditions in the greenhouse with a relative humidity of 60% and 110 klxh $d^{-1}$. The mean temperature was 20 ºC during the day and 15 ºC during the night. Pots of 8.5 l (28 cm in diameter and 20 cm long) were filled with 11 kg of soil, which was mechanically compressed to a density of 1.3 g $cm^{-3}$. Two plants per pot were cultivated with six replicates per treatment. At every sampling date, 6 pots with labelled Frisson, 6 pots with labelled P2 and the same number of pots with unlabelled plants of Frisson and P2 were harvested. All pots were inoculated with 5 g "rootgrowth™ professional", placed directly below the seeds to ensure AMF symbiosis. Once a week, the pots were weighed and watered with deionized water, to keep the water holding capacity between 60 and 80%.

The plants were labelled with a 0.5% $^{15}$N-urea (95 atom %) and 2% $^{13}$C-glucose (99 atom %) solution, using the cotton wick stem feeding technique (Russel and Fillery, 1996; Wichern et al., 2007b). The stem of each plant was drilled with a 0.5 mm diameter drill, approximately 3 cm above the ground. Then, a cotton wick was passed through the hole in the stem. The ends of the wick were passed through silicon tubes and through the lid of a 2 ml vial, which contained 1 ml of the labelling solution. During the labelling period, each plant received 5 ml of the labelling solution in total. To prevent evaporation losses, the connections between wick and plant or wick and lid were sealed with kneading mass (Teroson, Henkel). The labelling solution was produced using deionized water and then sterile filtrated (< 0.2 µm). All material used for labelling was steam sterilized for at least 20 min at a temperature of 121 ºC. For approximating continuous labelling, the plants were labelled fortnightly, five times during the experiment, beginning 14 days after sowing (DAS), with three leaves unfolded. Solution uptake varied in time. Plants needed at least 24 hours to take up 1 ml solution. Often solution uptake was incomplete and the residual solution remained in the vials until the next labelling event. After solution uptake, the

empty vials were filled with 0.5 ml deionized water to guarantee complete solution uptake. Pots were covered with mesh (1 mm) to prevent soil contamination from falling leaves. For measuring the natural abundance of $^{13}C$ and $^{15}N$ of the pea plants, as background values for calculating rhizodeposition, unlabelled control plants of Frisson and P2 were cultivated.

*3.2.3. Sampling and analyses*

At harvest, labelling systems were carefully removed from peas and stored at -18°C for analyses of remaining $^{13}C$ and $^{15}N$. Frisson and P2 plants were harvested at four different growth stages, depending on plant development, beginning 45 DAS (first petals visible, flowers still closed; beginning of flowering), 63 DAS (end of flowering), 71 DAS (pods have reached typical size; green ripeness) and 95 DAS (fully ripe: all pods are dry and brown; seeds are dry and hard; maturity/dry ripeness). All pea plants were separated into roots, stem and leaves (incl. pods 63, 71 and 95 DAS) and grain. After weighing, plant parts were stored for at least 48 h at 60 ºC to estimate dry matter. The dry plant parts were ground with a ball mill, weighed into tin cups and analyzed for total C, total N and the isotope ratio of $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

At harvest, the soil was weighed and a subsample was dried for 24 h at 105 ºC to estimate dry matter. Another subsample was taken for analyzing total C and N content and the isotope ratio of $^{13}C/^{12}C$ and $^{15}N/^{14}N$. For this, the soil was sieved (< 2 mm) and all visible roots were removed manually, and the soil was dried, ground and weighed into tin cups (as described above for plant parts). Total C and total N were analyzed with an elemental analyzer (Fisons-Instruments, Rodano, Milano, Italy). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios were determined by isotope ratio mass spectrometry (Finnigan MAT, Bremen, Germany).

To calculate the total recovery of the isotopes, the vials and labelling systems were extracted with 200 ml of 0.05 M $K_2SO_4$ and organic C and total N in the extracts were analyzed using a CN Analyzer (Multi N/C 2100S, Analytik Jena, Germany). The remaining amount of $^{13}C$ and $^{15}N$ in the extracts was calculated, assuming that the measured C and N in the extracts are solely derived from the isotope solution.

Microbial biomass C and N were determined by the fumigation extraction method (Brookes et al., 1985; Vance et al., 1987), including a pre-extraction step to remove living roots (Mueller et al., 1992; Mayer et al., 2003a,b). For the pre-extraction, 30 g of soil (after removing visible roots) and 70 ml 0.05 M $K_2SO_4$ were horizontally shaken for 30 min and 200 rev $min^{-1}$. Then, the soil solution was centrifuged for 10 min at 4000 g and 6 ºC. The supernatant was filtered to remove

the living roots and frozen for organic C, total N, inorganic N, and isotope ratio analysis. Then, 15 g of the pre-extracted soil was fumigated with $CHCl_3$ for 24 h at 25 ºC in the dark. After removal of the $CHCl_3$, the fumigated soil was extracted with 60 ml 0.05 M $K_2SO_4$ (Potthoff et al., 2003). While fumigation commenced, 15 g of the pre-extracted soil was extracted with 0.05 M $K_2SO_4$, providing the non-fumigated samples. Organic C and total N in the extracts were analyzed using a CN Analyzer (Multi N/C 2100S, Analytik Jena, Germany). After freeze-drying, the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios in the extracts (now dry salts) were also determined by isotope ratio mass spectrometry. Microbial biomass C was calculated as $EC/k_{EC}$, where EC = (organic C extracted from fumigated soils) – (organic C extracted from non-fumigated soils) 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 soils) - (total N extracted from non-fumigated soils) and $k_{EN}$ = 0.54 (Joergensen and Mueller, 1996).

*3.2.4. Calculation and statistical analyses*

The percentage (1 and 2) and the amount of C and N derived from rhizodeposition (CdfR and NdfR, respectively) were calculated with a mass balance approach as described in Hupe et al. (2016a).

$$^{13}C_{tracer}dfR\ (\text{in }\%) = \frac{^{13}C_{\ tracer}\ dfR}{^{13}C_{\ tracer}\ \text{in total}} \times 100 \qquad (1)$$

$$^{15}N_{tracer}dfR\ (\text{in }\%) = \frac{^{15}N_{\ tracer}\ dfR}{^{15}N_{\ tracer}\ \text{in total}} \times 100 \qquad (2)$$

The distribution of the tracer in the plant corresponds to the distribution of total plant C and N, respectively. For this reason, the rhizodeposition of $^{13}C_{tracer}dfR$ (%) and $^{15}N_{tracer}dfR$ correspond to the total C and N rhizodeposition (CdfR and NdfR, respectively). Microbial C and N, extractable C and inorganic N derived from rhizodeposition were calculated using an isotope mass balance approach.

Statistical analyses were performed using IBM SPSS Statistics 22. Differences of the means between the date of harvest (depending on plant development) were tested by a one-way analysis of variance (ANOVA) using the Scheffé post-hoc test. Student`s t-test was performed on pairs of means to determine differences between both the CdfR and NdfR of the pea varieties Frisson and P2, respectively. Data were normally distributed and variances were homogeneous.

## 3.3. Results

### *3.3.1. Tracer uptake, plant enrichment and tracer recovery*

Three weeks after the beginning of labelling, at beginning of flowering, pea plants had taken up between 4 mg (Frisson) and 6 mg (P2) $^{13}C$ (Table 3.1) and 2 mg $^{15}N$ (Table 3.2). At maturity (9 weeks after beginning of labelling), the $^{15}N$ uptake increased to 9 mg (Frisson) and 7 mg (P2). At the same time, the $^{13}C$ uptake increased to 21 mg (Frisson) and 17 mg (P2), indicating that the wick technique can be used for multiple pulse labelling of plants.

During plant growth, the multiple pulse labelling resulted in a homogeneous enrichment of $^{13}C$ and $^{15}N$ tracers in roots and shoots, with no substantial changes in plant enrichment over time. Regardless of time, the $^{13}C$ and $^{15}N$ enrichment of the P2 grain was in most cases twice as high in comparison with the Frisson grain. The $^{13}C$ tracer recovery was similar for Frisson and P2 and varied between 39 and 57% of the applied amount. Approximately 75% of the recovered $^{13}C$ amount was found in the plant, 5% in the soil and roughly 20% in the labelling systems. In contrast, 80 to 100% of the $^{15}N$ applied was recovered, with most of it found in the plant, about 4% in the soil, and roughly 30% in the labelling systems.

**Table 3.1**: Uptake (in mg plant-1), recovery (in plant, soil, wick system and total, in % of applied) and enrichment (of various plant parts and soil in atom%excess) of 13C in pea (Frisson and P2, harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). Values show means ± standard error of the mean (Frisson: n=4 for 45 and 63 DAS and n=6 for 71 and 95 DAS; P2: n=6;

| $^{13}C$ | Uptake (mg plant$^{-1}$) | Recovery (% of applied) | | | | Atom%$_{excess}$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | In plant | In soil | In wick system | In total | Soil | Root | Grain | Shoot |
| Frisson | | | | | | | | | |
| 45 DAS | 4 ± 0.8 | 25 ± 4.6 | 2 ± 0.9 | 12 ± 2.4 | 39 ± 6.5 | 0.001 a | 0.5 a | NA | 0.9 a |
| 63 DAS | 13 ± 1.8 | 35 ± 5.8 | 3 ± 0.4 | 7 ± 1.0 | 46 ± 5.8 | 0.002 bc | 0.6 a | 0.2 a | 0.8 a |
| 71 DAS | 11 ± 0.8 | 30 ± 2.2 | 2 ± 0.2 | 7 ± 0.5 | 39 ± 2.2 | 0.001 ac | 0.4 a | 0.1 b | 0.4 a |
| 95 DAS | 21 ± 1.7 | 47 ± 4.1 | 3 ± 0.3 | 7 ± 0.3 | 57 ± 4.0 | 0.002 b | 0.5 a | 0.2 c | 0.8 a |
| P2 | | | | | | | | | |
| 45 DAS | 6 ± 1.1 | 32 ± 6.5 | 3 ± 0.2 | 8 ± 1.3 | 43 ± 5.6 | 0.001 a | 0.6 a | NA | 0.9 a |
| 63 DAS | 12 ± 0.7 | 34 ± 2.1 | 2 ± 0.2 | 8 ± 0.8 | 43 ± 1.9 | 0.001 a | 0.5 a | 0.4 a | 0.7 a |
| 71 DAS | 13 ± 2.3 | 35 ± 7.5 | 3 ± 1.0 | 8 ± 0.4 | 45 ± 6.7 | 0.001 a | 0.5 a | 0.2 b | 0.5 a |
| 95 DAS | 17 ± 0.6 | 38 ± 1.5 | 2 ± 0.4 | 6 ± 0.7 | 47 ± 1.7 | 0.001 a | 0.6 a | 0.4 a | 0.9 a |

NA = not applicable

**Table 3.2**: Uptake (in mg plant-1), recovery (in plant, soil, wick system and total, in % of applied) and enrichment (of various plant parts and soil in atom%excess) of 15N in pea (Frisson and P2, harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). Values show means ± standard error of the mean (Frisson: n=5 for 45DAS, n=4 for 63 DAS and n=6 for 71 and 95 DAS; P2: n=6; except 71 DAS: n=5). Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$).

| $^{15}N$ | Uptake (mg plant$^{-1}$) | Recovery (% of applied) | | | | Atom%$_{excess}$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | In plant | In soil | In wick system | In total | Soil | Root | Grain | Shoot |
| Frisson | | | | | | | | | |
| 45 DAS | 2 ± 0.2 | 36 ± 3.8 | 4 ± 0.9 | 45 ± 8.3 | 86 ± 8.3 | 0.002 a | 1.9 a | NA | 4.0 a |
| 63 DAS | 5 ± 0.2 | 50 ± 2.2 | 5 ± 0.8 | 29 ± 2.6 | 84 ± 2.9 | 0.005 b | 2.5 a | 2.9 a | 3.8 a |
| 71 DAS | 6 ± 0.3 | 62 ± 3.3 | 3 ± 0.3 | 32 ± 2.4 | 97 ± 3.3 | 0.003 ab | 1.7 a | 1.7 b | 2.7 a |
| 95 DAS | 9 ± 0.5 | 78 ± 4.4 | 3 ± 0.3 | 29 ± 2.5 | 110 ± 5.4 | 0.004 ab | 2.2 a | 2.4 a | 4.1 a |
| P2 | | | | | | | | | |
| 45 DAS | 2 ± 0.2 | 44 ± 4.9 | 4 ± 0.7 | 33 ± 4.7 | 81 ± 2.8 | 0.002 a | 2.0 a | NA | 4.3 a |
| 63 DAS | 5 ± 0.3 | 49 ± 2.8 | 4 ± 0.2 | 32 ± 3.4 | 85 ± 3.2 | 0.004 a | 1.9 a | 4.7 ab | 4.9 a |
| 71 DAS | 5 ± 0.4 | 54 ± 4.8 | 4 ± 0.9 | 33 ± 2.1 | 90 ± 4.3 | 0.004 a | 1.8 a | 3.3 a | 3.6 a |
| 95 DAS | 7 ± 0.2 | 60 ± 1.9 | 3 ± 0.5 | 29 ± 1.2 | 93 ± 2.1 | 0.004 a | 2.1 a | 5.1 b | 5.0 a |

NA = not applicable

*3.3.2. Development of Frisson plants and rhizodeposition*

Between beginning of flowering and maturity, C distribution among the different plant parts was similar to that of N (Fig. 3.1 and 3.2). Until green ripeness, C and N in shoots showed a roughly fourfold increase, followed by a 23% and 58% decrease, respectively, between green ripeness and maturity. Between green ripeness and maturity, the amounts of C and N in grains increased more than five times to 2,370 mg and 230 mg plant$^{-1}$. The amount of root C and N roughly doubled until green ripeness. Then, the root C showed a 20% decrease, whereas root N remained constant between green ripeness and maturity.

The amount of CdfR showed a significant fivefold increase between beginning of flowering and maturity, (Table 3.3), whereas NdfR only doubled between beginning of flowering and end of flowering. Subsequently, the amount of NdfR remained roughly constant at 12 mg plant$^{-1}$. The contribution of BGP-C and BGP-N to total plant C and plant N significantly decreased with time from 15% to roughly 6% at maturity.

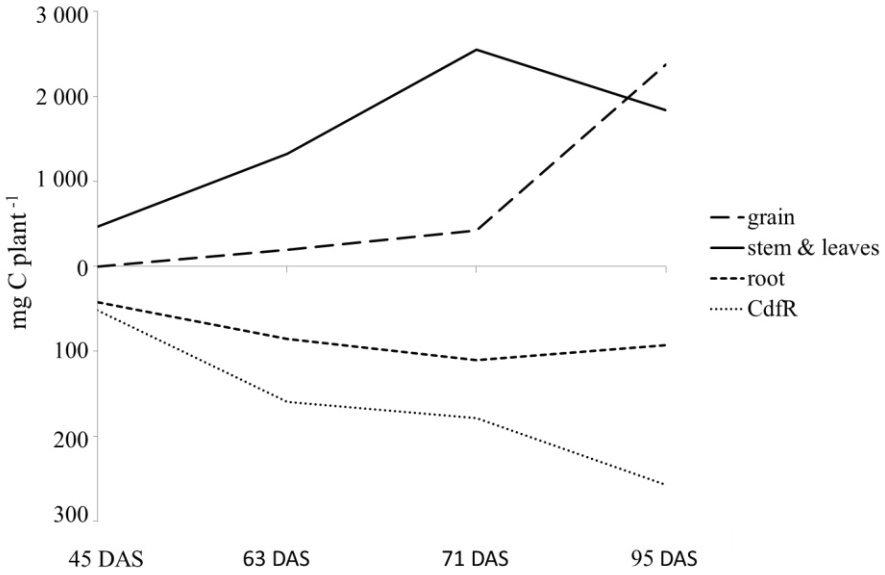


**Fig. 3.1**: Distribution of C (in mg plant$^{-1}$) in various plant parts of Frisson (grain, shoot and root) and N derived from rhizodeposition (in soil), harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS).

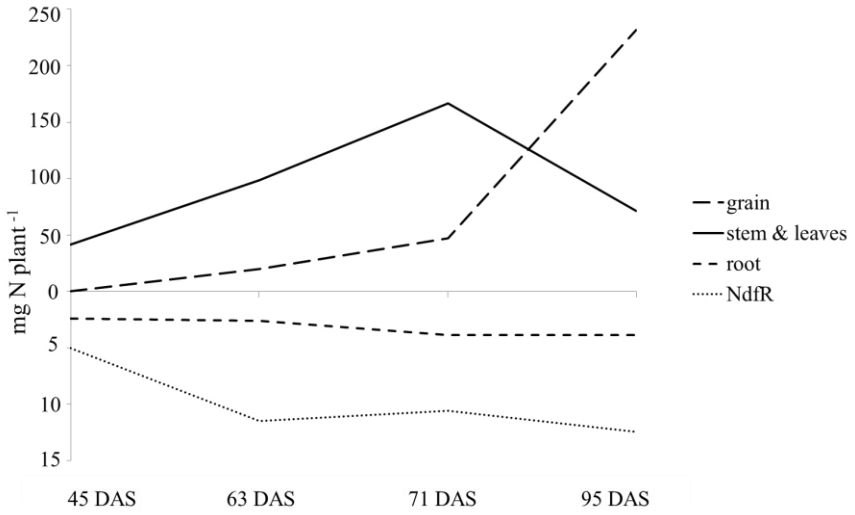


**Fig. 3.2**: Distribution of N (in mg plant$^{-1}$) in various plant parts of Frisson (grain, shoot and root) and N derived from rhizodeposition (in soil), harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS).

**Table 3.3**: Mycorrhization of Frisson (in % of total root numbers), N and C derived from rhizodeposition (NdfR & CdfR) in percentage of total plant N / C and in mg plant-1 and below-ground plant (BGP) N / C (NdfR plus N in roots; CdfR plus C in roots) in percentage of total plant N / C of Frisson and P2, harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$). Values with * indicate significant differences between the means of Frisson and P2 (t-test, pairwise comparison, $p < 0.05$).

| | Mycorrhization (% of total root numbers) | NdfR | | BGP-N | CdfR | | BGP-C |
|---|---|---|---|---|---|---|---|
| | | % of total N | mg plant$^{-1}$ | % of total N | % of total C | mg plant$^{-1}$ | % of total C |
| Frisson | | | | | | | |
| 45 DAS | 32 ± 5 a | 10 ± 1.0 a | 5 ± 0.7 a | 16 ± 1.5 a * | 8 ± 3.1 a | 52 ± 25.7 a | 15 ± 2.7 a |
| 63 DAS | 21 ± 4 ab | 9 ± 1.3 a | 12 ± 2.0 b | 11 ± 1.5 b | 9 ± 2.1 a | 160 ± 46.8 ab | 12 ± 2.2 ab |
| 71 DAS | 17 ± 2 b | 5 ± 0.3 b | 11 ± 1.1 b | 6 ± 0.4 bc | 6 ± 0.6 a | 179 ± 30.7 ab | 7 ± 0.6 b |
| 95 DAS | 17 ± 3 b | 4 ± 0.3 b | 13 ± 1.3 b * | 5 ± 0.4 c | 6 ± 0.7 a | 257 ± 40.5 b | 7 ± 0.8 b |
| P2 | | | | | | | |
| 45 DAS | - | 8 ± 1.0 a | 4 ± 0.5 a | 12 ± 1.0 a | 9 ± 1.2 a | 54 ± 7.4 a | 14 ± 1.2 a |
| 63 DAS | - | 7 ± 0.4 a | 7 ± 0.7 a | 9 ± 0.4 ab | 6 ± 0.7 a | 107 ± 19.9 a | 8 ± 0.7 a |
| 71 DAS | - | 7 ± 2.2 a | 9 ± 2.4 a | 9 ± 2.3 ab | 9 ± 3.2 a | 240 ± 77.5 a | 11 ± 3.3 a |
| 95 DAS | - | 4 ± 0.8 a | 6 ± 1.4 a | 6 ± 0.8 b | 6 ± 1.0 a | 162 ± 36.1 a | 8 ± 1.0 a |

Values show means ± standard error of the mean (N in Frisson: n=5 for 45 DAS, n=4 for 63 DAS and n=6 for 71 and 95 DAS; C in Frisson: n=4 for 45 and 63 DAS and n=6 for71 and 95 DAS; N and C in P2: n=6; except 71 DAS: n=5).

*3.3.3. Mycorrhization and rhizodeposition*

AMF colonization of Frisson roots significantly decreased with time, starting from 32% at beginning of flowering to 17% at green ripeness and maturity (Table 3.3). No AMF colonization of P2 roots was detected and also no nodulation, as expected. Only at maturity was the NdfR of Frisson (13 mg plant$^{-1}$) significantly higher than that of P2 (6 mg plant$^{-1}$). CdfR in mg plant $^{-1}$ or mg g$^{-1}$ plant dry matter did not differ between Frisson and P2, regardless of plant development or root mycorrhization (Fig. 3.3). As Frisson produced more dry matter than P2 (differently than expected; cf. Mahieu et al., 2009), the NdfR in mg g$^{-1}$ plant dry matter differed significantly between Frisson and P2 at beginning of flowering, i.e. the period of highest mycorrhization (Fig. 3.4). This difference disappeared with decreasing mycorrhization of Frisson.

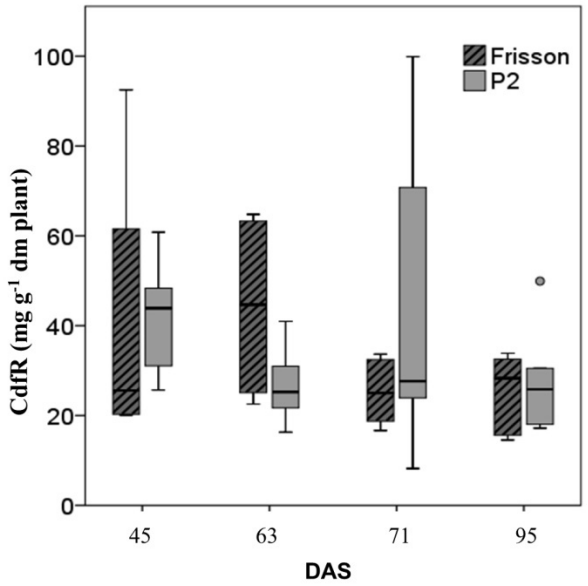


**Fig. 3.3**: C derived from rhizodeposition (CdfR) in mg g$^{-1}$ dry matter (dm) of Frisson and P2, harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). There were no significant differences between the means (t-test, pairwise comparison, $p < 0.05$). Values show means (Frisson: n = 4 for 45 and 63 DAS and n = 6 for 71 and 95 DAS; P2: n=6; except 71 DAS: n = 5).

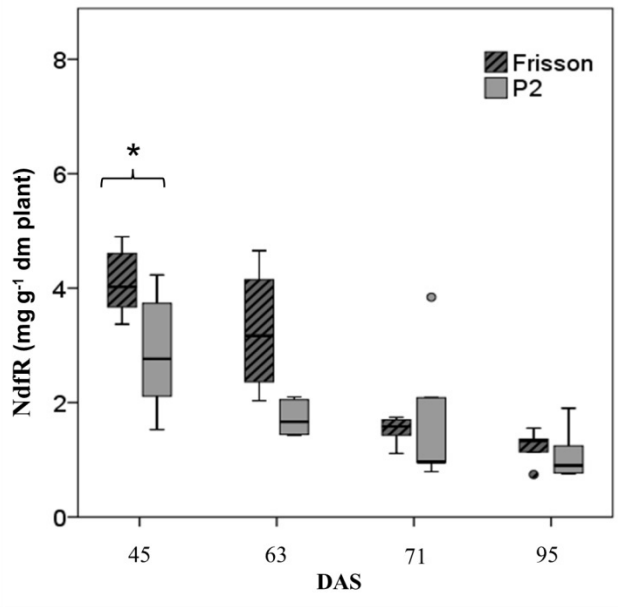


**Fig. 3.4**: N derived from rhizodeposition (NdfR) in mg $g^{-1}$ dry matter (dm) of Frisson and P2, harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$). Values show means (Frisson: n = 5 for 45 DAS, n = 4 for 63 DAS and n = 6 for 71 and 95 DAS; P2: n = 6; except 71 DAS: n=5).

### *3.3.4. Transfer of rhizodeposits into microbial biomass, inorganic N and extractable C*

At beginning of flowering, about 20% of CdfR was incorporated into the microbial biomass, which remained roughly constant until maturity (Table 3.4). Between 1% of microbial biomass C at beginning of flowering and 6% at maturity were derived from rhizodeposition. At beginning of flowering, about 17% of NdfR was incorporated into the microbial biomass, which insignificantly increased to 24% at maturity (Table 3.5). In total, between 1 and 3% of microbial biomass N originated from rhizodeposition.

Roughly 9% of CdfR was recovered in the extractable organic C pool during plant growth (Table 3.4). The proportion of this C pool derived from rhizodeposition increased from 1% at beginning of flowering to 5% at maturity. At beginning of flowering 11% of NdfR was recovered in the inorganic N pool and this percentage increased to 17% at maturity (Table 3.5). Between 0.2 and 0.4% of the inorganic N pool in soil resulted from rhizodeposition throughout plant growth.

**Table 3.4**: C in microbial biomass (MBC) and in extractable C in µg $g^{-1}$ soil, their percentage derived from rhizodeposition [dfR] and the percentage of CdfR found in MBC and extr. C, respectively, when plants were harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$). Values show means ± standard error of the mean (n=4 for 45 and 63 DAS and n=6 for 71 and 95 DAS).

| | Microbial biomass C | | % of CdfR | Extractable C | | % of CdfR |
|---|---|---|---|---|---|---|
| | µg $g^{-1}$ soil | dfR (%) | in MBC | µg $g^{-1}$ soil | dfR (%) | in extr. C |
| Frisson | | | | | | |
| 45 DAS | 165 a | 0.9 a | 20 a | 92 a | 0.8 a | 9 a |
| 63 DAS | 205 ab | 3.5 a | 20 a | 104 ab | 3.2 a | 9 a |
| 71 DAS | 217 b | 3.5 a | 22 a | 115 b | 3.0 a | 10 a |
| 95 DAS | 187 ab | 5.6 a | 22 a | 104 ab | 4.7 a | 10 a |

**Table 3.5**: N in microbial biomass (MBN) and in inorganic N in µg $g^{-1}$ soil, their percentage derived from rhizodeposition [dfR] and the percentage of NdfR found in MBN and inorg. N, harvested at beginning of flowering (45 DAS), end of flowering (63 DAS), green ripeness (71 DAS) and maturity (95 DAS). Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$). Values show means ± standard error of the mean (n = 5 for 45 DAS, n = 4 for 63 DAS and n = 6 for 71 and 95 DAS).

| | Microbial biomass N | | % of NdfR | Inorganic N | | % of NdfR |
|---|---|---|---|---|---|---|
| | µg $g^{-1}$ soil | dfR (%) | in MBN | µg $g^{-1}$ soil | dfR (%) | in inorg. N |
| Frisson | | | | | | |
| 45 DAS | 20 a | 0.8 a | 17 a | 95 a | 0.2 a | 11 a |
| 63 DAS | 29 b | 1.4 a | 18 a | 123 a | 0.3 a | 17 a |
| 71 DAS | 25 ab | 1.8 a | 22 a | 91 a | 0.3 a | 12 a |
| 95 DAS | 20 a | 3.2 a | 24 a | 105 a | 0.4 a | 17 a |

## 3.4. Discussion

### *3.4.1. Tracer uptake, plant enrichment and tracer recovery*

The strategy of multiple pulse labelling as an approximation of continuous labelling used in our experiment allowed pea plants to be sufficiently enriched with $^{13}C$ and $^{15}N$ tracer and labelled homogeneously over time. This is also the case in plant roots (Tables 3.1 and 3.2), which is an important prerequisite for estimating rhizodeposition quantitatively, especially when using the traditional Janzen and Bruinsma (1989) approach (Hupe et al., 2016a; Mayer et al., 2003b). Differences in grain enrichment between end of flowering and green ripeness, as well as between green ripeness and maturity show that $^{13}C$ and $^{15}N$ provided via the wick system is preferentially transferred into the sink organs of the plant during the reproductive phase. Additionally, some of the previously assimilated $^{13}C$ and $^{15}N$ might also be relocated within the plant (e.g. $^{15}N$ from roots). C and N rhizodeposition were calculated with a mass balance approach to prevent over- or underestimation caused by heterogeneous label distribution because of tracer relocation processes. A mass balance approach is less sensitive to heterogeneous enrichment (Hupe et al., 2016a). As mentioned above, plant roots were labelled homogeneously over time. This means that rhizodeposition can also be calculated using the Janzen and Bruinsma equation (Janzen and Bruinsma, 1989), although this method will lead to a larger overestimation of rhizodeposition if tracer leakage occurs (Gasser et al., 2015; Hupe et al., 2016a).

The formation of fungi and algae within the labelling system was observed during plant labelling, disturbing tracer uptake and causing C losses from microbial respiration. Consequently, the labelling system had to be replaced four times in total to facilitate multiple pulse labelling and reach high plant enrichment. The recovery of $^{13}C$ varied between 40 and 60% of the applied $^{13}C$ tracer. Between 13 and 30% of the recovered $^{13}C$ were found in the labelling system. A possible reason for the low recovery of $^{13}C$ is the loss of $^{13}C$ as $CO_2$ by rhizo-respiration (Jones et al., 2009). Losses of up to 30% of the applied $^{13}C$ tracer were observed by Yasmin et al. (2010). Even higher amounts of between 26 and 52% of the recovered $^{15}N$ were found in the labelling system, as the gaseous loss of $^{15}N$ is expected to be small under the experimental conditions. Consequently, the recovery of the applied tracer is similar to other publications (Mayer et al., 2003b; Wichern et al., 2007b; Fustec et al., 2010; Arcand et al., 2013a).

Multiple pulse labelling using the wick method provides a tool for labelling plants with multiple tracers (e.g. $^{13}C$, $^{15}N$) at relatively high concentrations, as shown earlier (Wichern et al., 2007a, b). In the present study, we were able to homogeneously label plant roots over time,

which allows estimation of rhizodeposition, assuming that root fragments and fine roots are a large part of rhizodeposits (McNeill and Fillery, 2008). However, it remains unclear whether roots have the same enrichment as rhizodeposits.

*3.4.2. Belowground plant C and N*

The contribution of BGP-C and N to total plant C and N both varied around 15% at beginning of flowering and around 6% at maturity. Compared with other studies, this value seems to be very low. Fustec et al. (2010) found that between 14 and 71% of total plant N of peas was BGP-N. For Frisson at maturity, Mahieu et al. (2007) found a contribution of 26% BGP-N to total plant N under greenhouse conditions. Wichern et al. (2007b) found a contribution of BGP-C to total plant C of 34% for peas at maturity under field conditions in microcosms. The low percentages in the current study were probably due to a shift in the ratio of aboveground plant C (AGP-C) to root C in comparison with field conditions (Mayer et al., 2003b). Nitrogen fertilization and pot size are the likely reasons for this observation. Wichern et al. (2007a) achieved an AGP-N to root N ratio of 17 with a pot volume of 4.3 l per pea plant. In the current study, an AGP-N to root N ratio of 20 was observed with the same soil volume and a similar vegetation period. Due to the high N fertilization, pea plants developed a smaller root system, leading to a larger AGP-N to root N ratio (Nguyen, 2003). The amount of rhizodeposition largely depends on the size of the root system (Shamoot et al., 1968; de Graaff et al. 2007; Wichern, 2007), considering the fact that rhizodeposition consists to a large extent of sloughed root cells and decomposed root material (Uren, 2007). The effects of N fertilization on rhizodeposition are often inconsistent. Arcand et al. (2013b) observed no N fertilization effects on the amounts of $NO_3^-$ and $NH_4^+$ released by pea roots, whereas Nguyen (2003) found that N fertilization increased C rhizodeposition, contrasting the results of Kuzyakov and Domanski (2000), who observed a decrease of C rhizodeposition.

Limitation of root growth due to pot size can also influence the amount of rhizodeposition (Poorter et al., 2012). Pot experiments can lead to a lower root to shoot ratio in comparison with field experiments, leading to a reduction in rhizodeposition (Wichern et al., 2008). Anglade et al. (2015) found significantly less NdfR as a percentage of BGP-N in pot experiments (56%) than in field trials (82%). Consequently, the results from our study cannot be directly transferred to field conditions. Nevertheless, as proposed by Pausch et al., (2013), the ratio of rhizodeposited C (or N) and root C (or N) can be used to predict rhizodeposition under field conditions using root biomass data. However, as roots are very plastic in their response to nutrient availability (Hodge,

2004), it remains questionable whether experimental conditions under controlled conditions can resemble field conditions, where nutrients are much more dynamic.

*3.4.3. Amount and fate of rhizodeposition over the course of time*

The amount of N rhizodeposition per plant (cv. Frisson) increased during flowering and remained constant afterwards until harvest. This indicates an increasing release of root exudates during flowering. It is already known that the quality and quantity of root exudates changes over the course of time (Grayston et al., 1997). For many annual crops it is known that the root system of plants increases until flowering and decreases thereafter (Jakobsen, 1986; Pritchard and Rogers, 2000). Therefore, N rhizodeposition probably does not increase until the end of flowering due to decomposed root material, but rather due to increased root exudation.

Grayston et al. (1997) found a decreasing release of amino acids by plants under N deficient conditions, whereas increasing $NO_3^-$ contents led to an increased release of carboxylates. On the other hand, an increasing $NH_4^+$ content caused a decline in the release of carboxylates (Neumann and Römheld, 2007). Therefore, the high N fertilization in the current study probably caused an increasing release of root exudates, especially carboxylates, during flowering. The C rhizodeposition constantly increased during the vegetation period and differed significantly only between beginning of flowering and maturity. After flowering, the decomposition of root material and the reduction in AMF most likely increased C rhizodeposition (Engels and Körschens, 2010), which was then mostly derived from root fragments, sloughed root cells and fungal hyphae.

No significant differences could be determined for the incorporation of rhizodeposition into the microbial biomass, the extractable organic C or inorganic N pool in soil over the course of time. The C and N rhizodeposition was similarly incorporated into these three pools at beginning of flowering and at maturity. In total, between 11 and 17% of total NdfR was found in the inorganic N pool, which is similar to the roughly 12% observed by Arcand et al. (2013b). However, this is only half the levels obtained by Mayer et al. (2003b) and Wichern et al. (2007a). Up to 24% of total NdfR was incorporated into the microbial biomass, somewhat above the 18% measured by Mayer et al. (2003b). The 20% of total CdfR in the microbial biomass was similar to Wichern et al. (2007b). In the current study, more labile N compounds, e.g. amino acids, peptides, were apparently released by the pea roots and incorporated into the microbial biomass.

Our results show that root exudation (including lysates, exudates, mucilage, etc.) may only be an important process of rhizodeposition up to the reproductive growth phase of plants. After flowering, senescence of roots becomes a more important part of rhizodeposition than before, as more roots die. Nevertheless, throughout plant growth rhizodeposition is obviously just a minor source of C and N for the soil microbial biomass, as indicated by the small part derived from rhizodeposition (less than 6%, Table 3.4 and 3.5). On top of this, only about 20% of the rhizodeposits end up in the microbial biomass, leaving about 50 to 70% of the rhizodeposited C and N unaccounted for. This may represent slowly available C and N in root fragments, which will be mineralised later. A substantial contribution of microbial residues to this unaccounted for fraction is not to be expected, as substantial microbial turnover during the short period of the experiment is unlikely (Mayer et al., 2003a; Wichern et al., 2007a,b).

### *3.4.4. Influence of mycorrhiza on rhizodeposition*

It is well known that mycorrhiza can influence the rhizodeposition of plants (Jones et al., 2009; Rillig and Mummey, 2006; Leifheit, 2014). In our study, AMF did not affect C rhizodeposition. Differences in the amount of C rhizodeposition may be caused by an additional C input of dying AMF hyphae (Engels and Körschens, 2010). At the time of the highest AMF colonization, Frisson had significantly higher N rhizodeposition than P2. With decreasing mycorrhization during pea maturation (Zhang et al., 1995), these differences in N rhizodeposition decreased too. This may be caused by differences in root exudation between mycorrhizal and non-mycorrhizal plants. Hyphal exudation of mycorrhizal fungi has been found to increase root respiration (Jones et al., 2004).

The high mycorrhization of Frisson at the beginning of flowering may cause an increasing exudation of various N compounds from roots to soil. Mada and Bagyaraj (1993) mentioned that plants with mycorrhizal fungi release less potassium, phosphate and glucose, but more N, especially as protein. Pea roots and pea root nodules are highly active during flowering (Dupont et al., 2012). AMF also support the N transfer from $N_2$ fixing plants to non-fixing plants (Hamel et al., 1991). However, these differences in plant morphology may also cause increasing N rhizodeposition (Fustec et al., 2010). Despite the high N fertilization, Frisson roots formed nodules. Therefore, we cannot estimate whether differences in rhizodeposition are caused by mycorrhiza or N-fixation through nodulation, when comparing Frisson and P2.

### 3.5. Conclusions

Obviously rhizodeposition is not an easily available source of C and N for soil microorganisms. This assumption may be true for root exudates only. However, rhizodeposition also contains particular components, such as root fragments, that are only slowly degraded. Consequently, when investigating C and N dynamics in agricultural systems quantitatively, the term rhizodeposition has to be used in its broad definition, including root fragments. Especially during the reproductive phase, root senescence makes a greater contribution to rhizodeposition than during vegetative growth, which is relevant when assessing total belowground plant C and N input. Mycorrhiza did not influence C rhizodeposition, as expected, but increased N rhizodeposition at flowering, indicating the relevance of mycorrhiza for nutrient transfer. Future experiments should be conducted under real field conditions, without a limitation of root growth, to estimate true amounts of C and N rhizodeposition.

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# 4. Get on your boots: Estimating root biomass and rhizodeposition of peas under field conditions reveals the necessity of field experiments

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**Abstract**

More sustainable agricultural systems, which contribute to C sequestration and biological N fixation, require accurate estimation of plant C and N input into soils. To estimate the true amount of below-ground plant C (BGC) and N (BGN), experiments have to be conducted under field conditions. Therefore, we wanted to quantify BGC and BGN and especially the rhizodeposition of peas during the vegetation period under field conditions, in particular the spatial distribution and the transfer into different soil compartments. Pea plants were labelled with $^{13}$C-glucose and $^{15}$N urea using the cotton wick method. Plants were harvested at four dates depending on plant development. Our results indicate that the quantity of C and N rhizodeposition did not change between flowering and maturity. About one third of the C and N rhizodeposits were integrated into microbial biomass and extractable C or the inorganic N pool of soil. When comparing this field study to a former pot experiment, a higher root-to-shoot ratio was detected; also the rhizodeposition-to-root ratio was altered in the field. Due to a higher BGC and BGN input compared to pot experiments, studies aiming at quantifying BGC and BGN input will have to be conducted under field conditions.

Key words: $^{13}$C; $^{15}$N; below-ground biomass; field conditions; *Pisum sativum*; rhizodeposition

## 4.1. Introduction

Carbon sequestration as a means of counteracting greenhouse gas emissions, the build-up of organic matter to increase the resilience of soils to physical stress and prevent erosion, nitrogen fixation and immobilization, and the increase of a diverse soil microbial community, all of these processes are affected by the root systems of plants (Rasse et al. 2005; de Graaf et al. 2013). Consequently, assessment of the root biomass, its C and N content and its effects on soil physical, chemical and microbiological properties is important when considering more sustainable agricultural systems. Moreover, in sustainable and organic farming systems, legumes are seen to be a crucial source of N input (Rasmussen et al. 2012). In this respect, proper assessment of the N fixed in symbiosis with rhizobacteria, the amount stored in plant biomass and the amount left on the field as a source of subsequent crop nutrition is required (Askegaard and Eriksen 2007). Therefore, quantification of below-ground N is important, as it harbors a substantial part of total plant N, which is not accounted for when estimating the above-ground biomass only (Peoples et al. 2009). Below-ground C and N is often quantified as root biomass by sieving out roots from a defined soil volume in pot or microcosm experiments. Under field conditions, root biomass is assessed from a representative volumetric soil sample and calculated on a hectare basis (Johnson and Morgan 2010). Roots are separated from soil by hand picking and/or wet sieving on sieves of different mesh sizes (Bolinder et al. 1997; Pierret et al. 2005). This leaves quantitative root estimation subjective to a certain extent. Furthermore, all root sampling and sieving cannot account for smaller root fragments, root debris, root exudates and smaller molecular compounds or even ions released from plants during the vegetation period. The process of release of all of this is often referred to as rhizodeposition and assessed as C or N released from roots during the vegetation period (Wichern et al. 2008).

Quantitative assessment of this C and N released is usually done by labelling plants with stable ($^{13}C$, $^{15}N$) or radioactive ($^{14}C$) isotopes and tracing the isotopes in the soil. The assumption that the stable isotopes are distributed within the plant like all other C and N allows the assessment of C and N in the below-ground biomass, including the rhizodeposits (Hupe et al. 2016a). Various labelling approaches have been used to apply isotopes to living plants, as summarized earlier (Wichern et al. 2008). Overall, a quantitative assessment of below-ground plant C and N requires homogeneously enriched plant roots and rhizodeposits (Hupe et al. 2016a). Usually, these isotope studies are conducted under controlled conditions in pots or microcosms with limited root space and soil disturbance from placement of the

microcosms (e.g. Wichern et al. 2007a, b; Hupe et al. 2016b). The reason for this is that accurate estimation of total root biomass and isotopes released from this biomass is only possible if a separated space is used.

In pot experiments rhizodeposits accounted for as much as 10 to 30% of total plant C and N. Data vary substantially between plant species (Kuzyakov 2001; Nguyen 2003; Wichern et al. 2008; Fustec et al. 2010). For peas (*Pisum sativum* L.) rhizodeposition values are between 4 and 71% of total N (Wichern et al. 2008) and below-ground C and N accounted for 7-64% and 5-74% of total plant C (Wichern et al. 2007a; Hupe et al. 2016b) and N (Fustec et al. 2010; Hupe et al. 2016a). Rhizodeposition is known to be closely related to root biomass (Shamoot 1968; de Graaf et al. 2007). However, in pot experiments the root-to-shoot ratio differs compared to field conditions (Poorter et al. 2012). As the process of rhizodeposition is closely related to root biomass, it can be expected that beside a larger root biomass under field conditions also amounts of C and N released from roots are higher. Moreover, abiotic conditions such as soil moisture and temperature usually vary more strongly under field compared to controlled pot conditions. As plant roots have a high plasticity, they respond to these changes with altered root development (Poorter et al. 2012) and rhizodeposition (Wichern et al. 2008).

Consequently, studies estimating below-ground biomass C or N solely by assessing the root biomass in pot experiments not only disregard rhizodeposits as an important C and N pool but also do not consider the larger below-ground biomass under field conditions compared to pot experiments. Therefore, accurate assessment of the C sequestration potential of crops and N input in cropping systems requires experiments that investigate below-ground plant biomass including rhizodeposition under field conditions without restriction of root growth. Another approach was suggested by Pausch et al. (2013) who proposed transfering the rhizodeposition C-to-root C ratio as derived from pot experiments to field conditions, where only the root biomass has to be measured. Nevertheless, it remains unclear whether conditions of pot and field experiments yield a similar rhizodeposition-to-root ratio.

Rhizodeposition is often expected to be an easily available source of energy and nutrients for soil microorganisms (Dennis et al. 2010). As it consists to a large extent of root debris and root fragments, C and N transfer from roots into the microbial biomass was shown to be only a minor pathway during plant growth (Mayer et al. 2003; Wichern et al. 2007a, b; Hupe et al. 2016b), contradicting the widely held belief that all rhizodeposits are highly available to soil microorganisms (Kuzyakov 2002; Paterson 2003; Wichern et al. 2008). Under field conditions, where root turnover can be expected to be higher due to higher variability of

abiotic factors, C and N transfer from plant roots into the microbial biomass can also be expected to be higher.

As a consequence of this, we conducted a field experiment with the objectives (i) to quantify below-ground plant C and N of peas (*Pisum sativum* L.) under field conditions without any restriction of root growth, (ii) to quantify C and N rhizodeposition during plant growth and (iii) to estimate the particular distribution of C and N, especially the transfer from plant roots into the soil microbial biomass, elucidating the question of how bioavailable rhizodeposits are.

## 4.2. Materials and Methods

### *4.2.1. Study site*

The experiment took place at the research station of the University of Kassel in Neu-Eichenberg, Germany (51º23´N, 9º 55´E 220 m asl), which is has been managed organically since 1998 according to European regulations (EC 834/2007). In 2013, the total annual rainfall was 711 mm. During the study time period, the rainfall was 240 mm and the mean average temperature was 15.3 ºC (min.: 5.6 ºC; max.: 25.0 ºC). The clayey silt soil was classified as a Haplic Luvisol according to the FAO Reference Base for Soil Resources, with a pH of 6.1, 1.2% soil organic C and 0.13% total N. At 0–30 cm soil depth, the soil contained 20 µg Ca-acetate lactate extractable P $g^{-1}$ soil, 54 µg K $g^{-1}$ soil, and 90 µg Mg $g^{-1}$ soil. The preceding crops before the experiment started were wheat (*Triticum aestivum* L.) in 2012 and oilseed rape (*Brassica napus* L.) in 2011.

### *4.2.2. Experimental design and labelling*

In April 2013, the peas (*Pisum sativum* L. "Santana") were sown manually in 40 microplots (0.50 m x 0.38 m). In every microplot, 12 pea plants were cultivated at a distance of 12.5 cm between the seedlings and between the rows, to reach achieve an optimal seeding rate (64 plants $m^{-2}$). To investigate the development of rhizodeposition over the course of time, plants were harvested at four different times, depending on plant development, beginning at BBCH 59 (first petals visible, flowers still closed) and 62 days after sowing (DAS), BBCH 69 (flowering)/ 68 DAS, BBCH 79 (pods have reached typical size; green ripe)/ 82 DAS and at BBCH 89 (fully ripe: all pods are dry and brown; seeds are dry and hard)/ 103 DAS (Lancashire et al. 1991). To figure out the best method for labelling plants in the field, a leaf feeding and a stem feeding method (cotton wick method) were compared in the greenhouse regarding their handling and applicability in the field. It was found, that the leaf feeding

method was not suitable for a multiple pulse labelling in the field, because of the greater effort required compared with the cotton wick method. A new leaf has to be used for every labelling interval. To approximate continuous labelling using a multiple pulse labelling approach, one new leaf has to be used at least every week and will be destroyed. This strong influence on plant biomass was an argument against the leaf feeding method to be used in our experiment.

Therefore, in half of the microplots (20) twelve plants were labelled with a $^{13}C$ glucose (99 atom%) and $^{15}N$ urea (95 atom%) solution, using the cotton wick method (Russel and Fillery 1996; Wichern et al. 2007b). For this technique, the stem of each pea was drilled with a 0.5 mm drill, approximately 3 – 4 cm above the ground. Then, a small cotton wick was passed through the hole. To prevent evaporation losses and contamination, the cotton wick was put through small silicon tubes. These silicon tubes (and the cotton wick) were passed through two holes in the lid of a 2 ml vial, which contained the labelling solution. A kneading mass (Teroson, Henkel), was placed between the stem or the lid and the silicon tubes to prevent evaporation losses of the labelling solution (supplementary material Fig. S1). Plants usually took up most of the solution within the first 48 h. The labelling solution was produced by mixing deionized water and the stable $^{13}C$-glucose and $^{15}N$-urea and then sterile filtered (< 0.2 µm). All material used for producing the labelling solution was steam sterilized for 20 min at 121 ºC beforehand. To reach a homogeneous enrichment of $^{13}C$ and $^{15}N$ in the plant, a continuous isotope uptake is important. For this reason plants were labelled beginning at BBCH 13 (3 leaves unfolded), 41 days after sowing (DAS), with 0.5 ml labelling solution. Solution uptake was documented every two days. If plants had taken up the solution completely, the vials were refilled with 0.5 ml labelling solution. In case of contamination of the labelling system (in some cases fungi or algae formation could be observed), a new sterile labelling system was installed. To prevent contamination and plant losses during installation of the labelling system, pea plants were labelled using an adapted multicarrier manual weeding device tool (supplementary material Fig. S2). This multicarrier consisted of a flatbed with a hole for the head, installed on a large wooden board with four rubber tires (with an appropriate width to cross the microplot without damaging the plants). For the later refilling of the vials with the isotope solution or the replacement of the labelling system, the multicarrier was height-adjustable. The soil of the microplots was covered with a 1 mm mesh to prevent soil contamination from falling leaves, which were collected regularly. The described labelling approach approximated continuous labelling by application of multiple pulses at high frequency.

*4.2.3. Sampling and analyses*

At each harvest date, the labelling system was carefully removed from the plants and frozen for analysis of the remaining labelling solution, which allows the determination of the isotope recovery. After this, one pea plant in the middle of the microplot was harvested completely above-ground, separated into flower/grain, stem and leaves. Then, the remaining 11 plants were harvested, also separated into flower/grain, stem and leaves.

For calculating the root biomass, at first, soil samples were first taken in at 0 – 30 cm and 30 – 60 cm depth with a HUMAX, a special soil sampling drilling tool (supplementary material Fig. S3a), in a sampling pattern (Fig. 4.1) based on the study of Anthes (2005). Directly at the position of the single harvested pea one soil sample was taken (α). The next sample was taken exactly between two peas within the row (β) and the last sample was taken in the middle of four peas between two rows of peas (γ).

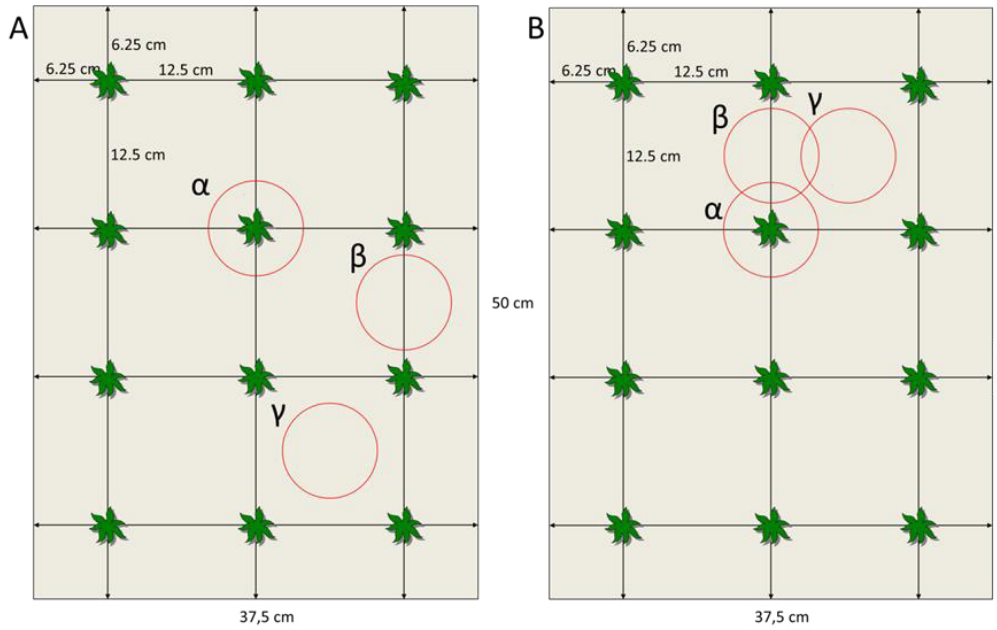


**Fig. 4.1**: Soil sampling scheme: (A) Soil samples were taken at three defined points in each microplot (α: directly on one single pea; β: between two peas in the row; γ: in the middle of 4 peas between two rows). (B) Theoretical scheme for calculating the complete root dry matter of one pea plant.

**($DM_{total} = \alpha \times 0.75 + \beta \times 0.75 + \gamma \times 1.5$)**

The soil was automatically filled in a plastic cylinder (with a volume of 1.5 l), which prevented a carry-over of the labelled soil from one microplot to the next (supplementary material Fig. S3b). This exact soil sampling was possible due to the hand sowing of peas. In a further step, the remaining pea roots of the 11 peas were excavated and soil samples were taken in at 0-30, 30-60 and 60-90 cm depth, to analyze the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratio. This was done at every harvest date for five labelled and five non-labelled control microplots.

After weighing the fresh matter, all plant parts were dried for 48 h at 60 ºC. With a ball mill, dry plant parts were ground to a fine powder and then weighed into tin cups for analyzing the total C, total N and the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios. To estimate the dry matter (DM), plant parts were dried for 48 h at 60 ºC and a soil subsample was dried for 24 h at 105 ºC until constant weight. A subsample of each soil sample was taken for isotope analysis. Therefore, the soil was sieved (< 2 mm) and all visible roots and root fragments (> 2 mm) were collected by hand and removed. Then, the soil was dried, ground with a ball mill to a fine powder and weighed in as described for the plant parts. Total C and N of plant and soil material was determined using an elemental analyzer (Fisons, Milano, Italy). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios were determined with isotope ratio mass spectrometry (Finnigan MAT, Bremen, Germany). To estimate recovery of isotopes, the vials and labelling systems were extracted with 200 ml of 0.05 M $K_2SO_4$ and the extractable organic C and total N was measured as described below. The remaining amounts of $^{13}C$ and $^{15}N$ in the extracts were calculated, assuming that the C and N were solely derived from the isotope solution.

Microbial biomass C and N were estimated using the fumigation extraction method (Brookes et al. 1985; Vance et al. 1987) including a pre-extraction step to remove living roots (Mueller et al. 1992). For the pre-extraction 30 g of soil (after removing remaining roots) and 70 ml 0.05 M $K_2SO_4$ were horizontally shaken for 30 min at 200 rev $min^{-1}$. Then, the soil solution was centrifuged for 10 min. and 4000 g at 6 ºC. The supernatant was filtered and frozen for measuring extractable organic C, total extractable N, inorganic N and the isotope ratios. After pre-extraction, 15 g of the extracted soil was fumigated for 24 h at 25 ºC with ethanol-free $CHCl_3$. After removal of the $CHCl_3$, the fumigated and 15 g of the non-fumigated soil samples (from pre-extraction) were extracted with 60 ml 0.05 M $K_2SO_4$ (as described above for the pre-extraction) and extracts were frozen until the measurements. Organic C and total N in the extracts were analyzed using a CN Analyzer (Multi N/C 2100S, Analytik Jena, Germany). After freeze-drying a subsample of the extracts, the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios were determined by isotope ratio mass spectrometry.

### *4.2.4. Calculation and statistical analyses*

The complete root dry matter was calculated as the sum of root dry matter, found in the three sectors (1). To prevent over- or underestimation of root dry matter, because of the theoretical overlapping of the three sectors, each sector was weighted differently, depending on microplot size and diameter of the drilling tool. The area of the theoretical overlapping of the

three sectors (Fig 4.1.) was evenly distributed on each sector. Therefore, only 75% of sector α and β were integrated into the calculation approach.

$$DM_{total} = \alpha \times 0.75 + \beta \times 0.75 + \gamma \times 1.5 \quad (1)$$

Microbial biomass C (MBC) was calculated as $E_C / k_{EC}$, where $E_C$ = (organic C extracted from fumigated soils) – (organic C extracted from non-fumigated soils) and $k_{EC}$ = 0.45 (Wu et al. 1990). Microbial biomass N (MBN) was calculated as $E_N / k_{EN}$, where $E_N$ = (total N extracted from fumigated soils) - (total N extracted from non-fumigated soils) and $k_{EN}$ = 0.54 (Joergensen and Mueller 1996).

C and N derived from rhizodeposition were calculated with a mass balance approach (2 and 3) as described in Hupe et al. (2016a).

$$^{13}C_{tracer}dfR\ (\text{in }\%) = \frac{^{13}C_{\ tracer}\ dfR}{^{13}C_{\ tracer}\ \text{in total}} \times 100 \quad (2)$$

$$^{15}N_{tracer}dfR\ (\text{in }\%) = \frac{^{15}N_{\ tracer}\ dfR}{^{15}N_{\ tracer}\ \text{in total}} \times 100 \quad (3)$$

The distribution of C and N tracer in the plant corresponds to the distribution of total plant C and N. For this reason, the rhizodeposition of $^{13}C$ tracer (%) and $^{15}N$ tracer (%) corresponds to the total C and N rhizodeposition (4 and 5).

$$^{13}C_{tracer}dfR\ (\text{in}\%) = CdfR\ (\text{in }\%) \quad (4)$$

$$^{15}N_{tracer}dfR\ (\text{in }\%) = NdfR\ (\text{in }\%) \quad (5)$$

The calculation of C derived from rhizodeposition (CdfR) and N derived from rhizodeposition (NdfR) in microbial biomass, in organic C and in mineral N was done using an isotope mass balance approach (Hupe et al. 2016a).

Statistical analyses were performed by t-test, repeated measures or one-way analysis of variance (ANOVA) with Scheffé post-hoc test, using IBM SPSS Statistics 22.

## 4.3. Results

### *4.3.1 $^{13}C$ and $^{15}N$ enrichment, uptake and recovery*

With the cotton wick technique, a homogeneous enrichment of pea plants with $^{13}C$ and $^{15}N$ was reached during the complete vegetation period (Tab. 1). For $^{13}C$, the highest enrichment was achieved 103 DAS in leaves and stem (0.57 atom% excess). The $^{13}C$ enrichment of soil

was constant during plant growth at each of the three soil depths. Similar to $^{13}C$, the highest enrichment with $^{15}N$ was achieved 103 DAS in leaves and stem (4.94 atom% excess). Differences could be observed at the subsoil. At 30 – 60 cm depth, there was a higher $^{15}N$ enrichment 82 DAS compared to 68 DAS. At 60-90 cm depth, the soil was more enriched 103 DAS compared with 62, 68 and 82 DAS.

**Table 4.1**: Enrichment of $^{13}C$ and $^{15}N$ in various plant parts of pea (Santana) and soil 62, 68 82 and 103 days after sowing (DAS). Values show means (n=5; except DAS 103: n=4). Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$).

| | Leaves + stem | grain | roots | soil | | |
|---|---|---|---|---|---|---|
| | | | | 0-30 cm | 30-60 cm | 60-90 cm |
| | | (Atom%excess) | | | | |
| $^{13}C$ | | | | | | |
| DAS 62 | 0.48 a | NA | 0.23 a | 0.0020 a | 0.0010 a | 0.0023 a |
| DAS 68 | 0.37 a | 0.20 a | 0.22 a | 0.0012 a | 0.0012 a | 0.0028 a |
| DAS 82 | 0.36 a | 0.15 a | 0.23 a | 0.0008 a | 0.0013 a | 0.0018 a |
| DAS 103 | 0.57 a | 0.25 a | 0.26 a | 0.0018 a | 0.0012 a | 0.0064 a |
| $^{15}N$ | | | | | | |
| DAS 62 | 3.15 a | NA | 1.12 a | 0.0069 a | 0.0041 ab | 0.0090 b |
| DAS 68 | 3.24 a | 3.00 a | 1.19 a | 0.0052 a | 0.0019 b | 0.0013 b |
| DAS 82 | 3.16 a | 2.83 a | 1.23 a | 0.0037 a | 0.0075 a | 0.0092 b |
| DAS 103 | 4.94 a | 3.41 a | 1.83 a | 0.0077 a | 0.0032 ab | 0.0240 a |

NA = not applicable

During plant growth, one pea plant took up between 11 and 37 mg $^{13}C$ and between 5 and 11 mg $^{15}N$ (Tab. 2). Approximately 2% of the applied $^{13}C$ and $^{15}N$ remained in the wick system. A large proportion of $^{13}C$ and $^{15}N$ was found in the plant, increasing during plant growth. Between 32 and 89% of the applied $^{13}C$ and between 59 and 117% of the applied $^{15}N$ were recovered in stem, leaves, grain and roots. The proportion of $^{13}C$ and $^{15}N$ recovered in soil decreased during plant development from 11 and 17% of $^{13}C$ and $^{15}N$ at beginning of flowering to 5 and 8% at maturity In total, between 45 and 94% of the applied $^{13}C$ and between 72 and 127% of the applied $^{15}N$ were recovered in total.

**Table 4.2**: Uptake (in mg plant$^{-1}$) and recovery (in plant, soil, wick system and total; in % of applied tracer) of $^{13}C$ and $^{15}N$ in pea (Santana) 62, 68, 82 and 103 days after sowing (DAS). Values show means ± standard error of the mean (n = 5; except DAS 103: n=4).

| | Uptake (mg plant$^{-1}$) | Recovery (% of applied tracer) | | | |
|---|---|---|---|---|---|
| | | Plant | Soil | Wick system | Total |
| $^{13}C$ | | | | | |
| DAS 62 | 12.1 ± 1.5 | 39 ± 5 | 11.1 ± 2.1 | 2.1 ± 0.2 | 53 ± 5 |
| DAS 68 | 11.1 ± 1.8 | 32 ± 4 | 10.1 ± 4.3 | 2.1 ± 0.4 | 45 ± 7 |
| DAS 82 | 37.1 ± 4.3 | 89 ±12 | 3.1 ± 0.6 | 2.1 ± 0.1 | 94 ±12 |
| DAS 103 | 30.1 ± 3.0 | 54 ± 6 | 5.1 ± 1.1 | 2.1 ± 0.5 | 60 ± 6 |
| $^{15}N$ | | | | | |
| DAS 62 | 5.1 ± 0.3 | 59 ± 3 | 17.1 ± 3.3 | 2.1 ± 0.2 | 80 ± 2 |
| DAS 68 | 5.1 ± 0.7 | 60 ±10 | 8.1 ± 1.1 | 5.1 ± 0.5 | 72 ±10 |
| DAS 82 | 14.1 ± 0.9 | 117 ± 8 | 7.1 ± 1.0 | 2.1 ± 0.1 | 127 ± 8 |
| DAS 103 | 11.1 ± 1.0 | 67 ± 8 | 8.1 ± 1.4 | 2.1 ± 0.3 | 76 ± 8 |

*4.3.2 C and N derived from rhizodeposition during plant development*

Until the end of flowering, root C increased to 382 mg plant$^{-1}$ (Tab. 3). Then, root turnover started and the amount of root C decreased to 53 mg plant$^{-1}$. The amount of C derived from rhizodeposition (CdfR) did not change significantly between beginning of flowering and maturity. Between 426 and 879 mg C plant$^{-1}$ in the soil were derived from rhizodeposition. Furthermore, there were no significant differences between the three soil depths; CdfR was integrated equally at 0-30, 30-60 and 60-90 cm depth.

**Table 4.3**: C and N in roots of pea (Santana), C and N derived from rhizodeposition (dfR) and total plant C and N (in mg plant $^{-1}$) 62, 68, 82 and 103 days after sowing (DAS). Values show means ± standard error of the mean (n = 5; except DAS 103: n = 4). Different letters (a, b) indicate significant differences between DAS (Scheffé). No differences were found between CdfR and NdfR in 0-30 cm, 30-60 cm and 60-90 cm (repeated measures).

| | Roots | dfR (mg plant $^{-1}$) | | | | Total plant |
|---|---|---|---|---|---|---|
| | (mg plant $^{-1}$) | total | 0-30 cm | 30-60 cm | 60-90 cm | (mg) |
| C | | | | | | |
| DAS 62 | 258 ± 40 b | 588 ± 147 a | 259 ± 81 | 119 ± 19 | 210 ± 129 | 2,472 ± 140 c |
| DAS 68 | 382 ± 6 a | 879 ± 414 a | 212 ± 63 | 212 ± 94 | 455 ± 415 | 3,402 ± 685 bc |
| DAS 82 | 147 ± 17 c | 426 ± 116 a | 154 ± 41 | 178 ± 53 | 94 ± 48 | 10,506 ± 952 a |
| DAS 103 | 53 ± 9 c | 499 ± 147 a | 104 ± 12 | 48 ± 20 | 347 ± 138 | 6,558 ± 1012 b |
| N | | | | | | |
| DAS 62 | 17 ± 3 b | 35 ± 7 a | 16 ± 5 | 8 ± 2 | 12 ± 4 | 157 ± 4 b |
| DAS 68 | 23 ± 1 a | 20 ± 3 a | 15 ± 3 | 4 ± 2 | 2 ± 1 | 166 ± 19 b |
| DAS 82 | 8 ± 1 c | 27 ± 6 a | 9 ± 3 | 11 ± 3 | 7 ± 3 | 435 ± 42 a |
| DAS 103 | 3 ± 1 c | 27 ± 5 a | 9 ± 1 | 2 ± 1 | 16 ± 5 | 271 ± 38 b |

After flowering, the proportion of C in roots decreased from 14% to 1% of total plant C at maturity. During flowering, the proportion of CdfR was stable between 23 and 22%. Until green ripe, the proportion of CdfR decreased significantly to 4% of total plant C. Until green ripe, no differences between the three soil depths were observed. At maturity, the proportion of CdfR at 60-90 cm depth (5%) was higher compared to 30-60 cm depth (1%).

Total plant N increased until green ripe to 435 mg N $plant^{-1}$ and thereafter decreased substantially by 162 mg $g^{-1}$ plant, leaving only 271 mg N $plant^{-1}$ plant (Tab. 3). Until the end of flowering, root N increased to 23 mg $plant^{-1}$. Because of root turnover, the amount of root N decreased to 3 mg $plant^{-1}$ until maturity. The amount of N derived from rhizodeposition was equal at 0-30, 30 -60 and 60-90 cm depth, between beginning of flowering and maturity. The proportion of N in roots increased until the end of flowering to 15% of total plant N and decreased to 1% until maturity because of root death and turnover. The proportion of NdfR was significantly higher at the beginning of flowering compared to green ripe. At each soil depth, the proportion of NdfR was equal during plant development. At the end of flowering, more NdfR was found at 0-30 cm depth (10% of total plant N) compared to 30-60 and 60-90 cm depth (2 and 1% of total plant N). At maturity, significantly more NdfR was found at 60-90 cm depth (6% of total plant N) compared to 30-60 cm depth (1% of total plant N). There were no differences in the proportion of NdfR on total plant N at the beginning of flowering and at green ripe.

*4.3.3 Transfer of rhizodeposits into the microbial biomass, inorganic N and extractable C*

The proportion of MBC derived from rhizodeposition and the proportion of rhizodeposition in MBC was constant over time at both depths (Tab. 4). At 0-30 cm depth, between 2 and 5% and at 30-60 cm depth between 4 and 10% of MBC were derived from rhizodeposition. Approximately one quarter of the CdfR was integrated into MBC. At 0-30 cm depth, the amount of extractable C increased during flowering from 81 to 136 µg $g^{-1}$ soil. At 30-60 cm depth, significantly more extractable C was found at the end of flowering and green ripe compared to the beginning of flowering. The proportion of extractable C derived from rhizodeposition and the proportion of CdfR in extractable C was constant over time and soil depth. Between 2 and 5% of the extractable C at 0-30 cm depth and between 3 and 7% of the extractable C at 30-60 cm depth were derived from rhizodeposition. At 0-30 cm depth, between 10 and 19% of CdfR were integrated into extractable C. This proportion was similar at 30-60 cm depth. Between 6 and 18% of CdfR were integrated into extractable C.

**Table 4.4:** C and N in microbial biomass (MBC; MBN), in extractable C / inorganic N in µg g-1 soil, in percentage derived from rhizodeposition [dfR] and in percentage of dfR in MB and extr. C / inorganic N. Soil samples were taken at 0-30 and 30-60 cm depth 62, 68, 82 and 103 days after sowing (DAS). Different letters indicate significant differences between DAS (Scheffé, $p < 0.05$). No significant differences between the means of MBC dfR, % of CdfR in MBC, extr. CdfR and % of CdfR in extr. C 103 DAS were found (t-test, pairwise comparison, $p < 0.05$). Values show means (n = between 3 and 5).

| | MBC | | % of | Extractable C | | % of | MBN | | % of | Inorganic N | | % of |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | µg $g^{-1}$ soil | dfR (%) | CdfR in MBC | µg $g^{-1}$ soil | dfR (%) | CdfR in extr. C | µg $g^{-1}$ soil | dfR (%) | NdfR in MBN | µg $g^{-1}$ soil | dfR (%) | NdfR in inorg. N |
| 0-30 cm | | | | | | | | | | | | |
| DAS 62 | 168 c | 5.4 a | 22 a | 81 b | 5.2 a | 10 a | 27 b | 1.3 a | 15 a | 4.1 a | 6.0 a | 11 a |
| DAS 68 | 265 a | 2.7 a | 21 a | 136 a | 3.2 a | 10 a | 42 a | 0.8 a | 15 a | 3.1 a | 6.7 a | 8 a |
| DAS 82 | 274 a | 4.3 a | 42 a | 137 a | 4.0 a | 19 a | 41 a | 1.3 a | 33 a | 3.1 a | 5.8 a | 11 a |
| DAS 103 | 217 b | 1.7 a | 21 a | 121 a | 1.5 a | 10 a | 29 b | 1.3 a | 24 a | 4.1 a | 7.9 a | 22 a |
| 30-60 cm | | | | | | | | | | | | |
| DAS 62 | 63 a | 3.7 a | 12 a | 32 b | 3.3 a | 6 a | 7 c | 1.3 | 11 | 4.1 a | 2.0 | 9 |
| DAS 68 | 74 a | 10.0 a | 39 a | 51 a | 6.7 a | 18 a | 13 ab | n.d. | n.d. | 2.1 b | n.d. | n.d. |
| DAS 82 | 99 a | 8.3 a | 23 a | 55 a | 6.9 a | 10 a | 14 a | 1.0 | 7 | 2.1 b | 2.6 | 3 |
| DAS 103 | 71 a | 4.2 a | 14 a | 44 ab | 2.5 a | 6 a | 8 bc | n.d. | n.d. | 2.1 b | n.d. | n.d. |

n.d.: not determined

At 0-30 cm depth, the amount of MBN increased until green ripe (Tab. 4) from 27 to 41 µg $g^{-1}$ soil and decreased to 29 µg $g^{-1}$ soil until maturity. At 30-60 cm soil depth, the amount of MBN increased from 7 to 13/14 µg $g^{-1}$ soil until green ripe and decreased to 8 µg $g^{-1}$ soil until maturity. The proportion of the MBN derived from rhizodeposition was constant during plant development at about 1%. Similarly to CdfR, the proportion of NdfR integrated into MBN was approximately one quarter of total NdfR at 0-30 cm depth. At 30-60 cm depth, the proportion of NdfR incorporated into MBN was between 7 and 11%. The inorganic N content in soil was stable during plant development at 0-30 cm depth. At 30-60 cm depth inorganic N decreased from 4 µg $g^{-1}$ soil at beginning of flowering to 2 µg $g^{-1}$soil at maturity. Similarly to C, the proportion of inorganic N derived from rhizodeposition and the proportion of NdfR integrated into inorganic N was constant over the course of time and soil depth. Between 5 and 8% of inorganic N at 0-30 cm depth and between 2 and 3% of inorganic N at 30-60 cm soil depth were derived from rhizodeposition. The proportion of NdfR integrated into the inorganic N pool of soil was between 8 and 22% at 0-30 cm depth and between 3 and 9% at 30-60 cm depth.

### *4.3.4 Pot versus Field*

We compared the results of the present study with a pot experiment conducted earlier (Hupe et al. 2016a, b). Under controlled conditions, pea plants (*Pisum sativum* L. cv. Frisson) were cultivated in soil taken from the same location as the field experiment was performed. The $^{13}C$ and $^{15}N$ double labelling method and also the time of harvest were similar in the present study. During flowering, root biomass in pots was significantly lower compared to field conditions (Fig. 4.2). After flowering, the dry matter of roots became more similar in pot and field experiments. After flowering, the C rhizodeposition of peas grown under controlled conditions in pots was stable between 1,690 and 1,664 mg $g^{-1}$ root DM (Tab. 5). In the present study, C rhizodeposition increased between the beginning of flowering and maturity to almost 4,500 mg $g^{-1}$ root DM. During flowering, C rhizodeposition as a percentage of total plant C was more than twice as high in the field as compared to pot conditions. Almost one quarter of total plant C came from rhizodeposition under field conditions.

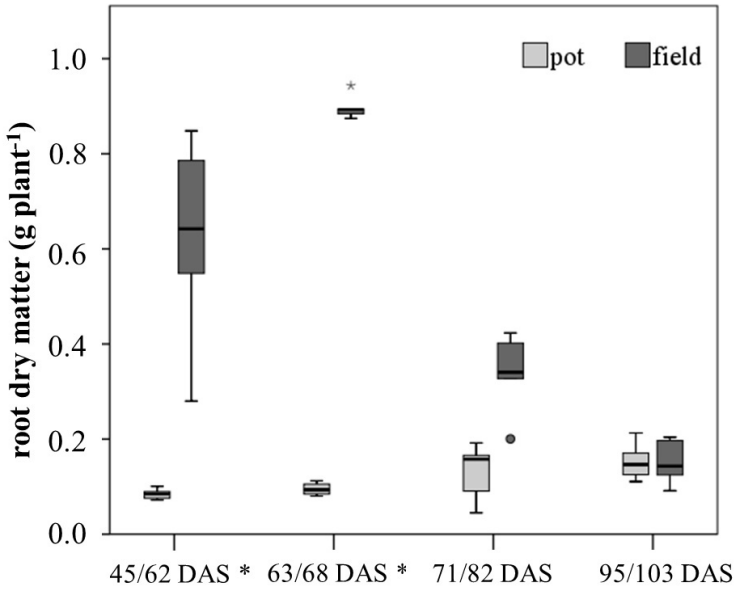


**Fig. 4.2**: Root dry matter in pot experiment (pea: Frisson) and field experiment (pea: Santana) in g plant$^{-1}$ at different harvest dates (45, 63, 71 and 95 days after sowing [DAS] for Frisson and 62, 68, 82 and 103 DAS for Santana). Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$).

In contrast, N rhizodeposition (mg g$^{-1}$ root DM) was significantly higher under controlled conditions at the end of flowering. However, at the beginning of flowering and at maturity, N rhizodeposition as a percentage of total plant N was significantly higher under field conditions, compared to the pot experiment. During flowering, the below-ground C (or N)-to-above-ground C (or N) ratio (BGC/AGC; BGN/AGN) was twice as high under field conditions compared to the pot experiment (Fig. 4.3 a and b). Compared to above-ground C and N, more rhizodeposition and root C and N were formed under unrestricted root growth in the field. The rhizodeposition-to-root ratio differed at the end of flowering (significantly for N) and at maturity between field and pot conditions (Fig. 4.3 c and d). At the end of flowering the rhizodeposition-to-root (C and N) ratio was higher in the pot experiment compared to the field experiment and at maturity the ratio was higher in the field.

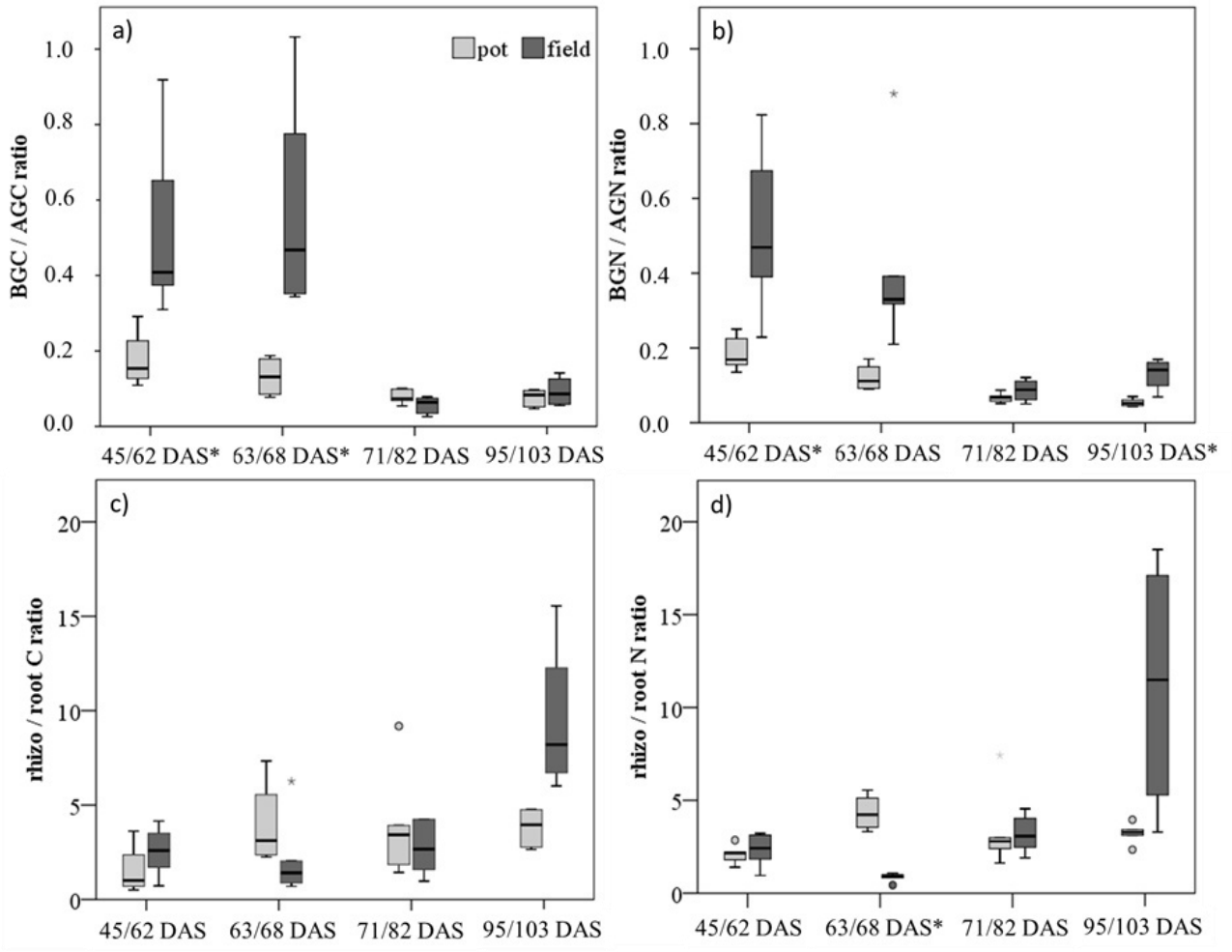


**Fig. 4.3**: a) Below-ground C / above-ground C (BGC / AGC), b) BGN / AGN, c) C derived from rhizodeposition (CdfR) / root C and d) NdfR / root N ratios of peas (Frisson for pot experiment and Santana for field experiment) at different harvest dates (45, 63, 71 and 95 days after sowing [DAS] for Frisson and 62, 68, 82 and 103 DAS for Santana). Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$).

**Table 4.5**: C and N derived from rhizodeposition (dfR) in pot experiment (pea: Frisson) and field experiment (pea: Santana) in mg $g^{-1}$ root drymatter (DM) and % of total plant C and N, at different harvest dates (45, 63, 71 and 95 days after sowing for Frisson and 62, 68, 82 and 103 days after sowing for Santana). Values with * indicate significant differences between the means (t-test, pairwise comparison, $p < 0.05$). Values show means (Frisson: n = 5 for BBCH 59, n = 4 for BBCH 69 and n = 6 for BBCH 79 and 89; Santana: n = 5; except BBCH 89: n = 4).

| | CdfR (mg $g^{-1}$ root DM) | | NdfR (mg $g^{-1}$ root DM) | | CdfR (% of total plant C) | | NdfR (% of total plant N) | |
|---|---|---|---|---|---|---|---|---|
| | Pot | Field | Pot | Field | Pot | Field | Pot | Field |
| DAS 45/62 | 635 ± 298 | 1,047 ± 245 | 60 ± 7 | 61 ± 11 | 8.4 ± 3.1* | 23.2 ± 4.6 | 10.4 ± 1.0* | 22.3 ± 4.1 |
| DAS 63/68 | 1,690 ± 521 | 995 ± 477 | 121 ± 21* | 22 ± 3 | 9.1 ± 2.1 | 22.1 ± 5.6 | 8.7 ± 1.3 | 13.0 ± 2.6 |
| DAS 71/82 | 1,617 ± 445 | 1,172 ± 270 | 95 ± 23 | 77 ± 11 | 5.5 ± 0.6 | 3.8 ± 0.9 | 6.8 ± 2.2 | 6.0 ± 1.1 |
| DAS 95/103 | 1,664 ± 163 | 4,454 ± 1016 | 82 ± 6* | 262 ± 65 | 5.6 ± 0.7 | 7.5 ± 1.6 | 3.9 ± 0.3* | 10.3 ± 1.9 |

## 4.4. Discussion

### *4.4.1 Labelling plants under field conditions*

The careful labelling of plants resulted in homogeneous and high plant enrichment with $^{13}C$ and $^{15}N$. Labelling of plants under field conditions is a challenge, especially when using the cotton wick method. Therefore, until now this method has been used only in experiments in which plants were grown in pots or columns with restricted root growth. However, the advantage of the cotton wick method is the possibility of a continuous labelling, without influencing plant growth. The enrichment in the present study is comparable to other labelling experiments under controlled conditions (Mayer et al. 2003; Mahieu et al. 2007; Arcand et al. 2013; Hupe et al. 2016b) or under field conditions with restricted root growth (Wichern et al. 2007b). The same holds true for the $^{13}C$ and $^{15}N$ isotope recovery. However, it should be noted, that at green ripe the recovery of $^{13}C$ and $^{15}N$ in plants is too high. Due to root respiration, a recovery of 89% of $^{13}C$ in plants is not possible.

In the field, the labelling system was renewed when plants stopped taking up solution, caused by a possible contamination of the labelling system or by the formation of callus on the plant stem. We discussed to digging a trench around the microplots, where we could stand to perform the labelling. However, the possible influence of a trench on plant stock and soil conditions was too great, due to the fact that a trench can also limit root growth. Moreover, soil moisture can be affected through a larger soil surface area. Therefore, a multicarrier for labelling plants from above was developed, based on the principle of a cucumber harvester. With this, the possible influence on plant growth and soil was limited to the time of labelling, whereas a trench would have affected the plants over the whole vegetation period.

Nevertheless, we observed an effect of the labelling approach on plant development. The dry matter of labelled and non-labelled pea plants differed significantly (data not shown). At the end of flowering and at maturity, the non-labelled plants formed more above- ground dry matter compared to the labelled peas. No differences could be detected for root dry matter. In contrast, Wichern et al. (2009) and Mahieu et al. (2009) found no differences in below-ground, above-ground and total dry matter or plant biomass, when using the cotton wick technique. Differences in dry matter may have been caused by mechanical damages during plant labelling or documentation of plant development and solution uptake. However, in a second field experiment conducted at the same time under similar conditions and investigating the transfer of C and N

from peas to triticale, no effect of labelling on pea biomass was observed (Hupe et al. unpublished). Therefore, we conclude that labelling with the multicarrier is better than using a trench.

Estimating root dry matter under field conditions is another challenge. Especially for estimating rhizodeposition, it is important to detect the exact root biomass of a plant. In the present study, the sampling of the whole microplot was not an option. Therefore, a sampling scheme was developed, based on Anthes (2005). With this, root dry matter could successfully be estimated down to 60 cm. Because of their small size and near transparency, fine roots are more difficult to detect. Therefore, the root biomass is often underestimated (Pierret et al. 2005). In the present study, with soil washing, no pea roots were detectable deeper than 60 cm. However, pea roots can be found deeper than this. Voisin et al. (2002) detected pea roots at 80 cm depth, Thorup-Kristensen (1998) at 100 cm depth, Fan et al. (2016) at 110 cm depth and Armstrong et al. (1994) found pea roots at almost 160 cm depth.

### *4.4.2 Rhizodeposition in time and space*

The amount of C and N rhizodeposition did not change between flowering and maturity of pea. In fact, differences between the development of CdfR and NdfR were to be expected. C and N are transported in plants differently, C as carbohydrates, especially sucrose, in the phloem (Kühn and Grof 2010) and N mainly as urea (Witte 2011) and amino acids (Lalonde et al. 2003) in phloem tissue, and mainly as $NO_3^-$ and partly as $NH_4^+$ in the plant xylem. In a pot experiment, it has already been shown, that C rhizodeposition constantly increased during the vegetation period and that N rhizodeposition increased during flowering and remained constant afterwards (Hupe et al. 2016b). However, under field conditions no differences between C and N rhizodeposition could be found. Nevertheless, differences in quality/composition of rhizodeposition can be expected, even though not investigated in the present study. The development of the pea root system is at its maximum during flowering (Gavito et al. 2001; Thorup-Kristensen 1998). This implies that until flowering root fragments are only a minor component of rhizodeposition. After flowering, between 40 and 50% of the root system dies (Gavito et al. 2001). An increasing amount of root fragments, originating from root turnover, can lead to changes in the quality of rhizodeposition.

The absence of more studies, dealing with rhizodeposition over the course of time and using a similar experimental setup, makes it difficult to compare present results with other data. Arcand et al. (2013) investigated the rhizodeposition of pea and used the same labelling method

and similar labelling intensity. However, they focused only on N rhizodeposition. Wichern et al. (2007 a, b) investigated C and N rhizodeposition with a $^{13}C$ and $^{15}N$ double labelling. However, there are considerable differences could be found in the frequency and duration of labelling and the time of harvest. These differences in experimental setup can influence the rhizodeposition, as reflected by the large range of values, reported for rhizodeposition in the literature (Wichern et al. 2008; Arcand et al. 2013).

Regardless of the plant development stage, C and N rhizodeposition could be found in similar amounts at 0-30, 30-60 and 60-90cm depth. Compared with other crops, pea roots can be found in deeper soil layers (Fan et al. 2016) with a maximum at 160 cm depth (Armstrong et al. 1994). Due to this, the finding of C and N rhizodeposition at 60-90 cm depth is not surprising. From May to August, the time of plant labelling, there is no downward water movement. Therefore, a shift of rhizodeposition from topsoil to subsoil due to leaching can be excluded.

The horizontal distribution of C and N rhizodeposition is shown in the supplementary material (Tab. S1 and S2). It was found, that the rhizodeposition is evenly distributed in the three sectors (α: directly to the single harvested pea; β: exactly between 2 peas in the row; γ: in the middle of 4 peas between two rows). This demonstrates also the evenly distribution of pea roots, which is important for calculating the complete root biomass.

Due to inhomogeneous field conditions, differences in the total amount of microbial biomass were found during plant development. Differences in microbial biomass due to microbial growth can be excluded, because of the constant MBC to SOC ratio during plant development (Ø 1.96 at 0-30 cm and 1.71 at 30-60 cm). At 0-30 cm depth, about one third of C and N rhizodeposition was integrated into microbial biomass and extractable C or the inorganic N pool. At 30-60 cm depth, the proportion of N rhizodeposition in the microbial biomass and inorganic N pool of soil is even lower. This indicates that rhizodeposits consist of substantial particular amounts, such as root residues or root fragments, which take longer to be mineralized and are not quickly incorporated into microbial tissue. Wichern et al. (2007a) recovered between 11 and 21% of C rhizodeposition and between 1 and 23% of N rhizodeposition of pea in microbial biomass, depending on time of plant labelling. Mayer et al. (2003) found 18% of N rhizodeposition integrated into MBN at pea maturity. In the present study, between 21 and 42% of C and between 15 and 33% of N released by pea roots were integrated into microbial biomass at 0-30 cm depth, depending on plant development. Because of a higher variability of abiotic factors, the root

turnover under field conditions will be higher compared to controlled conditions. This can cause a higher C and N transfer from plants to the microbial biomass in the field.

Beside of the plant development or the date of plant labelling, the integration of rhizodeposition into microbial biomass may depend on plant species. Pausch et al. (2013) found only 7% of C released by maize roots integrated into microbial biomass 44 days after germination (16 days after labelling). It is known, that plant species influences microbial community composition (Marschner et al. 2001; Paterson 2003; Chen et al. 2008; Dennis et al. 2010). It was found, that legumes (alfalfa and white clover) had a more dominating effect on the microbial community compared to Sudan grass and tall fescue (Chen et al. 2008). De Graaff et al. (2007) found differences in the integration of NdfR in MBN between wheat and maize. The $^{15}N$ recovery in microbial biomass was lower in maize compared to wheat. The rhizodeposits released by different plant species differ in amount and quality and are further affected by physical and chemical soil properties (e.g. nutrient deficiency, drought) ( (Neumann and Römheld 2007; Hartmann et al. 2009). Therefore, the amount of rhizodeposition used by soil microorganisms is influenced directly or indirectly by a lot of factors, such as the composition of microbial biomass, the amount and composition of plant rhizodeposits, as well as soil and environmental factors.

### *4.4.3 Pot versus field conditions*

As the process of rhizodeposition is closely related to root biomass (Shamoot 1968; de Graaf et al. 2007) and more root biomass will be developed under field conditions, the amount of C and N rhizodeposition of pea (in mg per g root DM) was two to three times higher at maturity under field conditions compared to controlled conditions.

Most of the studies that deal with rhizodeposition were conducted under controlled conditions as pot experiments with restricted root growth. Estimating rhizodeposition under field conditions is very challenging due to the labor intensity of plant labelling and sampling (Pausch et al. 2013). Therefore, if possible, the best option would be to transfer results from pot to field. However, the results of the current study imply that this will probably not be possible.

Under field conditions, biotic and abiotic stress factors (e.g. light intensity, temperature, pathogen attack, drought or nutrient deficiency) can influence plants, especially root development and rhizodeposition (Neumann and Römheld 2007; Jones et al. 2004). The intensity of stress factors is lower in greenhouse experiments under controlled conditions compared to experiments under field conditions. Moreover, pot size can influence plant development, especially the root system

and the root-to-shoot ratio (Poorter et al. 2012), with lower root biomass often observed in pot experiments. Therefore, the amount of root fragments, border cells, root hairs, and fine roots, which are part of the rhizodeposits (Kuzyakov and Domanski 2000; Wichern et al. 2008; Jones et al. 2009; Pausch et al. 2013) can differ between field and greenhouse experiments.

By comparing plant growth in the current study and in a similar pot experiment under controlled conditions in the greenhouse, with restricted root growth (Hupe et al. 2016b), a shift of the root-to-shoot ratio is evident. Depending on time, field-grown peas developed differently compared to peas grown under controlled conditions. Under field conditions, the development of a strong root system took a longer time, compared to controlled conditions. This led to high BGC/AGC and BGN/AGN ratios in the field until the end of flowering. After flowering, AGB rapidly increased in the field experiment. Due to this, the BGC/AGC and BGN/AGN ratios in the field and pot experiment became more similar.

Pausch et al. (2013) proposed using the ratio of rhizodeposition C-to-root C of maize as derived from a pot experiment to estimate the C rhizodeposition of maize under field conditions from root biomass assessment only, assuming a constant rhizodeposition-to-root ratio. Certainly, this approach would simplify total below-ground biomass assessment in the field, as determination of rhizodeposition in the field is even more laborious and expensive than under controlled conditions. However, the differences in plant development, especially the root growth dynamics including root death and turnover, between field and pot experiments lead us to the conclusion that the rhizodeposition-to -root ratio will also be different under field conditions compared to pot experiments under controlled conditions and cannot be transferred from pot experiments to field experiments. First of all, altered soil structure from sieving the soil affects root growth patterns and abrasion of root cells, consequently altering rhizodeposition patterns (rhizodeposition-to-root ratio). At the same time, soil moisture, soil temperature and nutrient availability are usually change under field conditions, whereas they are often kept constant in pot experiments under controlled conditions. This will ultimately also affect root growth and death in response to water and nutrient availability and soil temperature and thus alter the rhizodeposition patterns as well. Additionally, rhizodeposition might also be altered qualitatively, e.g. by higher mucilage production under water limiting conditions or soils with higher penetration resistance (Brimecombe et al. 2007).

Besides the differences in BGP/AGP ratio, a different rhizodeposition/root ratio can be observed when comparing results of field and pot experiments. During the vegetative growth of

peas in the field, more root dry matter was formed. This led to a lower rhizodeposition/root ratio in the field compared to the pot. Because of a higher root turnover in the field and due to the fact, that rhizodeposition includes tissue root fragments as well as sloughed cells and root exudates, at maturity this ratio shifted towards rhizodeposition in the field experiment. At this time, the rhizodeposition to root ratio was more than twice as high under field conditions compared with the pot experiment. Therefore, due to differences in the duration/intensity of root development and a higher root turnover in the field, the rhizodeposition/ root ratio and the BGP/AGP ratios differ between field and pot grown peas, depending on plant development.

## 4.5 Conclusions

The present study has clearly shown that, depending on plant development, the C and N rhizodeposition of peas differs between pot and field experiments. Reasons can be found in the restricted root growth in pot experiments. Moreover, biotic and abiotic factors under field conditions can influence the amount and quality of rhizodeposition. For estimating rhizodeposition, a continuous labelling is required. For the first time, pea plants were successfully labelled with the cotton wick method under field conditions without restricted root growth. This allowed the estimation of the true quantity of plant rhizodeposition. Comparable future studies will help to give more exact information about the C sequestration potential of plants or to develop more exact N balances by quantifying the N input more precisely. A transfer of results from pot experiments to field conditions is not recommended. Therefore, for estimating rhizodeposition, we recommend to carrying out more experiments under field conditions, without restricted root growth.

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# 5. General conclusions and future research needs

In preparation for performing a pot experiment, it was noticeable that there is a huge variation of results dealing with the quantification of C and N rhizodeposition. Differences in the amount of rhizodeposition can be explained through the use of different plant species, due to the fact that rhizodeposition correlates with the root system and this differs between plant species. But there were also differences in rhizodeposition within one species, caused by differences in the method used for labelling plants.

We developed the mass balance approach, a less sensitive way of calculating rhizodeposition. If this approach is adopted, studies dealing with the estimation of rhizodeposition will be more comparable in future. By comparing the two different calculation methods, it could be shown that the duration of labelling has a direct impact on the calculation, especially when using the Janzen and Bruinsma equation. This approach is considerably more sensitive to tracer dilution or relocation processes related to plant development. Janzen and Bruinsma focuses only on enrichment of root and soil. Therefore, this approach is highly sensitive to tracer dilution in roots and can lead to an overestimation of rhizodeposition. It could be shown that the results of both calculation approaches become more similar with increasing label homogeneity.

The assumption that rhizodeposition represents an easily available source of C and N for microorganisms could not be confirmed; only a small part (one third) of rhizodeposition was integrated into the soil, independently of time. This means that a large part of rhizodeposition is accounted for by root fragments that originate from root death and turnover. If the definition of rhizodeposition is restricted to root exudates, the larger part of the C and N input through plants will not be disclosed. Especially the reproductive phase of legumes is characterized by an increasing root turnover. During this time, the C and N input through rhizodeposition can be greatly underestimated. Therefore, we recommend using the term belowground plant biomass, which includes the amount of C and N derived from rhizodeposition and also C and N originating from the root system that remains in the soil after harvest.

We showed that the C and N rhizodeposition have developed differently during plant growth. For estimating rhizodeposition of peas, the time of flowering is an important moment. Until this date, a large part of N rhizodeposition can be already found in soil. Future studies should concentrate on the vegetative plant growth. More harvest dates (and a shorter period between the harvest dates) can help to model a more exact development of rhizodeposition.

A possible influence of mycorrhiza on rhizodeposition, especially on nitrogen rhizodeposition, cannot be excluded by the present study. However, the influence of nitrogen fertilization is probably even bigger. In the pot experiment, the root dry matter was on a low level, due to the application of nitrogen fertilizer at the beginning. This led to a small amount of rhizodeposition originating from root turnover. Future research should investigate the influence of different fertilizers used in conventional or organic farming (e.g. manure or compost) on the amount and quality of rhizodeposition and differences in the incorporation into soil pools.

The transfer of results from pot experiments to the field is difficult. The present studies show clear differences between pea development in pot and field. These differences, especially in root development, lead to a shift in the root to shoot ratio and the rhizodeposition to root ratio. A large amount of rhizodeposition originates from root turnover. Therefore, differences in the root dry matter influence the amount of rhizodeposition. In the present study, controlled conditions in the greenhouse and the restricted root growth led to a smaller root system compared to field conditions. This resulted in more rhizodeposition under field conditions, due to a higher root turnover.

For the first time, pea plants were successfully labelled with the cotton wick method under field conditions without restricted root growth. This will allow a true estimation of the amount of plant rhizodeposition in future and will help to give more exact information about the C sequestration potential of plants or to develop more exact N balances. The determination of the complete amount of root dry matter under field conditions is difficult, as fine roots are small, nearly transparent and difficult to detect. Therefore, we developed a root sampling scheme to calculate the complete root dry matter of pea. Further studies are needed to improve this, especially the detection of roots in deeper soil layers.

Due to global warming, plants will grow under changing environmental conditions in the future (e.g. more frequent heavy rain events or spring drought). These changes can influence the amount and quality of rhizodeposition. Therefore, future studies should focus on the impact of different biotic and abiotic stress factors on the amount and quality of rhizodeposition.

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# 8. Supplementary material

**Table S1** (cf. 4.4.2 Rhizodeposition in time and space) C derived from rhizodeposition (CdfR) and percentage of CdfR in microbial biomass C (MBC) and extr. C. Soil samples were taken in 0-30 and 30-60 cm depth 62, 68, 82 and 103 days after sowing in three defined positions in the microplot (α: directly to the single harvested pea; β: exactly between 2 peas in the row; γ: in the middle of 4 peas between two rows). Different letters indicate significant differences between BBCH (Scheffé, $p < 0.05$). Values show means (n=between 3 and 5).

| | | CdfR (mg plant$^{-1}$) | % of CdfR in MBC | % of CdfR in extr. C | CdfR (mg plant$^{-1}$) | % of CdfR in MBC | % of CdfR in extr. C |
|---|---|---|---|---|---|---|---|
| | | 0-30 cm | | | 30-60 cm | | |
| 62 DAS | α | 71 a | 42 a | 19 a | 19 a | 4 a | 2 a |
| | β | 28 a | 50 a | 23 a | 18 a | 2 a | 1 a |
| | γ | 55 a | 32 a | 15 a | 17 a | 20 a | 9 a |
| 68 DAS | α | 736 a | 5 a | 2 a | 30 a | 12 | 7 |
| | β | 29 b | 31 a | 14 ab | 16 a | n.d. | n.d. |
| | γ | 71 b | 37 a | 17 b | 22 a | n.d. | n.d. |
| 82 DAS | α | 239 a | 36 a | 16 a | 31 a | 19 a | 9 a |
| | β | 146 a | 34 a | 15 a | 33 a | 27 a | 13 a |
| | γ | 236 a | 26 a | 12 a | 102 a | 15 a | 7 a |
| 103 DAS | α | 283 a | 38 a | 17 a | 13 a | 20 | 9 |
| | β | 32 a | 36 a | 16 a | 4 a | n.d. | n.d. |
| | γ | 126 a | 59 a | 27 a | 15 a | 20 | 27 a |

n.d. = not detectable

**Table S2** (cf. 4.4.2 Rhizodeposition in time and space) N derived from rhizodeposition (NdfR) and percentage of NdfR in microbial biomass N (MBN) and inorganic N. Soil samples were taken in 0-30 and 30-60 cm depth 62, 68, 82 and 103 days after sowing in three defined positions in the microplot (α: directly to the single harvested pea; β: exactly between 2 peas in the row; γ: in the middle of 4 peas between two rows). Different letters indicate significant differences between BBCH (Scheffé, $p < 0.05$). Values show means (n=between 3 and 5).

| | | NdfR (mg plant$^{-1}$) | % of NdfR in MBN | % of NdfR in inorg. N | NdfR (mg plant$^{-1}$) | % of NdfR in MBN | % of NdfR in inorg. N |
|---|---|---|---|---|---|---|---|
| | | 0-30 cm | | | 30-60 cm | | |
| 62 DAS | α | 3.4 a | 19 a | 13 a | 1.1 a | 12 a | 10 a |
| | β | 2.1 a | 25 a | 15 a | 1.0 a | 3 a | 12 a |
| | γ | 4.2 a | 18 a | 9 a | 1.9 a | 12 a | 11 a |
| 68 DAS | α | 3.6 a | 37 a | 7 a | 1.3 a | 27 | 8 |
| | β | 2.1 a | 31 a | 6 a | 1.3 a | n.d. | n.d. |
| | γ | 5.6 a | 34 a | 6 a | 0.7 a | n.d. | n.d. |
| 82 DAS | α | 7.3 a | 20 a | 4 a | 1.6 a | 10 a | 5 a |
| | β | 4.5 a | 20 a | 4 a | 1.7 a | 9 a | 4 a |
| | γ | 9.2 a | 18 a | 4 a | 3.3 a | 14 a | 4 a |
| 103 DAS | α | 7.9 a | 13 a | 11 a | 1.2 a | 10 a | 5 a |
| | β | 2.6 a | 16 a | 12 a | 0.2 a | n.d. | n.d. |
| | γ | 9.1 a | 17 a | 16 a | 1.4 a | 22 a | 5 a |

n.d. = not detectable

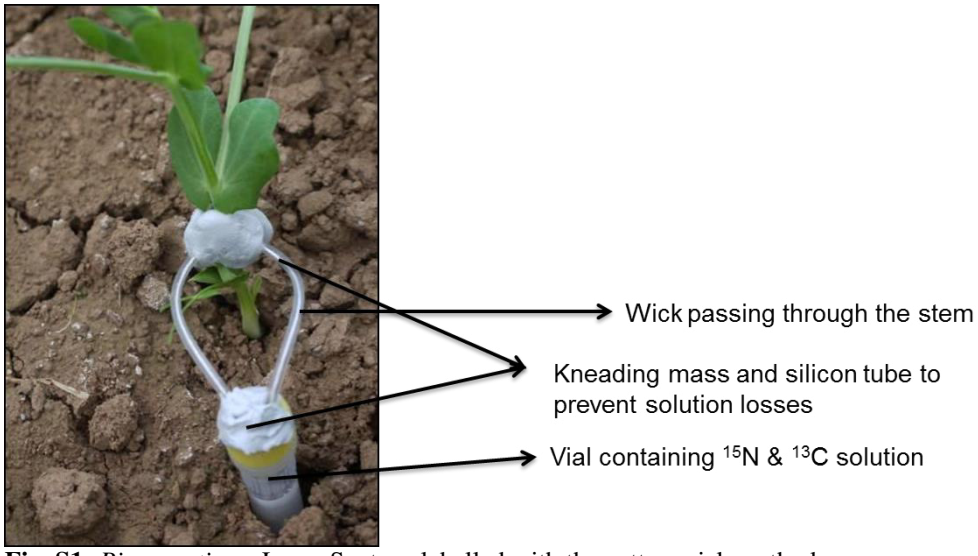


**Fig. S1:** *Pisum sativum* L. cv. Santana labelled with the cotton wick method

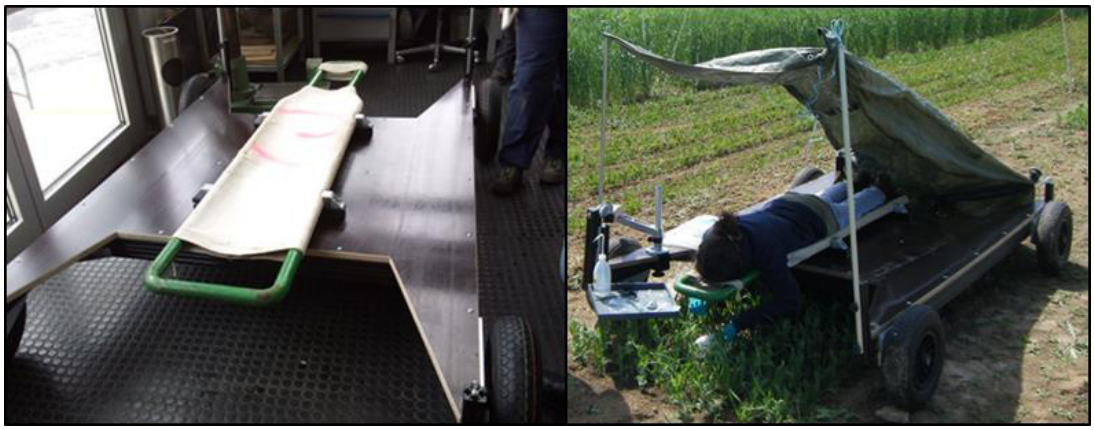

**Fig. S2** Multicarrier for labelling pea plants under field conditions


**Fig. S3** (a) HUMAX, a special soil sampling drilling tool
(b) The soil will be automatically filled in a plastic cylinder.