HOKKAIDO UNIVERSITY

Title	Studies of properties of microorganisms in bulk and rhizosphere soils following the application of cover crops
Author(s)	Chinta, Yufita Dwi
Citation	北海道大学. 博士(環境科学) 甲第14340号
Issue Date	2021-03-25
DOI	10.14943/doctoral.k14340
Doc URL	http://hdl.handle.net/2115/88939
Type	theses (doctoral)
File Information	Yufita_Dwi_Chinta.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Studies of properties of microorganisms in bulk and rhizosphere soils following the application of cover crops

(緑肥施用土壌の根域および根圏における微生物特性に関する研究)

Yufita Dwi Chinta

Dissertation
Graduate School of Environmental Science
Hokkaido University
2021

This doctoral dissertation is produced in partial fulfilment of the requirements for the Degree of Doctor of Philosophy of the Environmental Earth Science at a University of Hokkaido. The research results included have either already been published or are in manuscript at various stages (submitted or constructed).

Nulla difficultas, si in Iesum credo
Yufita Dwi Chinta
Sapporo, March 2021

Acknowledgment

Throughout the writing of this dissertation I have received a great deal of support and assistance from various people.

I would first like to thank my prior supervisor, Prof. Hajime Araki, in Niigata Agro-Food University who kindly accepted me into his project, through which I was able to study organic agriculture, a subject I am passionate about. I would also like to thank Dr. Yoshitaka Uchida, who has facilitated and enhanced my skills in research. I would like to thank my current supervisor, Prof. Yoichiro Hoshino, who has whole heartedly supported me academically and mentally especially in the final year of my PhD. My sincere gratitude to committee members of the dissertation review: Prof. Masaaki Morikawa, Prof. Toshihiko Yamada, and Dr. Toshiyuki Hirata, for their thoughtful comments and recommendations on the dissertation.

I would like to acknowledge all members of the Laboratory of Environmental Biogeochemistry in Research Faculty of Agriculture, Hokkaido University, who in various ways have supported me in achieving my research goals. I would also like to thank all students at the FSC for Northern Biosphere, Hokkaido University, who have followed my research progress and shared their advice and knowledge across various multidisciplinary topics. Special thanks to Akane C., Miyuki O., Yuto M., Yvonne M.M., Eva M., A. Sezawa, and R. Muchanga for their valuable help and discussions. Special thanks also to Mrs. Aoi Nakamura in Global Facility Center, Mr. Tsunoda, Mr. Ichikawa, and Mr. Nakano in FSC for Northern Biosphere, Mr. Muraoka in Wasamu town, and all members of the cover crop project under the Kakenhi in Ibaraki University and Niigata Agro-Food University for their great help.

I would like to sincerely thank my husband Atsushi Yagioka, whose constant love and support as a husband and fellow scientist was significant in completion of the dissertation, and my Indonesian and Japanese families for their immeasurable counsel and sympathetic ear during my study. Finally, I could not have completed this dissertation without the support of my good friends, M. Fukaya, Kakoli M., and Yvonne and her mom who through their encouragement have helped me mentally, inspired, and prayed for me during challenging times.

Abstract

Rye (*Secale cereale* L.) and hairy vetch (*Vicia villosa* Roth, HV) are cover crops, plants commonly applied in crop production with multifunction. One of the functions is supplying inorganic nitrogen (N) for subsequent plants. Inorganic N supply is abundant during the decomposition phase of cover crop residues which occurs within a month of incorporation. However, utilization of this inorganic N by subsequent plants during this period is not performed well. In bulk soil, bacteria and fungi play roles in the inorganic N supply from cover crop residues. However, their roles during the decomposition period are barely known. During interaction with the subsequent plant, microorganisms inhabiting the plant rhizosphere soils can assist in N utilization (N uptake, N_{up}) affecting plant yield. Plant–microbe interactions in the subsequent plant rhizosphere soil during cover crop decomposition is still not well understood. Therefore, to understand the effects of cover crops on horticultural systems, this study was carried out (1) to clarify N availability and its utilization by the subsequent plant, (2) to identify microorganisms contributing to N availability in the bulk soil, and (3) to evaluate plant–microbe interactions in the rhizosphere soil of subsequent plant cultivated during the decomposition periods of cover crop residues.

The research, which included field and pot experiments, was conducted for two years (i.e., 2017 and 2018). Treatments were soil without any cover crops (control) and soils with rye, HV, and mixed (rye+HV) cover crops. In the fields, two ammonium sulfate application rates (i.e., 0 and 2.5 g N m^{-2} in 2017 and 0 and 6 g N m^{-2} in 2018) were applied to compare the effects of cover crops and synthetic N fertilizer. The effects of cover crops were clarified in the pot. Red leaf lettuce (*Lactuca sativa* L. var. *crispa* cv. Red fire), the subsequent plant, was transplanted 5 days after incorporation (DAI) of cover crops and harvested at a mature stage in the field and at the mid growth and mature stages in the pot to evaluate lettuce N_{up} and yield. Bulk and rhizosphere soils were collected during the experiments (i.e., 5–38 DAI in 2017 and 3–31 DAI in 2018). The bulk soils were analyzed for soil inorganic N (i.e., NO_3^-N + NH_4^+-N), activity of β-glucosidase enzyme (BG), and carbon-based soil microbial biomass (SMB). Bulk and rhizosphere soils from the pots were subjected to DNA-based molecular analysis to quantify and identify bacteria and fungi. Influenced microbial taxa, whose relative abundance was affected by cover crops, were selected; and their relative abundance was correlated with values of BG activity and SMB or lettuce N_{up} and yield.

Results of N availability as concentration of soil inorganic N was as follows HV > rye+HV > rye = control. This clarifies the common effects of cover crops on N supply. In the fields, HV-0N and rye+HV-0N promoted lettuce yield and N_{up} over control-0N. Conversely, lettuce performances in rye depended on 2.5N or 6N fertilizer addition. This indicates that HV and rye+HV, but not rye, could alternate application of synthetic N fertilizer. However, in the pots, HV effect was initial soil carbon dependent, resulting in a suppression and promotion of lettuce performances in 2017 and 2018, respectively, at the plant mid growth stage. Specific analysis to rhizosphere soils showed that plant–microbe interactions had significant negative correlations between bacterial influenced taxa and N_{up} and yield in HV in 2017. On the contrary, vigorous lettuce growth in HV in 2018 showed that the roots recruited more beneficial microorganisms that positively direct and indirect effected on N_{up} and yield promotion.

In bulk soils, bacterial and fungal DNA was relatively higher in cover crop-treatments than control within 5–10 DAI, concomitant with active N mineralization periods, up to 15 DAI, of cover crops. Further, microorganisms in bulk soil sequentially promoted BG activity and SMB during the decomposition period, implying BG activity and SMB as indicators of microbial roles in N availability. Specifically, family *Parachlamydiaceae* and unidentified bacteria of class SAR202 were correlated with BG activity and SMB positively in HV and rye+HV or negatively in rye. This indicates that each cover crop enhances specific microbial roles relating to N availability.

This study demonstrated the technique to maximize utilization of N supplied from cover crops during the residual decomposition periods. The technique can be adapted in cover crop-horticulture rotation systems. Furthermore, microbial pathways of N supply and N utilization were molecularly revealed. In cover cropping system, thus, cover crops can be selected based on the N benefit and microbial feature.

Contents

	Page
Acknowledgement and abstract	i
Contents	iv
List of Figures	x
List of Tables	xii
List of supplementary material	xiii
Chapter 1. Introduction	
1.1. Background	1
1.2. Research objectives	2
1.3. Dissertation structure	2
Chapter 2. Literature Review	
2.1. Cover crops in agriculture	4
2.1.1. Cover crops	4
2.1.2. Types and function	6
2.1.2.1. Leguminous cover crops	6
2.1.2.2. Gramineous cover crops	6
2.1.3. Factors controlling cover crop biomass	7
2.2. Residual decomposition, N mineralization, and N-cycle	7
2.2.1. Quality of residue and pattern of decomposition and N mineralization	7
2.2.2. Factors controlling residual decomposition and N mineralization	8
2.2.3. Microbial properties related to residual decomposition and N mineralization in bulk soil	9
2.2.3.1. β-glucosidase enzyme (BG) activity	10
2.2.3.2. Soil microbial biomass (SMB)	12
2.2.3.3. Microbial community	13
2.2.3.3.a. Microbial richness	14
2.2.3.3.b. Microbial abundance	14
2.2.3.3.c. Microbial diversity	15
2.2.3.4. Microbial network	16
2.2.3.5. Microbial function	17

2.2.4. Bacterially-N cycle in bulk soils 18
2.2.4.1. Decomposition 18
2.2.4.2. N fixation 19
2.2.4.3. Nitrification 19
2.2.4.4. Denitrification 20
2.3. Utilization of N by the subsequent plant 20
2.3.1. Leaf lettuce and parameter of N utilization 21
2.3.2. Abiotic factors influencing plant N uptake (N_{up}) 22
2.3.3. Microbial properties in rhizosphere soils related to plant N_{up} and yield 22
2.3.3.1. Microbial community 24
2.3.3.2. Beneficial and harmful rhizosphere microbiomes 24
2.3.3.3. Microbe–microbe network 26
2.3.3.4. Plant–microbe interaction 26
2.4. Recommendation for research 28
2.4.1. Research 1 29
2.4.2. Research 2 29
2.4.3. Research 3 30

Chapter 3. Nitrogen supply from cover crops by soil microorganisms and its utilization by lettuce plants during the residual decomposition

Graphical Abstract and Highlights 31
3.1. Research gap and potential solution 32
3.2. Materials and Methods 33
3.2.1. Experimental levels and site 33
3.2.1.1. Field experiment 33
3.2.1.2. Pot experiment 33
3.2.2. Treatments and experimental designs 34
3.2.2.1. Field experiment 34
3.2.2.2. Pot experiment 35
3.2.3. Lettuce performances 37
3.2.3.1. Field experiment 37
3.2.3.2. Pot experiment 37
3.2.4. Soil samplings 38
3.2.4.1. Field experiment 38

3.2.4.2. Pot experiment 38
3.2.5. Analysis of soil biochemical properties 38
3.2.5.1. Soil chemical properties 38
3.2.5.2. Soil biological properties 39
3.2.6. Quantifying bacterial and fungal DNA 40
3.2.6.1. DNA extraction and purification 40
3.2.6.2. Quantitative PCR (qPCR) 40
3.2.7. Litter bag assay 41
3.2.8. Statistical data analyses 41
3.2.8.1. Field experiment 41
3.2.8.2. Pot experiment 42
3.3. Results 42
A. Field experiment 42
3.3.1. Climatic conditions during the preparation and experimental periods 42
3.3.2. Biomass and N input (N_{in}) of cover crops 42
3.3.3. Performances of lettuce plants 43
3.3.4. Changes on soil biochemical properties and microbial abundance 43
3.3.5. Cover crop residual decomposition, and N and C mineralization 47
B. Pot experiment 49
3.3.6. Performances of lettuce plants 49
3.3.7. Dynamics of soil biochemical properties and microbial abundance 49
3.3.8. Cover crop residual decomposition, and N and C mineralization 53
3.4. Discussion 55
A. Field experiment 55
3.4.1. Variation on biomass and N input (N_{in}) of cover crops 55
3.4.2. Effects of cover crops and synthetic N fertilizer on lettuce performances 56
3.4.3. Effects of cover crops and synthetic N fertilizer on soil biochemical properties and microbial abundance 58
3.4.4. Cover crops decomposition and N supply 59
B. Pot experiment 60
3.4.5. Effects of cover crops on lettuce performances 60

3.4.6. Significance of BG activity and SMB to N supply from cover crops 61
3.4.7. Significance of microbial abundance to N supply from cover crops 63
3.5. Conclusion 64
Supplementary Material 65

Chapter 4. Properties of bulk soil microorganisms and the roles in nitrogen supply from cover crops during the decomposition periods
Graphical Abstract and Highlights 68
4.1. Research gap and potential solution 68
4.2. Materials and Methods 70
4.2.1. Treatments and design 70
4.2.2. Soil sampling, DNA extraction, and DNA purification 70
4.2.3. Library preparation and sequencing of 16S rRNA and ITS 70
4.2.4. Processing data of sequence 71
4.2.5. Correlation network analysis 72
4.2.6. Functional analysis 72
4.2.7. Estimation of N budget 73
4.2.8. Quantifying bacterial N cycle-related gene abundance 73
4.2.9. Statistical data analyses 74
4.3. Results 75
4.3.1. Microbial richness and diversity 75
4.3.2. Microbial community structures and the influenced taxa 75
4.3.3. Correlation networks within and between bacteria and fungi 78
4.3.4. Correlation between influenced taxa and soil biochemical properties 78
4.3.5. N budget and abundance of bacteria with N cycle-related genes 81
4.4. Discussion 84
4.4.1. Effects of cover crops on microbial community 84
4.4.2. Microbe–microbe networks under different cover crops incorporation 85
4.4.3. Potential roles of influenced taxa in N supply from cover crops 86

4.4.4. N cycle-associated bacteria in cover crop-treated soils 87
4.5. Conclusion 90
Supplementary Material 90

Chapter 5. Properties of microorganisms in lettuce rhizosphere soil and the roles in plant nitrogen utilization at the mid growth stage
Graphical Abstract and Highlights 99
5.1. Research gap and potential solution 99
5.2. Materials and Methods 101
5.2.1. Treatments and design 101
5.2.2. Sampling of lettuce rhizosphere soil 101
5.2.3. DNA extraction and purification, and quantification of microbial abundance 101
5.2.4. Libraries preparation, sequencing, and processing data of sequence 101
5.2.5. The beneficial and harmful rhizosphere microbiomes 102
5.2.6. Directed network analysis 102
5.2.7. Statistical data analyses 102
5.3. Results 103
5.3.1. Bacterial and fungal abundance in lettuce rhizosphere soil 103
5.3.2. Community structures of rhizosphere microbiomes 103
5.3.3. The directed network 107
5.4. Discussion 109
5.4.1. Effects of cover crops on richness and diversity of rhizosphere microbiomes 109
5.4.2. Effects of cover crops on microbial community structures in lettuce rhizosphere soil 110
5.4.3. Microbe–microbe interactions in lettuce rhizosphere soil 111
5.4.4. Plant–microbe interactions in lettuce rhizosphere soil under different cover crop treatments 112
5.5. Conclusion 113
Supplementary Material 114

Chapter 6. General discussion
6.1. Supply of soil inorganic N from cover crops mediated by microorganisms existing in bulk soils 121
6.2. Effects of cover crops on lettuce productivity assisted by

microorganisms inhabiting lettuce rhizosphere soils 122

Chapter 7. Limitation and recommendation for further research

7.1. Application of cover crops in other soil types 125

7.2. Incubation and isotopic experiments 126

7.3. Different application methods of cover crops 127

List of references 129

List of Figures

Page

Chapter 2

2.1. Illustration of nitrogen (N) cycle based on Lindsay et al. (2010), Levy-Booth et al. (2014), and Ren et al. (2018) 18

2.2. Illustration of research recommendation 28

Chapter 3

3.1. (A) Lettuce yield and (B) estimation of specific nitrogen uptake (N_{up}) from cover crops in (1) 2017 and (2) 2018 field experiments 44

3.2. Trends of (A) residual decomposition, (B) nitrogen release, and (C) carbon release from cover crops in (1) 2017 and (2) 2018 field experiments 48

3.3. (A) Lettuce yield and (B) estimation of specific nitrogen uptake (N_{up}) from cover crops in (A) 2017 and (B) 2018 pot experiments 50

3.4. Dynamics of (A) soil inorganic nitrogen, (B) β-glucosidase enzyme (BG) activity, and (C) soil microbial biomass (SMB) during cover crop decomposition periods in (1) 2017 and (2) 2018 pot experiments 51

3.5. Dynamics of (A) bacterial and (B) fungal abundance during cover crop decomposition periods in (1) 2017 and (2) 2018 pot experiments 52

3.6. Trends of (A) residual decomposition, (B) nitrogen release, and (C) carbon release from cover crops in (1) 2017 and (2) 2018 pot experiments 54

Chapter 4

4.1. Heatmaps showing relative abundance of influenced taxa of (A) bacteria and (B) fungi in bulk soils at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively 77

4.2. Number of links in the networks (A) within bacteria (B–B), (B) within fungi (F–F), (C) between bacteria and fungi (B–F), and (D) total significant positive and negative links in bulk soils in 2017 and 2018 pot experiments 79

4.3. Canonical correspondence analysis (CCA) showing the correlations between influenced taxa of (A) bacteria and (B) fungi and soil biochemical properties at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively 80
4.4. Nitrogen (N) budget in g N pot^{-1} unit in bulk soil at 25 days after cover crops incorporation in 2017 pot experiments 82
4.5. Abundance of bacterial specific genes in bulk soil at 25 days after cover crops incorporation in 2017 pot experiments 82
4.6. Sum of relative abundance of bacteria with (A) *nifH*, (B) *chiA*, (C) AOB 16S rRNA, and (D) *nirS* genes in bulk soil at 25 days after cover crops incorporation in 2017 pot experiments 83

Chapter 5

5.1. Heatmaps showing relative abundance of influenced taxa of (A) bacteria and (B) fungi in family level in lettuce rhizosphere soil at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively 106
5.2. Directed networks of influenced microbial taxa and lettuce yield (Yield) and nitrogen uptake (Nup) in (A) control soil and soils with (B) rye, (C) mixed (rye+HV), and (D) hairy vetch (HV) cover crops at (1) 25 and (2) days after the incorporation in 2017 and 2018 pot experiments, respectively 108

List of Tables

Page

Chapter 3

3.1. Initial soil chemical properties in the field and pot experiments 34

3.2. Biomass and chemical characteristics of cover crops for the (A) field and (B) pot experiments each year 36

3.3. Soil biochemical properties in the fields with different cover crops and fertilizer application rates 45

3.4. Microbial abundance in the fields with different cover crops and fertilizer application rates 46

Chapter 4

4.1. The input and output factors used in estimation of nitrogen (N) budget 73

4.2. Sets of specific primers targeting the bacterial N cycle-related genes 74

4.3. Microbial richness, Shannon index, and differentiation of community structure of (A) bacteria and (B) fungi at the phylum and family levels in bulk soils of the pot experiments 76

Chapter 5

5.1. Microbial abundance, richness, diversity, and differentiation of (A) bacterial and (B) fungal community structures in lettuce rhizosphere soil at the mid growth stage of plant in the pot experiments 105

Chapter 7

7.1. List of soil types used for the applications of cover crops 126

List of supplementary materials

Page

Chapter 3

Figure

S3.1. Total carbon (TC) in cover crop-treated soils at lettuce mid growth stage in 2017 and 2018 pot experiments 65

S3.2. Correlation within soil biochemical properties at 5 days after cover crops incorporation in (A) 2017 and (B) 2018 pot experiments 67

Table

S3.1. *P*-value based on t-test of the rates of residual decomposition, nitrogen release, and carbon release between 2017 and 2018 up to 35 days after cover crops incorporation in the (A) field and (B) pot experiments 65

S3.2. Redness values of lettuce plants at the mature stage in the fields in 2017 66

S3.2. Estimation of nitrogen (N) mineralization for different cover crop components, based on the concentration of soil inorganic N at 5 days after cover crops incorporation in the pot experiments 66

Chapter 4

Figure

S4.1. Venn diagram of fungal family in bulk soils in 2018 pot experiments 96

Table

S4.1. Soil biochemical properties at 25 and 20 days after cover crop incorporation in 2017 and 2018 pot experiments, respectively (Chapter 3) 90

S4.2. List of bacteria families with specific genes: (A) *nifH*, (B) *chiA*, (C) AOB 16S rRNA, and (D) *nirS* 91

S4.3. *P*-value based on t-test of the rates of residual decomposition, nitrogen (N) release, and carbon (C) release between 2017 and 2018 experimental years up to 25 days after cover crops incorporation in the pot experiments 96

S4.4. General functions of (A) bacteria and (B) fungi in bulk soils at 25 and 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively 97

S4.6. Correlations between specific gene abundance and sum of relative abundance of bacteria with specific genes and the nitrogen (N) availability at 25 days after cover crops incorporation in 2017 pot experiments 98

Chapter 5

Figure

S5.3. Relative abundance of selected beneficial (A) bacteria) and (B) fungi in lettuce rhizosphere soils in family level at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively 119

S5.4. Relative abundance of selected plant pathogen of lettuce rhizosphere soils at (A) 25 and (B) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively 120

Table

S5.1. Lists of rhizosphere microbiomes with specific functions related to plant growth: (A) IAA-producing bacteria, (B) IAA-producing fungi, (C) plant growth promotor, and (D) plant pathogen 114

S5.2. Lettuce yield and nitrogen uptake (N_{up}) at the mid growth stage of plant in the pot experiments (Chapter 3) 118

S5.3. Number of links in the microbe–microbe correlation networks in lettuce rhizosphere soils 120

CHAPTER 1

Introduction

1.1. Background

This PhD dissertation is structured to summary the effects of cover crop on properties of soil microorganisms related to the inorganic nitrogen (N) supply, the available N for plants, and production of vegetable plant that is subsequently rotated with cover crops.

Cover crops are plants that can maintain soil fertility that is defined as soils with high concentration of inorganic N and high functional levels of soil microorganisms. In N supply process, organic N contained in cover crop residues is transformed into inorganic N by soil microorganisms. Due to the microbial mediation, functional levels of soil microorganisms are improved following cover crop application in bulk soils. The inorganic N is then taken up by the subsequent plants, wherein N uptake (N_{up}) process may be assisted by microorganisms inhabiting rhizosphere soils of the plant roots. Both N supply from cover crops and N_{up} by subsequent plants can dynamically occur during decomposition of cover crop residues, leading to dynamics microbial functions in bulk and rhizosphere soils.

Cover cropping systems are practiced in agriculture worldwide, including in the crop production areas in Japan. The types of cover crops, such as leguminous and gramineous, are commonly applied as a single or in combination within them. There are huge numbers of researches separately focus on the effects of cover crops on soil inorganic N supply, microbial functions, and the subsequent plant N_{up} and yield. However, the connections between N supply, microbial roles, and N utilization are limitedly studied, particularly during the cover crop decomposition periods. Therefore, this dissertation provides research results of (1) how bulk soil microorganisms play roles in inorganic N supply from different cover crop types; and (2) how rhizosphere soil microorganisms interact with subsequent plants thus affecting N_{up} during the cover crop decomposition periods.

1.2. Research objectives

This research was aimed to address the effects of different cover crops [i.e. rye – gramineous, hairy vetch (HV) – leguminous, and mix of rye and HV (rye+HV)] on properties of soil microorganisms related to the N supply from cover crop residues and the N_{up} by subsequent plant.

To accomplish the aims, the researches was divided into three researches with each goal:

a. to clarify the effects of different cover crops on soil inorganic N supply and N_{up} and yield of lettuce as the subsequent plant.
b. to evaluate the changes on properties of microorganisms existing in bulk soil that relate to the soil inorganic N supply from different cover crops.
c. to understand plant–microbe interactions in lettuce rhizosphere soils that relate to the plant N_{up} and yield.

1.3. Dissertation structure

Chapter 1	Research background and objectives, and dissertation structure.
Chapter 2	General knowledges about (1) cover crops application in crop production sector of agriculture, (2) microbially mediated-decomposition of cover crop residues evaluated through microbial activity, biomass, abundance, and network, (3) N cycle and N related-microbial functions in bulk soils, (4) N utilization by subsequent plant, and (5) rhizosphere microbiomes of the subsequent plant that may assist the N utilization. In the end of the chapter, there is a section, describing the general reasons of performing the three researches.
Chapter 3	Discussion about the effects of different cover crops on productivity of lettuce plants, concentration of soil inorganic N, microbial activities as β-glucosidase enzyme (BG) activity, and soil microbial biomass (SMB). A field-based experimental

results are discussed to compare the effects of N supplied from cover crops and synthetic N fertilizer. A pot-based experimental results are discussed to clarify the effects of cover crops under limit weather factors.

Chapter 4 Discussion about how bacteria and fungi in bulk soils play roles in inorganic N supply from different cover crops. The roles are evaluated as the changes on bacterial and fungal community structures and networks within and between bacteria and fungi. In addition, relative abundance of specific microbial taxa is linked to BG activity and SMB, thereby specific microbial functions in the inorganic N supply are addressed. The discussion is based on the results of pot experiments at specific timing, i.e., middle of residual decomposition periods.

Chapter 5 Discussion about how bacteria and fungi in rhizosphere soils play roles in the N_{up} of lettuce plants under different cover crop amendments. The roles are evaluated as the changes on bacterial and fungal community structures and plant–microbe interactions as correlations between specific microbial taxa and plant N_{up} and yield. The discussion is based on the results of pot experiments at the mid growth stage of lettuce plants, when HV cover crop once inhibits plant performance.

Chapter 6 General discussion of the clarifications and findings in the three researches described in Chapter 3–5.

Chapter 7 Recommendations for further researches based on limitations of the current study.

CHAPTER 2

Literature Review

Cover crops are plants that have been used since ancient times and have been civilized for serving multiple ecosystem services (Mauro et al., 2013). However, the numbers of adoption are still limited, due to the expensive cost and lack of robust scientific results on the net effect of cover crops (Daryanto et al., 2019). The outcomes of this dissertation, therefore, are expected to augment the knowledge about cover crops in relation to the soil, horticulture, and agronomy aspects. In a broader expectation, the results may promote the practices of cover cropping system.

2.1. Cover crops in agriculture

2.1.1. Cover crops

Sustainable agriculture system is agriculture in which the methods are environmentally friendly, and the effects on improving plant production would be sustained for long-term. Cover crops have been introduced as one of tools to achieve sustainable agriculture. In general, cover crops have multitude functions, such as (1) covering and protecting soils from wind and water erosion, (2) serving as sinks for plant nutrients that might otherwise be lost by volatilization or leaching, (3) controlling weeds through competition and allelopathy, and (4) controlling diseases, insects, and nematodes by reducing the population and dispersal (Doran and Smith, 1991; Robačer et al., 2016; Sharma et al., 2018). These functions head to the sustainable agriculture system.

Cover cropping system in Japan has been developed in several regions to overcome the agronomy and environment problems as pointed above, e.g., to maintain soil fertility of paddy fields in the southwest region (Ehime prefecture, Asagi and Ueno, 2009a), to prevent soil erosion and nitrogen (N) leaching in the northeast region (Ibaraki prefecture, Komatsuzaki, 2011), and to maintain soil fertility of tomato cultivation in the northernmost region (Hokkaido prefecture, Araki et al., 2009).

In term of the functions to serve plant nutrients, such as N, cover crops can alternate synthetic N fertilizer in vegetable cultivation systems (Sainju et al., 2001; Araki et al., 2009; Lee et al., 2014; Muchanga et al., 2017). By the late nineteenth century, vital roles of N as plant nutrient has been understood. Thus, the supply has been endeavored. Prior to industrial process of N production as synthetic N fertilizer, crop production in agriculture relied on N input (N_{in}) from plant residues and manures. However, the increase in human population increases food demands. This situation increases the use of synthetic N fertilizers that has been industrialized and frequently practiced since 1945. As the consequence, crop production has depended on N_{in} from synthetic N fertilizers (Widdison and Burt, 2010). The dependency has led to an excessive application amount of synthetic N fertilizers. The bad news is that accumulation of nitrate (NO_3), the easily leached N form, pollutes a drinking water causing a fatal human health (i.e., blue-baby syndrome and gastric cancer, Widdison and Burt, 2010). Thus, it is required to control the application of synthetic N fertilizers without declining plant production. Cover crops are suitable, since the consisted N is gradationally released from the residues to soils; and the residues are organic materials which are save for soil ecosystems. In Japan, moreover, cover crop applications are clearly recommended by the Ministry of Agriculture, Forestry, and Fisheries (Annual report of food, agriculture, and rural areas in Japan in 2017, https://www.maff.go.jp/e/data/publish/attach/pdf/index-93.pdf), following the N pollution issue in soils and water in the current ten years (Komatsuzaki, 2020).

In the technique practiced worldwide, cover crops are grown during the non-production periods of vegetables and rotated with vegetable plants by incorporation, as mulch, or as living cover crops (Mauro et al., 2013). Hokkaido region has short cultivation periods, due to the short periods of spring and summer (average 5 months). Thus, incorporation method is popularly done by farmers to maximally achieve the cover crop benefits. This cover crop-vegetable rotation become the popular system to build organic and environmentally friendly vegetables production (Robačer et al., 2016; Chahal and Eerd, 2019; Brust, 2019), such as those practiced in cover crop-tomato rotation in Hokkaido (Araki et al., 2009; Sugihara et al., 2016; Muchanga et al., 2017; Muchanga et al., 2020).

2.1.2. Types and function

The most common cover crop species belong to three botanical families: *Fabaceae*, *Poaceae*, and *Brassicaceae* (Mauro et al., 2013). *Fabaceae* and *Poaceae* are leguminous and gramineous types, respectively. *Brassicaceae* includes species of mustard-family crops (Mauro et al., 2013; Brust, 2019). Hereafter, the review is focused on leguminous and gramineous types of cover crop.

2.1.2.1. Leguminous cover crops

The famous species of leguminous cover crops are vetches (*Vicia* spp.), clovers (*Trifolium* spp.), and medics (*Medicago* spp.). They are mostly able to fix the atmospheric N via root–rhizobia association (Mauro et al., 2013; Brust, 2019), thus the residue is rich on N content. In consequence, leguminous cover crops can provide a high amount of N for the subsequent vegetable plants. Among the species, hairy vetch (*Vicia villosa* Roth; HV) is commonly applied in agriculture.

The HV is also functioning to control weeds in fields. HV residue produces allelochemical, named cyanamide (CH_2N_2), which toxically suppresses root growth of weeds (Ercoli et al., 2007). Unfortunately, the allelopathic effects of HV can also decline growth of subsequent vegetable plants (Soltys et al., 2012), when vegetable roots develop in allelopathic zone of HV residue (Robačer et al., 2016).

2.1.2.2. Gramineous cover crops

The gramineous cover crops include rye (*Secale* spp.), oats (*Avena* spp.), wheat (*Triticum* spp.), and barley (*Hordeum* spp.). The plants can scavenge N from soils by absorbing and keeping it in their mass, thus reducing N loss via leaching (Mauro et al., 2013; Brust, 2019). Winter rye (*Secale cereale* L.) is the example of gramineous cover crops and cultivated worldwide (Sapirstein and Bushuk, 2016).

Rye also can control weeds by being a competitor for space, nutrient, water, and solar radiation in fields. Further, rye contains allelochemical, called DIBOA (4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one), that toxically reduce seed germination (Ercoli et al., 2007; Geddes et al., 2015) and slow root growth of weeds (Ercoli et al., 2007). Up to date, allelopathic effects of rye have not known to disserve growth

of subsequent vegetable plants, rotated after rye cover crop.

2.1.3. Factors controlling cover crop biomass

Each cover crop plant has characteristic of seed germination and plant development. Rye seeds easily germinate, while HV seeds hardly germinate due to the high dormancy (Lee et al., 2014). During their growing periods, rye plants adapt climates quicker than HV plants (Kuo and Sainju, 1998). For instance, the adaptation to scarcity of rainfall (Fraiser et al., 2017). Regarding to these characteristics, rye plants have more opportunity to grow, survive, and produce high biomass than HV in the nature.

In cover cropping system, mixing rye and HV is established to collaborate their functions on N service in soils (Kuo and Sainju, 1998; Brennan and Acosta-Martinez, 2017; Drost et al., 2020; Nevins et al., 2020). However, the adaptable traits of rye plants often lead to competitions with HV plants for spaces, nutrients, and water during their growth periods (Lawson et al., 2012), as shown by Sorensen and Thorup-Kristensen (2003) in the fields with legume and non-legume mixture. As the significance of the competition, various biomass production in legume + non-legume cover crop plots are obtained every year. It causes various amount of N supply each year.

2.2. Residual decomposition, N mineralization, and N-cycle

2.2.1. Quality of residue and pattern of decomposition and N mineralization

Plant residues consist of carbon (C) and N. Proportion of C to N (C:N ratio) is commonly considered to point out the residual quality. Residue of HV is rich in N (i.e., > 2%), leading to a low C:N ratio (i.e., 9–13) and considering as a high-quality residue. In contrast, rye residue contains less than 2% N, causing a high C:N ratio (i.e., 22–37) and considering as a low-quality residue (Kuo and Sainju, 1998; Kuo et al., 2001; Sainju et al., 2001; Lawson et al., 2012; Fraiser et al., 2017). Mixing of rye and HV result in an intermediate C:N ratio between rye and HV, which varies depending on biomass ratio of each cover crop (Kuo and Sainju, 1998).

It is unarguable that decomposition pattern of cover crop residues is determined

by the residual C:N ratio. Specifically, the lower C:N ratio of cover crop residues, the faster decomposition rates (Kuo and Sainju, 1998; Kuo et al., 2001; Sainju et al., 2001; Lawson et al., 2012; Fraiser et al., 2017). Along with residual decomposition, N from the residues is mineralized to soils (Rosecrance et al., 2000). Therefore, N mineralization rates of the high-quality cover crops are also fast and the residues are reasonably preferred in agriculture.

On the other hand, the low-quality cover crop residues are hardly decomposable and slowly mineralize the N; but, high C content of the residues can increase C sequestration in soils (Kuo and Sainju, 1998; Komatsuzaki and Ohta, 2013; Sharma et al., 2018). Accumulation of C in soils can improve soil physical properties, e.g., soil aggregates, thus improving soil aeration and water infiltration to support root growth of the subsequent plants (Diacono and Montemurro, 2010; Brust, 2019). By mixing legume and non-legume, thus, farmers and agriculture practitioners expect to benefit both sustainable N supply and accumulation of C in the soils.

2.2.2. Factors controlling residual decomposition and N mineralization

Process of residual decomposition and N mineralization in soils are mediated by microorganisms. Despite of residual quality, duration of the processes is changeable, depending on the residue conditions and environmental factors in soils (Chen et al., 2014). Cover crop residues in oven-dried condition are decomposed within 21–30 weeks in soils (Kuo and Sainju, 1998; Lawson et al., 2012). Thus, fresh residues are expectedly degraded in less than 21 weeks in soils and the N supply can be quickly accelerated. The environmental factors in soils, e.g., soil moisture and temperature, and initial soil chemical properties, can affect microbial counts and activities during degrading plant residues.

In the microbially-decomposition process, microorganisms require an optimal soil moisture for their growth and enzymatic activity (Borowik and Wyszkowska, 2016). They have demonstrated that colony count of decomposers is affected by soil moisture: the highest colony of *Azotobacter*, and Actinomycetes, and organotrophic fungi are in soils with 20%, 40%, and 60% soil moisture, respectively. In addition, microbial enzymatic activity, e.g., β-glucosidase (BG) activity peaks in

bare soils with 20% of soil moisture (Borowik and Wyszkowska, 2016) or in residual-amended soils with 10% or less of soil moisture (Geisseler et al., 2012).

Microbially-decomposition rates increase exponentially with soil temperature, according to the Arrhenius kinetic theory. An optimal soil temperature (i.e., 10–28°C) is required by microorganisms for their growth and enzymatic activity (Conant et al., 2008), including the BG activity. Therefore, optimal rates of residual decomposition and N mineralization can be achieved. In an open field, range of soil temperature is seasonal depending, e.g.,10.3–21.5°C in the spring and 19.9–29.8°C in the summer (Moinet et al., 2020). They have concluded that those temperature ranges could increase soil microbial respiration, implying the high activities of microorganisms to decompose soil organic matter. In a month observation during the spring and summer, soil temperature may fluctuate, thus varying microbial growth and activities on decomposition and N mineralization of cover crops.

A high initial soil N content may indicate a high active organic material pool, which means an abundant initial food for soil microorganisms to result in a higher turnover of residual N (Doran and Smith, 1991). For instance, soils with 9.08% of initial total nitrogen (TN) facilitate N mineralization from clover leaves twice faster than soils with 2.56% of initial TN (Frøseth et al., 2015).

2.2.3. Microbial properties related to residual decomposition and N mineralization in bulk soils

Soil microorganisms, including bacteria and fungi, are the actors on decomposition of complex cover crop residues and mineralization of inorganic N (Drury et al., 1991; Carrera et al., 2007; Chen et al., 2014; Fernandez et al., 2016; Brennan and Acosta-Martinez, 2017; Drost et al., 2020; Khan et al., 2020). Properties of soil microorganisms including microbial activity, biomass, abundance or composition in community, richness, diversity, function, and interaction within soil microorganisms, are measurable and analyzable. To understand the roles of soil microorganisms in the process of decomposition and N mineralization of cover crops, understanding of soil microbial properties is required.

As the measurement technique, microbial activity (e.g., BG activity) and microbial biomass [e.g., C-based of soil microbial biomass (SMB)] can be chemically examined. Microbial abundance is molecularly measured through a DNA-based molecular analysis. Microbial community (i.e., microbial diversity, and richness), function, and network can be yielded through bioinformatic analyses of the results of the DNA-based molecular analysis.

2.2.3.1. β-glucosidase enzyme (BG) activity

In the decomposition process, complex or high molecular weight substances (e.g., polysaccharides and olygosaccharides) of plant residues are enzymatically broken down into simpler or low molecular weight substances (e.g., monosaccharides). The enzymes, for instance BG that particularly degrade cellobiose (oligosaccharide) to glucose (monosaccharide, Li et al., 2009; de Almeida et al., 2015).

In general, BG activity has been used to imply microbial function in C cycling. Furthermore, BG activity is strongly positive correlated with soil inorganic N in cover cropping system, implying the significance of BG activity in N cycling (Nevins et al., 2020). Thus, BG activity can be one of indicators of soil fertility and microbial roles in N supply from cover crops.

Activity of BG in soil can be measured by reacting soils with 4-nitrophenyl α-D-glucopyranoside (ρNPG) in McIlvaine buffer to produce yellow color. The present of β-glucosidase in soils converts ρNPG as ρ-nitrophenol (ρNP). The higher concentration of ρNP, the darker yellow color of the extracts; in which the color intensity indicates the level of BG activity (Hayano et al., 1973). Related to the decomposition process, the higher BG activity in soil, the higher microbially-decomposition activity may occur (Liang et al., 2014; Mbuthia et al., 2015; Nevins et al., 2020). Thus, BG activity in soils would be increased, following input of plant residues, such as cover crops.

Cover cropping system is practiced in short- and long-terms using various kinds of cover crop and various methods. This varies the changes on BG activity. In the long-term, BG activity has been increased up to 11% and 33% after 1 year (Liang

et al., 2014) and 31 years (Mbuthia et al., 2015) HV inputs, respectively. In the short-term, BG activity is greater in soils with mulch of rye and mix of rye and HV over the soils with mulch of HV at 24–75 days (Nevins et al., 2020). Incorporation method of fresh cover corps is chosen for Hokkaido region (sub-section 2.1.1). Therefore, the changes on BG activity during the decomposition periods, i.e., within a month, can be different from those of the previous studies. As the impacts, the results are expected to highlight the importance of microorganisms on early decomposition period of different cover crops.

On the contrary to the organic materials input, application of inorganic salt such as synthetic N fertilizer reduces BG activity in soils (Eivazi and Tabatabai, 1990; Mbuthia et al., 2015). In more specific, Mbuthia et al. (2015) state that synthetic N fertilizers are not inducers of BG activity. It stresses that there is a strong chain between plant residues and BG activity.

In the nature, BG activity can be declined when soil pH (Eivazi and Tabatabai, 1990) and soil moisture (Adetunji et al., 2017) are declined. In contrary, soil TC and TN in range of 1.77%–3.74% and 0.19%–0.31%, respectively, are appropriate for soil microorganisms, thus increasing BG activity (Eivazi and Tabatabai, 1990). Soil chemical properties (e.g., soil pH, TC, and TN) are possibly changed during cover crop decomposition. In consequence, BG activity is dynamically changed.

Groups of microorganisms, bacteria and fungi, produce BG. The BG-producing bacterial families are e.g., *Microbacteriaceae* and *Streptomycetaceae* of phylum Actinobacteria, *Flavobacteriaceae* of phylum Bacteroidetes, *Lactobacillaceae* of phylum Firmicutes (Zang et al., 2018), *Sinobacteraceae* of class Gammaproteobacteria (Zhou et al., 2014), and *Mycobacteriaceae* of phylum Actinobacteria (Lévy-Frébault et al., 1982). The BG-producing fungal families are e.g., *Trichocomaceae* of phylum Ascomycota, *Phanerochaetaceae* of phylum Basidiomycota (Zang et al., 2018), and *Mortierellaceae* of phylum *Mortierellomycota* (Li et al., 2018). The effects of cover crops on BG activity and existence of BG-producing microorganisms have been independently and progressively studied over the last 2 years, but connections between those two

microbial properties remain unclear (Kim et al., 2020). A further analysis is needed, so that specific microbial roles in residual decomposition and N supply from cover crops via BG activity can be addressed.

2.2.3.2. Soil microbial biomass (SMB)

Soil microbial biomass (SMB) emphasizes the living storage of C and N (Sugihara et al., 2010; Gonzalez-Quiñones et al., 2011; Li et al., 2018). Specifically, microorganisms enlarge their biomass by assimilating hydrolytic results of the enzymatic decomposition process, such as glucose that is degraded using BG. Thus, microbial biomass is marked in a high value (Adetunji et al., 2017).

SMB in C- and N-based explains microbial roles in C and N cycling, respectively. Regarding to the concurrently demand of soil microorganisms on C and N (Kuzyakov and Blagodatskaya, 2015), both nutrients-based can show microbial roles in both cycles. In the nature, SMB in C-based comprises up to approximately 5% of soil TC (He et al., 2003). The cover crops input increase simpler substances (sub-section 2.2.3.1). Thus, the living C storage can be increased, indicating an increase in soil fertility.

Application of cover crops in soil variously affect SMB in C-based, hereafter simply written as SMB. For instance, the 31 years of HV incorporation did not affect SMB (Mbuthia et al., 2015); but the 6 years of legume-rye incorporation increased SMB (Brennan and Acosta-Martinez, 2017). Further, in the short-term incorporation of maize straw and leaf residues, SMB is promoted in particular timings during 105 days of residual decomposition (Sugihara et al., 2010). These researches might show that cover crop types and/or timings after the application influence SMB.

Similar to the effects of cover crops, application of synthetic N fertilizers variably affect SMB: N, P, and other fertilizers negatively and non-significantly change SMB (Gonzalez-Quiñones et al., 2011); several fertilizer application rates (i.e., 0N, 34N, 67N, and 101N) combined with HV and wheat cover crops non-significantly affect SMB after 31 years of continual cover crop practices (Mbuthia et al., 2015).

To highlight the effects of cover crops on SMB during the residual decomposition, it is necessary to compare inputs of cover crops and synthetic N fertilizer.

He et al. (2003) state that SMB represents an important reservoir of labile nutrients, which can be declined or increased over the time. The declining pattern of SMB during the N supply is suggested as the periods of N release from microbial body (Sugihara et al., 2010). This has been clarified by Li et al. (2014), who conclude that in the final of decomposition, low molecular weight, including inorganic N, is derived from the decomposed plant residue and from microbial body to the soils. If the values and trends of SMB during cover crop decomposition can be determined, the significance of SMB in N supply will be possibly emphasized.

2.2.3.3. Microbial community

Nowadays, molecular analysis based on soil DNA has been sophisticatedly developed to quantify and identify soil microorganisms. In the nature, indigenous microbial community structures are shaped by initial properties of soils, the microbial habitats. The initial properties include initial soil nutrient sources, e.g., TC and TN contents and soil inorganic N concentration (Fernandez et al., 2016b; Barel et al., 2019). Therefore, soil microbial communities in different soils vary in their responses to cover crops input. For instance, soil bacterial community structure was neither altered by annual legume-rye and rye inputs based on the fatty acid methyl ester profiling (FAME, Brennan and Acosta-Martinez, 2017), nor by HV and rye inputs based on the terminal restriction fragment length polymorphism analysis (Maul et al., 2014). On the other hand, fungal community structure was sensitive to crown vetch (*Coronilla varia* L., Zheng et al., 2018), ryegrass (*Lolium perenne*, Detheridge et al., 2016), and mix of legume and non-legume cover crops (Schmidt et al., 2019) based on a next-generation sequencing (NGS) approach.

Changes on microbial community structure imply changes on components of the community, i.e., microbial richness, diversity, and relative abundance. Hereafter, the reviews focused on those components of microbial community.

2.2.3.3.a. Microbial richness

Microbial richness is number of identified bacterial or fungal groups (Dini-Andreote, 2019). To analyze microbial richness, DNA-based molecular assessment is conducted. In the technique, DNA from soils is extracted using the kits or manual procedure, such as chloroform-phenol method (Miller et al., 1999; Sagova-Mareckova et al., 2008). Afterward, the DNA is amplified to target specific region of bacteria and fungi. For instance, the v4 region of 16S rRNA (bacteria, Cai et al., 2013) and region 1 of fungal internal transcribed spacer (ITS, White et al., 1990). The amplified DNA was sequenced; and then, the sequences are aligned to the database of bacteria (e.g., GreenGenes) and fungi (e.g., UNITE) for resulting the identified bacteria and fungi. The alignment can be performed using the bioinformatic pipelines, such as Quantitative Insights into Microbial Ecology (QIIME, Caporaso et al., 2010). The identification can be assigned to several phylogenetic levels: phylum, class, order, family, genus, and species, presented from the highest to the lowest in order (Dini-Andreote and van Elsas, 2019).

Input of plant residues in soils can attract new species of microorganisms (Brahmaprakash et al., 2017), leading to increase in microbial richness. The increase, however, may not indicate an increase in residual decomposition and N mineralization, due to the inconsistent changes on microbial richness in cover crop-treated soils. For example, bacterial richness is not changed in fields with HV over the rye (Fernandez et al., 2016b) and in fields with legume cover crops over the non-legume cover crops (Nivelle et al., 2016), although N mineralization is high in fields with HV and legume cover crops.

2.2.3.3.b. Microbial abundance

Microbial abundance can be evaluated as relative abundance according to the sequence reads and the DNA or gene quantity. Results of alignment using the QIIME pipeline includes relative abundance of each identified bacteria and fungi. The relative abundance shows composition of a single group of soil microorganism in percent, which is relative to the sum of all groups in a treatment (community) reaching a 100% abundance as the total fraction.

Because microbial succession over the course of residual decomposition changes microbial community structure, relative abundances of specific microbial taxa either increase or decrease during the decomposition process (Banerjee et al., 2016). Those taxa are copiotrophs, i.e. bacteria that grow quickly in response to plant residues addition (Carrera et al., 2007). The copiotrophs can be assigned as decomposers, e.g., bacteria members of family *Cytophagaceae* of phylum Bacteroidetes that decompose the complex C of plants (McBride et al., 2014) and fungi members of phylum Basidiomycota that decompose the plant cellulose (Treseder and Lennon, 2015). It becomes a basic understanding to evaluate interactions between cover crop residues and relative abundance of soil microorganisms, including the decomposers involving in the N supply.

The microbial DNA or gene quantity can be measured using the real time quantitative PCR (qPCR, Filion et al., 2003). As the technique, extracted soil DNA is amplified to target bacterial and fungal regions. The quantity of DNA or genes are measured as fluorescent intensity of the target amplification by the point in time.

Along with the microbial activities, microbial abundance is also promoted by cover crops input at particular timings (Hu et al., 1999; Maul et al., 2014; Drost et al., 2020). Specifically, bacterial and fungal genes are increased in soils incorporated with HV and rye, over the soil covered with black poly (Maul et al., 2014). Additionally, HV, oat, radish, and mix of them promote copies of bacterial gene and biomass of fungi at different timings within 50 days after the incorporation (Drost et al., 2020). However, they do not emphasize how those promotion may affect cover crop residual decomposition. Thus, analyzing bacterial and fungal abundance during cover crop decomposition; and linking the trends of microbial abundance and residual decomposition and N supply will imply microbial roles.

2.2.3.3.c. Microbial diversity

Analysis of microbial diversity can be performed within microbial groups in a community (i.e., α-diversity) and between communities (i.e., β-diversity, Dini-Andreote, 2019). The α-diversity can be estimated by calculating the number (richness) and distribution (evenness) of microbial taxa in a community as an index,

such as Shannon index. The β-diversity can be quantitatively estimated by calculating the distances between each community, for example using the Bray-Curtis distance method.

A meta-analysis of 60 cover cropping systems by Kim et al. (2020) shows that cover crop types and soil order do not change Shannon diversity as demonstrated by Fernandez et al., 2016b in fields with HV or rye and Nivelle et al., 2016 in fields with legume or non-legume cover crops. However, vetch-oat-radish mixture potentially increase microbial diversity (Drost et al., 2020). Hence, comparing the effects of single and mixed cover crops on microbial diversity will be informative.

Similar with the microbial richness, microbial diversity may not easily determine microbial functions in the decomposition and N mineralization, because microbial diversity include the numbers and abundances of both beneficial and harmful microorganisms. Therefore, it is crucial to not only evaluate microbial diversity, but also detail evaluate relative abundance of potential microbial groups that may point out microbial function in decomposition and N mineralization from cover crop.

2.2.3.4. Microbial network

In a community, bacteria and fungi interact with each other during residual decomposition process (de Boer et al., 2005; Banerjee et al., 2016; Finlay and Thorn, 2019). The interaction types can be positive or negative, which indicate synergistic or antagonistic associations, respectively (Banerjee et al., 2016; Zheng et al., 2018). Synergistic interactions facilitate biodegradation, while antagonistic interactions slow down nutrient cycling because of competition of nutrients sources (Finlay and Thorn, 2019). Therefore, network analysis provides the tools for understanding the microbiomes as a system and how microbiome structure can influence pathway of N supply (Finlay and Thorn, 2019) and crop health (Poudel et al., 2016).

Although networks within and between bacteria and fungi can determine the available nutrient supply and soil fertility within the framework of soil biogeochemical cycles (e.g., N supply, Deveau et al., 2018), the role of microbe–microbe interactions in soil biogeochemical cycles have so far been neglected

(Finlay and Thorn, 2019). To our knowledge, there are limit reports about microbe–microbe networks during cover crop decomposition and N mineralization. Hence, the evaluation will respectfully enhance the understanding of microbial roles in N availability in soils with cover crops amendment.

2.2.3.8. Microbial function

Microbial function can be generally and specifically analyzed. The general function involves all of the identified bacteria and fungi; and the function is predicted using several software, e.g., Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software for bacteria (Langille et al., 2013) and Fungi Functional Guild (FUNGuild) software for fungi (Nguyen et al., 2016). The functions related to decomposition are, e.g., bacterial metabolism function determining the way of bacteria to degrade carbon, fatty acid, and aromatic compound [Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database; https://www.genome.jp/kegg/pathway.html], and fungal saprotroph and pathotroph modes determining the way of fungi to gain energy by breaking down residue and harming plant cells, respectively (Nguyen et al., 2016).

The PICRUSt (Ashworth et al., 2017) and FUNGuild (Detheridge et al., 2016; Schmidt et al., 2019) software have been used to assess bacterial and fungal general functions in soils amended with plant residues. Similar general functions possibly occurred during decomposition and N mineralization from cover crops.

The specific functions of soil microorganisms can be shown by specific microbial taxa, such as influenced taxa of bacteria and fungi. Influenced taxa are microbial groups whose relative abundances are significantly influenced by treatments, including amendment of different cover crops. These influenced taxa fill unique and important niches in the community (Fernandez et al., 2016b); and the roles can be explained as their reciprocal interactions with soil biochemical properties (e.g., BG activity, SMB, and soil inorganic N) that are the N mineralization indicators using a multivariate analysis (Fernandez et al., 2016a; Drost et al., 2020; Khan et al., 2020). The influenced taxa may include microbial groups that are specifically producing BG as those written in sub-section 2.2.3.1 and copiotrophs as describes

in sub-section 2.2.3.3.b.

2.2.4. Bacterially-N cycle in bulk soils

Sources of N can be from soils, atmosphere, synthetic N fertilizer, and plant residues. The cycle of N in the nature includes fixation of the atmospheric N, ammonification from plant residue, nitrification, and denitrification. The processes are controlled by soil microorganisms, particularly bacteria. Thus, we would focus on bacterially-N cycle hereinafter.

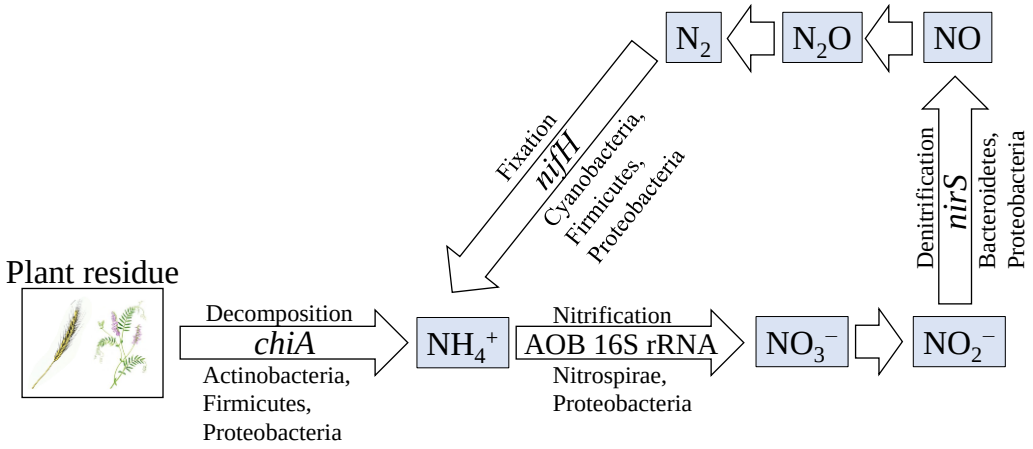


Fig.2.1. Illustration of nitrogen (N) cycle based on Lindsay et al. (2010), Levy-Booth et al. (2014), and Ren et al. (2018). The N cycle in soils is controlled by soil bacteria having specific genes (i.e., *chiA*, *nifH*, AOB 16S rRNA, and *nirS*) at each process (i.e., decomposition, fixation, nitrification, and denitrification) to produce the available inorganic N forms [i.e., ammonium (NH_4^+), nitrate (NO_3^-), and nitric oxide (NO)] for plants.

2.2.4.1. Decomposition

In order for N to be absorbable for plants, organic N contained in cover crop residues must be transformed into the available inorganic N: ammonia (NH_3), NH_4, nitrite (NO_2), and nitrate (NO_3, Fig.2.1). Ammonification is a reforming process from organic N to NH_4. It includes a complex transformation recruiting various enzymes, such as chitinase that degrade chitin to the low molecular weight chitooligomers (Hamid et al., 2013). Chitinase is produced by soil bacteria with *chiA* gene (Ramiah et al., 2000; Lindsay et al., 2010; Hamid et al., 2013), e.g., members of families *Yersiniaceae* and *Alteromonadaceae* of class Gammaproteobacteria, family *Bacillaceae* of phylum Firmicutes (Ramiah et al.,

2000), and family *Actinomycetaceae* of phylum Actinobacteria (Miyashita and Fujii, 1993). Bacteria with *chiA* gene contribute to the early step of C and N cycling (Ramiah et al., 2000; Lindsay et al., 2010; Hamid et al., 2013). Thus, quantification of *chiA* gene abundance and identification of bacteria with *chiA* gene will describe the crucial early contribution of microorganisms in N supply from cover crops.

2.2.4.2. N fixation

Approximately 79% of Earth's atmosphere is comprised by N, which was discovered in 1772 and has been fixed in industrial scale in early twentieth century (Widdison and Burt, 2010). The natural fixation process of the atmospheric N (N_2) is performed by bacteria that are dependently living in plant roots and free-living in bulk soils (Hayatsu et al., 2008; van Groenigen et al., 2015). The product is ammonium (NH_4), one of the available forms (inorganic) of N (Fig.2.1). The free-living N fixers are, e.g., bacteria members of families *Nostocaceae*, *Fortieaceae*, and *Scytonemataceae* of phylum Cyanobacteria (Levy-Booth et al., 2014; Brahmaprakash et al., 2017) and members of phyla Proteobacteria and Firmicutes (Ren et al., 2018). Their abundance can be predicted by measuring the abundance of *nifH* gene (Levy-Booth et al., 2014; Poly et al., 2001). The NH_4^+ increases N supply in soils. Additional N supply from N fixation might happen during cover crop decomposition and N mineralization, due to the activation of microbial community by cover crop residues. Thus, evaluation of *nifH* gene abundance and identification of bacteria with *nifH* gene will be valuable to know the potential of N fixer contributing to the N supply.

2.2.4.3. Nitrification

Nitrification is a transformation process from NH_4 to NO_3. The ammonia-oxidizing bacteria (AOB) with ammonia monooxygenase (*amo*) gene (Levy-Booth et al., 2014), which is also known as AOB 16S rRNA gene (Layton et al., 2005), are responsible in nitrification. The nitrifiers are, e.g., bacteria belong to family *Nitrosomonadaceae* of class Betaproteobacteria (Rotthauwe et al., 1997; Layton et al., 2005; Jin et al., 2010; Levy-Booth et al., 2014; Li et al., 2015) and family *Nitrospiraceae* of phylum Nitrospirae (Rotthauwe et al., 1997; Levy-Booth et al.,

2014; Ren et al., 2018). Nitrification is a crucial second step in N mineralization from cover crops residue. Therefore, nitrifier is necessarily evaluated by examining the AOB 16S rRNA gene abundance and identifying the bacteria.

2.2.4.4. Denitrification

The NO_3 in soils is easily moved upward (gaseous loss), downward (as leaching to groundwater), and lateral (via surface water flow, Widdison and Burt, 2010). Reformation from NO_3 to NO_2 and from NO_2 to the gaseous N (NO) are known as denitrification (Lindsay et al., 2010). There are several genes of bacteria that express their contribution as denitrifier (e.g., *nirS*, *nirK*, *nosZ*, *narG*, Ren et al., 2018). Bacteria with *nirS* gene, belonging to phyla Bacteroidetes and Proteobacteria (Ren et al., 2018), have been known to play roles in the transforming NO_2 to NO. Proteobacteria includes families *Rhodobacteraceae* (Throbäck et al., 2004; Levy-Booth et al., 2014) and *Bradyrhizobiaceae* (Wang et al., 2017) of class Alphaproteobacteria; families *Alcaligenaceae* (Braker et al., 1998), *Burkholderiaceae* (Rösch et al., 2002), *Zoogloeaceae, Oxalobacteriaceae*, and *Comamonadaceae* (Wang et al., 2017) of class Betaproteobacteria; and families *Pseudomonadaceae* (Throbäck et al., 2004) and *Thiotrichaceae* (Wang et al., 2017) of class Gammaproteobacteria. Bacteria members of family *Rhodospirillaceae* of class Alphaproteobacteria include *nifH* and *nirS* genes composedly (Rösch et al., 2002; Levy-Booth et al., 2014). Evaluate *nirS* gene abundance and identify the bacteria will fill other part of the N cycle puzzle, especially during decomposition and N mineralization from cover crops.

2.3. Utilization of N by the subsequent plant

In cover crop-vegetable rotation system, the available inorganic N in soils is supplied from cover crops for the subsequent plants (Drury et al., 1991; Carrera et al., 2007; Fernandez et al., 2016; Brennan et al., 2016; Chahal and Eerd 2019). The utilization of N by subsequent plants can be measured, thus the efficiency of N supply can be determined. In a broader impact, the efficiency of cover cropping system can be considered.

Demands of N by subsequent plants vary, depending on characteristics and cultivation periods of the plants. The maximum N demand of vegetable plants is shown in a bare field applied with synthetic N fertilizers. For instance, tomato plants require at least 184 kg N ha^{-1} for fruit production in 4 months (Muchanga et al. 2020); red pepper plants require at least 191 kg N ha^{-1} for fruit production in 4 months (Lee et al., 2014); and iceberg and romaine lettuce plants require at least 145 kg N ha^{-1} for leaves production in 9 weeks (Bottoms et al., 2012).

It is a common challenge to synchronize N supply from cover crops residue and the subsequent plant N demands. In addition, peaks of N supply from fresh residue of cover crops may occur during its residual decomposition, which can be less than 21 weeks (2.2.2 sub-section). Thus, lettuce plants with short production periods (i.e., 30–40 days) is suitable as the subsequent plant model in this study. This is aimed to observe the efficiency of N supply for subsequent plant during residual decomposition and N mineralization periods Hereafter, the review will be focused on cover cropping system in lettuce cultivation.

2.3.1. Leaf lettuce and parameter of N utilization

Leaf lettuce (*Lactuca sativa* L.) has been studied since decades, for example the study by Jones in 1927. Leaf lettuce contains vitamins and minerals which are good sources of human health. Popularity of leaf lettuce, especially the red one, is risen because 8% of 2.5 mg total phenolic content per g fresh biomass could against risk of cardiovascular disease by declining cholesterol in human body (Kim et al., 2016). The popularity progresses the production. In Japan, total production of lettuce has risen to 7.4% from 2006 to 2018 (https://japancrops.com/en/crops/lettuce/).

In general, N utilization by plants is widely assessed through the nitrogen use efficiency (NUE) parameter (Benincasa et al., 2011). This parameter depends on two factors: the amount of N_{in} and the ability of plants on absorbing the N (N uptake; N_{up}). High NUE indicates that N_{in} is efficiently absorbed by plants (Bottoms et al., 2012; Caliskan et al., 2014). Further, statistical overview shows that N_{up} is positively correlated with plant yield, highlighting dependency of plant biomass on N_{in} (Haque et al., 2010; Benincasa et al., 2011; Sapkota et al., 2019).

2.3.2. Abiotic factors influencing plant N uptake (N_{up})

Abiotic factors affect the natural ability of plants to absorb nutrients, such as N, from soils. Those are water, solar radiation, and soil acidity (pH). Water is essential for vegetables to grow. Thus, irrigation system is built in lettuce cultivation (Tei et al., 2000; Sorensen and Thorup-Kristensen, 2003; Bottoms et al., 2012). Cultivation system in an open field without any irrigation relies on rainfall, so that plant production fluctuates in each year (Fraiser et al., 2017).

Solar radiation is required by plants in the photosynthesis process. In an open field, solar radiation is possibly cut off naturally. A study of lettuce cultivation with 50% and 70% shadowing cut the solar radiation, so that disrupting lettuce production (Marrou et al., 2013).

In addition to the successfully lettuce cultivation system, soil pH is required to be maintained in approximately 7.8 (Caliskan et al., 2014). As ammonification and nitrification of cover crop residues decrease soil pH, due to the increase in H^+ ion (Lawson et al., 2012), lettuce N_{up} is affected (Caliskan et al., 2014).

The abiotic factors may singly or collectively affect lettuce production. For that reason, comparing lettuce performances (i.e., yield and N_{up}) in an open field and pot experimental level will benefit farmers to identify the essential abiotic factors that influence cover crop-lettuce rotation system.

2.3.3. Microbial properties in rhizosphere soils related to plant N_{up} and yield

Individual plants are complex systems whose productivity is a function of interactions with and among diverse organisms associated with them, as well as with the abiotic environment. These complex interactions have profound the effects on plant health, stress tolerance, growth dynamics, and yield. These effects include those that strongly relate to the ability of plants on taking up the available N from soils. The high-throughput identification approach ignores the likely connections that occur among the populations co-inhabiting the phytosphere. Therefore, understanding the interactions among microbial populations, their plant hosts, and the abiotic environment can open up new opportunities to better manage the crucial

of microbial communities to the plant health (Poudel et al., 2016).

Rhizosphere is derived from the Greek word *Rhiza* meaning roots. Rhizosphere soil is portion of soil adhering plant roots and contains high activity and diversity of microorganisms (Mendes et al., 2013; Brahmaprakash et al., 2017; Samad et al., 2019). Today, rhizosphere is redefined into three zones: endorhizosphere/root, rhizoplane, and ectorhizosphere/rhizosphere (Brahmaprakash et al., 2017). Endorhizosphere is the internal portion of root, the cortex and endodermis. Rhizoplane includes zone directly adjacent to the root surface. Ectorhizosphere/rhizosphere includes the extension from rhizoplane out to the soil. The soil is an actual portion of soil under the influence of plant roots (Brahmaprakash et al., 2017). In general, rhizosphere soil is selected as the "hot spot of microorganism" to show their roles in the plant nutrition uptake (Kuzyakov and Blagodatskaya, 2015; Samad et al., 2019).

In the technique, rhizosphere soil is determined as the attached soil obtained after manually shaking off plant roots to remove the loosely attached bulk soils (Gobran and Clegg, 1996; Mijangos et al., 2009; Samad et al., 2019).

Bacteria and fungi inhabiting plant rhizosphere soils are called rhizosphere microbiomes (Brahmaprakash et al., 2017). They are biotic factors influencing plant N_{up} by intimately associate with the plants (Gobran and Clegg, 1996; Nannipieri et al., 2003; Milansari, 2013). Similar to the microorganisms existing in bulk soils, properties of the rhizosphere microbiomes can be evaluated as microbial richness, diversity, community structure, function, and network.

Microbial properties in rhizosphere soils may be similar with or different from that in bulk soil, due to the major effect of plant root exudates (Samad et al., 2019). Simply, application of cover crop residue in bulk soils may or may not shift rhizosphere microbial properties of the subsequent plants. In fact, properties of rhizosphere microbiomes may relate to microbial services in plant health and productivity. Therefore, the evaluation will be knowledgeable not only for practitioners of cover cropping system, but also for the agriculture microbiologist and scientists in the related fields.

2.3.3.1. Microbial community

In plant rhizosphere soil, components of microbial community (i.e., microbial richness, diversity, and relative abundance) are one of shaped by compounds of root exudates. Most plants secrete about 5%–30% of their photosynthesis products into soil surrounding the roots (Samad et al., 2019). A healthier plant may excrete more abundant root exudates (Brahmaprakash et al., 2017), attracting more number and diverse rhizosphere microbiomes and promoting the relative abundance (Nannipieri et al., 2003).

In the nature, 'rhizosphere effect' has been termed to describe that microbial properties are more pronounced in rhizosphere soil in comparison with that in bulk soil. For instance, microbial richness is higher in rhizosphere soils than in bulk soils based on the phospholipid fatty acid analysis (PLFA, Ai et al., 2012) and denaturing gradient gel electrophoresis (DGGE, Smalla et al., 2001) method.

In cover cropping system, microbial community in plant rhizosphere soils can be changed (Li et al., 2016; Fernandez et al., 2016a; Zhang et al., 2017) or unchanged (Maul et al., 2014), although microbial properties in bulk soils have been influenced by cover crop input. Further, microbial community in rhizosphere soils can be either similar or dissimilar among different cover crops input in the bulk soils (Maul et al., 2014; Manici et al., 2018). It shows that rhizosphere microbiome in the hot spot may respond cover crops input and intimately relate to the associated plants at the same time. In more specific, influenced microbial taxa may become the significant taxa in plant growth, due to their flexibility to respond the treatments (sub-section 2.2.3.3.b). In plant rhizosphere soils, additionally, relative abundance of the influenced taxa is possibly affected by both plant rhizosphere and soil management.

Understanding microbial community in subsequent plant rhizosphere soils will give a deeper understanding of the effects of cover crops on the subsequent plant productivity, particularly plant N_{up} and yield.

2.3.3.2. Beneficial and harmful rhizosphere microbiomes

Rhizosphere microbiomes may have direct and indirect influences on plant N_{up}

and yield. Some of rhizosphere microbiomes beneficially interact with plants, thus promoting plant N_{up} and yield. Pathways of the beneficial actions can be (1) nutrient solubilizations to provide plant nutrients, (2) plant hormones and antimicrobial compound production to encourage plant growth and alleviate biotic and abiotic stresses, and (3) induction of plant resistance to against biotic stresses.

Beneficial rhizosphere microbiomes can be classified as plant growth promotor rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF), and bio-control agent (BCA). The PGPR are, e.g., Bacilales fam *Incertae sedis* of phylum Firmicutes and families *Rhodospirillaceae* of class Alphaproteobacteria and *Oxalobacteraceae* of class Betaproteobacteria. They produce an indole acetic acid (IAA, Duca et al., 2014; Brahmaprakash et al., 2017). In addition, fungi members of families *Nectriaceae*, *Hypocreaceae*, and *Trichocomaceae* of phylum Ascomycota produce IAA (Fu et al., 2015). The IAA hormone develops meristem cells, such as those of plant roots, thus elongating the roots and enhancing plant tolerance to stresses to improve plant N_{up} and growth (Etesami et al., 2015).

The AMF includes family *Helotiaceae* of phylum Ascomycota, functioning as ectomycorrhiza to support plant absorption ability on water and nutrient from soils (Cannon and Kirk, 2007).

The BCA has direct and indirect protection pathway to the associated plants. The direct protection can be a poisoning soil-borne phytopathogens. This action is shown by bacteria of families *Bacillaceae* of phylum Firmicutes and *Bulkhoderiaceae* of class Betaproteobacteria, and fungi of family *Bionectriaceae* of phylum Ascomycota. Indirect protection pathway is by inducing plant resistance against phytopathogens. This role is shown by bacteria family *Pseudomonadaceae* of class Gammaproteobacteria (Brahmaprakash et al., 2017). Both protection pathways can result in a healthy plant, thereby maximizing plant N_{up} and yield.

Nevertheless, some of rhizosphere microbiomes are phytopathogens that harm to plants, so that declining plant ability on N_{up} (Battacharyya and Jha, 2012; Brahmaprakash et al., 2017). The soil-borne phytopathogens, causing lettuce root rot disease, are *Erwinia carotovora* belonging to family *Enterobacteriaceae* of class

Gammaproteobacteria (Bohn, 1953) and *Fusarium oxysporum* f.sp. *lactucae* (FOL) belonging to family *Nectriaceae* of phylum Ascomycota (Matuo and Motohashi, 1967). As the mode of action, those phytopathogens enter to the xylem system of lettuce roots together with water and nutrients; and then, they destroy plant cells to obtain the energy. The cells are killed over the time, thus inhibiting plant N_{up} and suppressing plant yield.

2.3.3.3. Microbe–microbe network

A concept about how microbe–microbe interaction in rhizosphere soil of plant can determine the plant health has been introduced (Wallenstein, 2017). In general, rhizosphere microbiomes communicate among themselves, as shown in bulk soil (Haldar and Sengupta, 2015). The communication in microbe–microbe can be as negative, neutral, and positive (Farrar et al., 2014; Wallenstein, 2017). More complex, the communication is categorized as commensalism, competition, predation, cooperation, amensalism, and no interaction (Groβkopf and Soyer, 2014). Commensalism is a continuing process of producing metabolite by one microbe that is then utilized by another microbe. Competition occur when two microbes utilize the same metabolite. Predation occur when a microbe is inhibited by metabolite that is produced by another microbe. Cooperation is the two-way interaction of commensalism. Amensalism occur when one microbe produces metabolite to inhibit another microbe. If there are no connection between two microbes, they have no relationship.

Based on the communication of microbe–microbe, primary roles of them to the colonized plants are determined and illustrated as the plant–microbe interaction (Bonfante and Anca, 2009; Nihorimbere et al., 2011; Haldar and Sengupta, 2015; de Vries and Wallenstein, 2017). However, the analysis steps of microbe–microbe and plant–microbe interactions in the order are still undone in rhizosphere microbiome research, both in general agriculture and cover cropping systems.

2.3.3.4. Plant–microbe interaction

The plant–microbe interaction is considered to show the intimate relationships between plant and rhizosphere microbiome and microbial specific function on the

plant performance, i.e., plant N_{up} and yield (Fernandez et al., 2016a; Jiang et al., 2019). As the roles, specific microbial taxa are not individually promoting or inhibiting plant performance, but they work as a community. The scenario of plant–microbe interaction is based on Poudel et al. (2016) comprises: (1) a healthy plant, due to direct and indirectly beneficial microbial functions to plant yield; and (2) an unhealthy plant, due to direct effect of the highly existing phytopathogen and no action against the phytopathogens from the low existing beneficial taxa. The scenario can be adopted to emphasize roles of rhizosphere microbiome in the subsequent plant under cover cropping systems.

Specific microbial taxa, e.g., influenced taxa, can be identified in plant rhizosphere soils which the bulk soil is treated with cover crop residues (Tian et al., 2013; Maul et al., 2014; Fernandez et al., 2016b; Manici et al., 2018). However, function of influenced bacterial taxa to plant performance has not addressed (Maul et al., 2014; Fernandez et al., 2016b). To identify functions of influenced fungal taxa, Manici et al. (2018) culture them and directly inoculate the culture suspension to plant. Plant–microbe parameters are defined based on biomass changes of the inoculated and uninoculated plants: (a) a neutral plant–fungal relationship for ± 10% biomass changes; (b) a pathogenic plant–fungal relationship for < 9% biomass changes; and (c) a mutualistic plant–fungal relationship for > 11% changes. Manici et al. (2018) state that this technique can vividly demonstrate the effects of cover crops to the farmers. However, the technique is costly and time consuming. On the other hand, Jiang et al. (2019) performed multivariate analysis that simply and clearly show the relationships between influenced bacterial taxa in phylum and genus taxonomical levels and tomato root dry weight.

Roles of influenced taxa in rhizosphere soils to N_{up} and yield of subsequent plants under cover crop treatments are still unknown. Various effects of different cover crops on performances of the subsequent plants may be related to various plant–microbe interactions. Hence, it is importantly required to evaluate plant–microbe interactions in rhizosphere soils to understand the whole effects of cover crops on cover crop-vegetable rotation system.

2.4. Recommendation for research

Cover crops has been fallen into sustainable agriculture interest, because it can alternate synthetic N to improve horticultural products. Based on the literature review above, many previous studies clearly have demonstrated the benefits of cover crops, especially HV – leguminous cover crop, to improve N supply in the treated soil and its utilization by the subsequent plants. Several studies also have confirmed that soil microorganisms stand behind the improvements of N supply and plant N_{up}. However, specific roles of the microorganisms in N supply and plant N_{up} during cover crop decomposition and N mineralization periods are still limitedly explored (Fig.2.2).

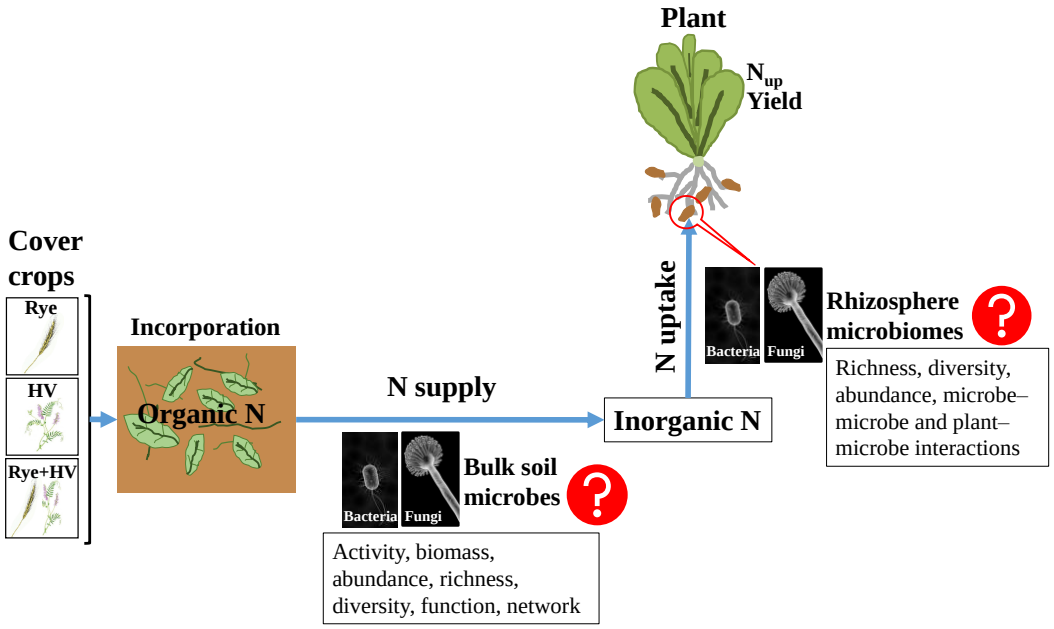


Fig.2.5. Illustration of research recommendation. Organic nitrogen (N) from cover crops residue is transformed into inorganic N via mediation of bulk soil microorganisms. The soil inorganic N is taken up by lettuce as the subsequent plant, affecting plant N uptake (N_{up}) and yield. The N_{up} can be assisted by rhizosphere microbiomes, microorganisms inhabiting lettuce rhizosphere soil. Thus, properties of bulk soil microorganisms and rhizosphere microbiomes and the roles in the N supply from each cover crop and N_{up} by lettuce plant are crucial. However, the evaluation during cover crop decomposition periods is limitedly performed (?).

The detail of research gaps has been mentioned in each sub-section in this chapter and are written as 'Research gap and potential solution' section in chapter 3–5. Following are summary of the research gaps.

2.4.1. Research 1

The first research was aimed to clarify the effects of N_{in} from different cover crops on N availability in soil and its utilization by subsequent plant. Although there are several numbers of previous research covering this aim, the effects during decomposition period of fresh cover crops residue are limitedly performed. In addition, it is poorly reported about microbial abundance that are expected to be more dynamic during the decomposition and N mineralization periods.

Results of research 1 are shown and discussed in Chapter 3. The cover crops tested were rye and HV as the gramineous and leguminous cover crop, respectively, and mix of rye and HV (rye+HV). Red leaf lettuce was selected as the subsequent plant. The experiments were conducted for two years in the field and pot experimental levels. The main results are plant performances (yield and N_{up}) and soil biochemical properties (i.e., concentration of soil inorganic N, soil pH, microbial activity as BG activity, and SMB) during cover crop decomposition period, i.e., up to 31 or 38 days.

2.4.2. Research 2

Research 2 was performed to answer the first question about how bulk soil microorganisms, bacteria and fungi, may play roles in N supply from each cover crop (Fig.2.2). Components of microbial community have been analyzed in several previous studies as the basic clarification of interaction between bulk soil microorganisms and cover crops input. Those properties may be more changeable at the middle period of the decomposition. Some recent studies demonstrate the potential roles of one or two of specific microbial taxa, such as influenced taxa. However, there are scarce numbers of study showing their roles in N supply during decomposition periods of cover crops. Additionally, microbial networks are still neglected in cover crop decomposition periods. Therefore, the results would be the novelties of this study.

Results of research 2 are shown and discussed in Chapter 4. The main results are microbial richness, diversity, abundance, network within and between bacteria and fungi, and functions of influenced microbial taxa related to the indicators of N

mineralization (soil inorganic N, soil pH, BG activity, and SMB) under rye, HV, rye+HV, and control at the middle of decomposition periods, i.e., 20 and 25 days.

As an addition, potential roles of soil bacteria in N cycle (i.e., decomposition, N fixation, nitrification, and denitrification), which may happen in the cover crops-treated soil, were emphasized as quantity of specific gene (*nifH*, *chiA*, AOB 16S rRNA, and *nirS*) and composition of the bacteria

2.4.3. Research 3

Research 3 was aimed to answer the second question about how rhizosphere microbiomes, bacteria and fungi, may play roles in lettuce plant N_{up} and yield (Fig.2.2). There are many reviews have described the intimate relationships between bellow- and above-ground. Nevertheless, there are few technical studies explore the relationships. Specifically, microbe–microbe and plant–microbe interactions in rhizosphere soils, whereas those networks are the vital reason of plant performance. Moreover, it is poorly understood that the effects of cover crops in bulk soils may pull up to the rhizosphere microbiome properties. Therefore, a direct network analysis was usefully adapted in this study to determine the roles of rhizosphere microbiome in lettuce performances.

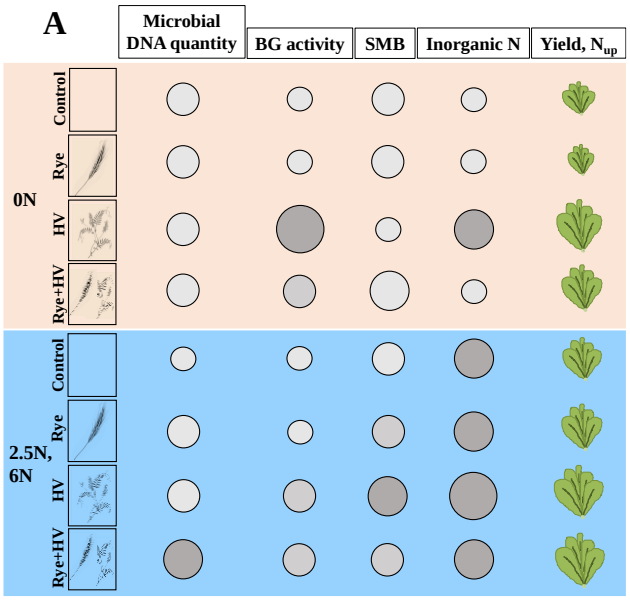
Results of research 3 are shown and discussed in Chapter 5. The main results are the microbial richness, diversity, abundance, microbe–microbe network, and plant–microbe interaction. The microbe–microbe network was recommended to know the primary functions (beneficial or harmful) served by rhizosphere microbiome. Plant–microbe network includes the direct service of influenced microbial taxa to plant performances (N_{up} and yield).

CHAPTER 3

Nitrogen supply from cover crops by soil microorganisms and its utilization by lettuce plants during the residual decomposition

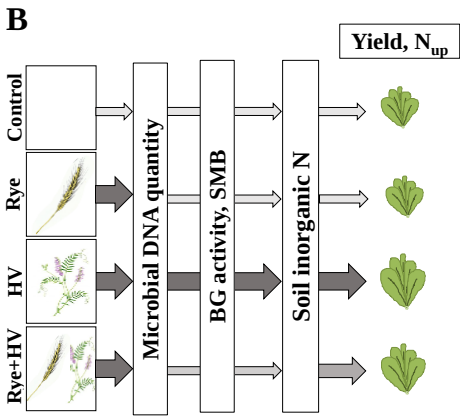
This chapter is rewritten based on the whole publication in Scientia Horticulturae 270:109415 (2020) https://doi.org/10.1016/j.scienta.2020.109415, and parts of the manuscript that has been submitted to Applied Soil Ecology.

Graphical abstract and highlight



Field-based research

- Hairy vetch (HV) with 0 g nitrogen (N) fertilizer per m^2 supplied high soil inorganic N and resulted in high lettuce performance.
- 2.5 and 6 g N fertilizers per m^2 relatively declined β-glucosidase enzyme (BG) activity and relatively increased soil microbial biomass (SMB) and fungal DNA quantity.



Pot-based research

- All cover crops promoted microbial abundance.
- HV promoted BG activity and SMB at sequential timing, emphasizing microbial roles in the inorganic N supply.

3.1. Research gap and potential solution

Synthetic nitrogen (N) fertilizers provide sufficient nitrogen input (N_{in}), resulting in high nitrogen uptake (N_{up}) of lettuce plants (Bottoms et al., 2012). Similar to N fertilizers, hairy vetch (HV) and mix of HV and rye cover crops in specific biomass ratios (i.e., HV:rye = 60:40 and 40:60, Kuo and Sainju, 1998) supply a large amount of N_{in}, thus achieving a high N_{up} and yield of the subsequent plants (Lee et al., 2014). Moreover, sustainable agriculture that is environmentally friendly agriculture can be achieved by limiting application of synthetic N fertilizers (Araki et al., 2009; Muchanga et al., 2017; Muchanga et al., 2020). It is necessary to compare the effects of N sources from synthetic fertilizer and cover crops on N_{up} and yield of the subsequent plants through a field experiment.

A long interval periods (e.g., 2 weeks) between application of cover crops and transplantation of tomato (Sainju et al., 2001) and red pepper (Lee et al., 2014) is commonly practiced to avoid allelopathic effects of cover crops, especially HV. However, these first 2 weeks can be the richest periods of soil inorganic N, considering that N mineralization from cover crop residues is coincidently occurred with the residual decomposition. Our groups have practiced one-day interval for cover crop-tomato rotation, which did not yield allelopathic effects (Araki et al., 2009; Muchanga et al., 2017). Thus, shortened interval periods from 2 weeks to 5 days is proposed to maximize the N utilization.

It has been reported that incorporation of HV (Liang et al., 2014; Mbuthia et al., 2015), rye, and mix of rye and HV residues (Nevins et al., 2020) promotes β-glucosidase enzymes (BG) activity in soil. Additionally, inputs of HV (Mbuthia et al., 2015) and mix of legume-rye residues (Brennan and Acosta-Martinez, 2017) increase carbon (C)-based soil microbial biomass (SMB). Dynamics of microbial properties, such as BG activity and SMB indicate the regulation of inorganic N (Sugihara et al., 2010). However, the dynamics during cover crop decomposition and the relations to soil inorganic N supply are still poorly explored. To obtain the possible explanation, a pot-based research is established.

Inputs of rye and HV (Maul et al., 2014) and mix of vetch-oat-radish residues

(Drost et al., 2020) stimulate bacterial and fungal genes. A long-term application of synthetic N fertilizer in 34N, 67N, and 101N rates increases total microbial fatty acid methyl ester, over the 0N rate (Mbuthia et al., 2015). Nonetheless, the effects of synthetic N fertilizer and cover crops and the significance on N mineralization in a short-term in the field-based research are unknown.

Therefore, the hypotheses of this research were that the easily decomposable HV cover crop (1) can alternate synthetic N fertilizers in the fields and (2) can promote BG activity, SMB, and microbial abundance that describe the roles of soil microorganisms in the decomposition and N mineralization.

3.2. Materials and Methods

3.2.1. Experimental levels and site

Field and pot experiments were conducted in the Experimental Farm and green house, respectively, located in Field Science Center for Northern Biosphere, Hokkaido University, Japan (43°3′N, 141°20′ E). The experiments were done in 2017 and 2018. The soil was classified as Calcaric, Eutric Fluvisol light clay, as detailed by Nakamoto et al. (2012) with initial soil chemical properties shown in Table 3.1.

3.2.1.1. Field experiment

Two different field locations were selected for each year to avoid an accumulation effects of cover crops. Precipitation, solar radiation, and temperature of soil and air in the fields were monitored per hour during the preparation (April–June) and experimental (July–August) periods using the Weather Bucket TA-WL-2U (SEC Corporation, Hakodate, Japan).

3.2.1.2. Pot experiment

In 2017, soils used were habitually prepared for vegetable seedlings. Fallow soils were mixed with vegetable wastes and incubated for 7 months during the winter. In 2018, fallow soils were chosen to show clear effects of the cover crops.

The soils were sieved through a 1 cm mesh to obtain fine soils. A 1.5 kg unfiltered

soils were placed in the bottom of a black plastic pot (diameter 30 cm; height 25 cm) for water flowing spaces; and a 7.5 kg moist fine soils were added on the top.

Table 3.1. Initial soil chemical properties in the field and pot experiments

	Field		Pot	
	2017	2018	2017	2018
TC (%)	2.93	2.90	6.89	4.74
TN (%)	0.24	0.25	0.59	0.37
Inorganic N (mg N kg^{-1} soil)	28.0	12.9	98.1	24.3
pH (H_2O)	5.99	5.30	5.98	6.21

The TC, TN, and N abbreviate total carbon, total nitrogen, and nitrogen, respectively.

3.2.2. Treatments and experimental designs

Rye var. Fuyumidori (Hokuren Federation of Agricultural Cooperatives, Sapporo, Japan), HV (Snow Brand Seed Co., Ltd., Sapporo, Japan), and mix of rye and HV (rye+HV) were prepared.

3.2.2.1. Field experiment

Treatments were bare plots (control) and plots with rye, HV, and rye+HV in the 3 × 7 m^2 plots. A 10 g rye seeds m^{-2} and 5 g HV seeds m^{-2} were separately sown for rye and HV plots on September a year before the examination as the recommendation of cover cropping system in Hokkaido region. A 50:50 sowing rate per m^{-2} was selected for rye+HV plots (i.e., 5 g rye seed+5 g HV seed). Cover crops were grown until the flowering stage on July, terminated using flail mower, and incorporated into soils using a rotary tiller up to 15 cm depth. In 2018, HV plants poorly grew (Table 3.2A). Thus, fresh HV from other fields was added to HV and rye+HV plots to adjust the applied biomass similar to that in 2017.

Sub-treatments were fertilizer application as ammonium sulfate [$(NH_4)_2SO_4$] in 2.5 g N m^{-2} (2.5N) in 2017 and 6 g N m^{-2} (6N) in 2018, compared with non-fertilized split plots (0N) in both experimental years. Fertilizer applications were at the same time of cover crop incorporation. The 2.5N and 6N were a quarter and a half of the recommended rates (i.e., 12 g N m^{-2}), respectively, for lettuce cultivation in Hokkaido region (Hokuren Agricultural Cooperation for Hokkaido, 2012). To

remove limiting factors of phosphorus (P) and potassium (K) in soils, a 12 g m^{-2} of sodium tripolyphosphate (P_2O_5) and 14 g m^{-2} of potassium sulfate (K_2SO_4) were applied following the recommendation.

Aboveground of cover crops were sampled in 0.25 m^2 area. The mass was oven-dried at 60°C for 2 days and ground for N and C content analysis using the Vario EL III CHNOS elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) according to the manufacturer's protocol. Acetanilide ($CH_3CONHC_6H_5$; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was arranged as the standard. Briefly, a 10–13 mg ground plants and 2–4 mg standards were subjected to dry combustion system at 950°C of furnace temperature under ± 1.3 Bar and ± 200 mL min^{-1} of O_2 and He gas pressure and flow, respectively. The O_2 index was 2, 1, and 3 for blank, standard, and plant samples, respectively. Cover crop N_{in} and C:N ratio in the fields each year (Table 3.2A) were based on the N and C content.

The experimental design was a split-plot with three replicates. Main plots included the treatments assigned to the first randomization. A separate randomization was conducted within each treatment to assign the sub-treatments.

3.2.2.2. Pot experiment

Treatments were soils without any cover crops (control) and soils with rye, HV, and mixed (rye+HV) cover crops. Aboveground of cover crops from the fields were freshly harvested, chopped into small pieces (i.e., 5–10 cm long), and mixed into the fine soils (sub-section 3.2.1.2). The applied biomass was based on the equal dry weight (DW) of each cover crop, i.e., 40 g DW of rye residue and 40 g DW of HV residue for rye and HV treatments, respectively. A 50:50 biomass ratio was selected as the rye+HV treatment (i.e., 20 g DW rye residue + 20 g DW HV residue). Cover crop N_{in} and C:N ratio in the pots (Table 3.2B) were based on the N and C contents analyzed using the Vario EL III CHNOS elemental analyzer.

The experimental design was a randomized block with three replicates. Each treatment in each replicate contained 10 pots, which 5 pots were used separately for 2 growth stage evaluations: mid growth and mature stages.

Table 3.2. Biomass and chemical characteristics of cover crops for the (A) field and (B) pot experiments each year

(A)

Cover crop	Biomass (g DW m^{-2})			N_{in} (g m^{-2})			C:N ratio	
	2017	2018	Added HV	2017	2018	Added N_{in}	2017	2018
Control	-	227	-	-	5.30	-	-	17.0
Rye	612	1142	-	7.70	15.2	-	37.8	34.2
Rye+HV	461+233	555+21	100	14.4	13.2	6.27	21.6	30.2
HV	572	317	117	22.2	9.65	5.31	10.7	12.7

(B)

Cover crop	N_{in} (g pot^{-1})		C:N ratio	
	2017	2018	2017	2018
Control	-	-	-	-
Rye	0.49	0.52	37.8	34.2
Rye+HV	0.81	0.94	21.6	16.8
HV	1.56	1.2	10.7	12.7

HV, DW, N_{in}, and C:N ratio abbreviate hairy vetch, dry weight, nitrogen input, and ratio of carbon to nitrogen, respectively. Data are the mean of 3 replicates.

3.2.3. Lettuce performances

The 4–5-week-old seedlings of red leaf lettuce (*Lactuca sativa* L. var. *crispa* cv. Red fire; Takii Seed Co., Tokyo, Japan) were prepared in a nursery commercial soil for preparing seedlings of flowering plant and vegetables (Takii & Co., Ltd, Kyoto, Japan). Seedlings with 3 true leaves were readily transplanted (Caliskan et al., 2014) at 5 days after incorporation (DAI) of cover crops.

3.2.3.1. Field experiment

In each split plot, 6 seedlings were used as samples and 14 seedlings were the borders. Cultivation spaces were 40 cm × 40 cm between seedlings. Hand watering was provided at the first week after the transplantation. Hand watering was run every 2 days until the harvest time in 2018, due to the rain scarcity. Pests were managed by applying Adion insecticide (Sumitomo Chemical Company, Ltd., Tokyo, Japan) as 1 mL per 3 L of water based on the general recommendation for lettuce plants in Japan. Hand weeding was applied.

Lettuce plants were harvested at the mature stage: at 38 and 31 DAI in 2017 and 2018, respectively.

3.2.3.2. Pot experiment

One pot received one seedling. A 0.6–1.8 L of water per pot was daily given to maintain soil moisture as 30%–35% during lettuce cultivation. Hand weeding was applied, but insecticide was not.

Lettuce mid growth stages were at 25 and 20 DAI; the mature stages were at 38 and 31 DAI in 2017 and 2018, respectively. At the harvest time, lettuce aboveground was cut, and fresh weight (FW) was measured to obtain the yields. Subsequently, the mass was oven-dried at 60°C for 5 days and ground for the N and C contents analysis using the Vario EL III CHNOS elemental analyzer.

The N_{up} was first calculated in general as the result of lettuce N content (%) multiplied by the DW. Then, specific N_{up} was calculated as a potentially taken up N from cover crops applied in the fields and pots.

Specific N_{up} from cover crop = general N_{up} in cover crop treatment-0N − general N_{up} in control-0N.

3.2.4. Soil samplings

3.2.4.1. Field experiment

Soils at 0–10 cm depth from 5 to 7 spots in each split plot were collected at the end of experiment (i.e., lettuce harvest time) and mixed thoroughly.

3.2.4.2. Pot experiment

Bulk soils were regularly sampled without disturbing the lettuce plants: at 5, 25, and 38 DAI in 2017 and at 3, 5, 10, 15, 20, 25, and 31 DAI in 2018 for further explanation. Sampled soils from each pot in each replicate with the same treatment were mixed thoroughly.

Sampled soils from the fields and pots were divided into two parts: one part was air-dried and analyzed for soil chemical properties; other part was stored at 5°C or –20°C until the analyses for soil biological properties and biomolecular.

3.2.5. Analysis of soil biochemical properties

3.2.5.1. Soil chemical properties

Soil inorganic N was sum of soil nitrate and ammonium (NO_3^--N+NH_4^+-N). Firstly, 1:5 (w:v) ratio of soil and 2M KCl solution was continuously mixed for 30 min; and then, the extract was filtered. The extract is subjected to the flow injection analyzer (FIA, AQLA-700; Aqualab, Tokyo, Japan). For nitrate measurement, the carrier solution contained of 0.24 g of EDTA · 4Na ($C_{10}H_{12}N_2Na_4O_8 \cdot 4H_2O$) and 0.90 g of ammonium chloride (NH_4Cl) in 300 mL miliQ; the color solution contained of 0.30 g of sulfanilamide ($C_6H_8N_2O_2S$), 0.03 g of N-1-Naphthylethylenediamine dihydrochloride ($C_{12}H_{14}N_2 \cdot 2HCl$), and 9 mL of Hydrochloric acid (HCl) in 300 mL miliQ; and the standards were 0.5, 1, 2.5, 5, and 10 ppm of NO_3^--N as potassium nitrate (KNO_3).

For ammonium measurement, the carrier solution consisted of 22.365 g of KCl in 300 mL miliQ; the first color solution consisted of 3.30 mL of phenol (C_6H_6O)

and 0.015 g of sodium pentacyanonitrosylferrate (III) dihydrate ($Na_2[Fe(CN)_5NO] \cdot 2H_2O$) in 300 mL miliQ; the second color solution consisted of 4.50 g of sodium hydroxide (NaOH) and 15 mL of sodium hypochloride solution (NaClO) in 300 mL miliQ; and the standards were 0.5, 1, 2.5, 5, and 10 ppm of NH_4^+-N as ammonium chloride (NH_4Cl).

Soil pH was examined in 1:5 of soil:miliQ mixture (w:v) (Hardie and Doyle, 2012) using the Docu-pH meter (Sartorius AG, Goettingen, Germany) after being shaken for 30–45 min.

3.2.5.2. Soil biological properties

The BG activity was examined based on method of Hayano (1973) modified by directly dissolving 150 mg of 4-nitrophenyl α-D-glucopyranoside ($C_{12}H_{15}NO_8$; Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) in 50 mL of McIlvaine buffer (McIlvaine, 1921) to obtain 10^{-2} M of the ρNPG solution. A 0.8 g moist soil was mixed with 3 mL of ρNPG solution, vortex for 10 sec, and incubated at 30°C for 3 hours, and then 8 mL of pure ethanol (C_2H_5OH; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to stop the reactions. Subsequently, the substrate was vortex for 10 sec and filtered. The standards were 0, 0.2, 0.4, 0.8, and 1 µmole mL^{-1} of ρ-nitrophenol (ρNP, $O_2NC_6H_4OH$; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Absorbance of ρNP was measured in 405 nm using the Wallac 1420 ARVO MX Multilable Counter (Perkin Elmer Inc., USA). Duplicate reactions were done for each sample and standard.

The SMB was evaluated on the basis of C. A 2 g moist soils were fumigated with chloroform ($CHCl_3$) for 24 hours. Dissolved total C was extracted in 10 mL of 0.5 M K_2SO_4 (Kanto Chemical Co. Inc., Tokyo, Japan) following the method of Vance et al. (1987). Subsequently, the dissolved C was converted into CO_2 with a converter solution consisted of 25 g of potassium persulfate ($K_2S_2O_8$) and 15 g of Trihydroxy(oxo)-λ 5-borane (H_3BO_4) in 500 mL miliQ (Doyle et al., 2004). Conversion methods were started by mixing an equal volume of dissolved C and converter solution in a bottle with a rubber lid under a −100 KPa (i.e., 0 KPa) of the air pressure. Afterward, zero air was added until the air pressure reached to a −00.8

KPa (i.e., the normal air pressure; 1.013×10^2 KPa). The mixture was reacted at 120°C for 20 min. Nonfumigated soils were directly subjected to the K_2SO_4 extraction and CO_2 conversion steps. Produced CO_2 was measured using an isotopic CO_2 analyzer (CCIA-46-EP; Los Gatos Research, Inc., CA, USA). In final, SMB was calculated following the method of Bailey et al. (2002).

$$\text{SMB} = \frac{\text{C in fumigated soil} - \text{C in nonfumigated soil}}{0.45}$$

3.2.6. Quantifying bacterial and fungal abundance

3.2.6.1. DNA extraction and purification

DNA was extracted from all collected bulk soils (sub-section 3.2.4.2) using either PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., CA, USA) or chloroform phenol methods (Miller et al., 1999; Sagova-Mareckova et al., 2008) in 2017 and 2018, respectively. The extracted DNA was purified with AMPure XP reagent (Nipon Genetics Co. Ltd., Tokyo, Japan); and the amount was measured using Qubit dsDNA HS Assay Kit (Invitrogen, Life Technologies Co., Oregon, USA).

3.2.6.2. Quantitative PCR (qPCR)

DNA templates were purified DNA that was 10^2 times diluted with Tris-EDTA buffer (TE) consists of 10 mM Tris base and 0.1 mM EDTA (Ion Torrent; Life Technologies Co.). Sets of primer targeting v4 region of bacterial 16S rRNA gene (515F and 806R) and region 1 of fungal internal transcribed spacer (ITS) (ITS1 and ITS2, White et al., 1990) were prepared. The reaction mixture was 10 µL of SYBR Fast qPCR Mix (Takara Bio Inc., Tokyo, Japan); 0.8 µL of 10 µM concentration of each primer; 0.4 µL of ROX reference dye II (Takara Bio Inc.); 6 µL of nuclease-free water (Applied Biosystem; Thermo Fisher Scientific Inc., CA, USA); and 2 µL of the DNA template. The standards were serial dilution of purified DNA (Oka and Uchida, 2018) from one of the three control replicates at 5 DAI in 2017 and 3 DAI in 2018. The qPCR was carried out on the Mx3005P qPCR system (Stratagene; Agilent Technologies Inc., CA, USA) following the manufacture's settings. Annealing temperature and cycle numbers were suitably adapted for the primers:

57°C and 30 cycles for bacteria; and at 55°C and 40 cycles for fungi. Duplicates reactions were conducted to each sample and standard.

Detected quantity of bacterial and fungal genes was automatically calculated by applying adaptive algorithms in the MXPro-Mx3005P qPCR software version 4.10 (Stratagene) of the cycle threshold (CT) values. The PCR efficiencies and correlation coefficients (R^2) of the standards were > 94.7% and > 0.95, respectively. DNA quantity was calculated relatively to DNA quantity of control soil at 5 DAI in 2017 or 3 DAI in 2018 used as the standards.

3.2.7. Litter bag assay

Three equivalent DW of fresh rye, HV, and rye+HV residues were placed into a 10 × 10 cm^2 of nylon mesh (bag) with 2 mm mesh size to allow microorganism passage, but omit macroorganisms entering (Bradford et al., 2002). Thus, influence of soil microbes can be expected. This assay was demonstrated in three replicates in 0N split plots in the fields and in the pots in which the soils were subjected to the same cover crops. Four bags were prepared per replicate.

One bag from each replicate was collected at 5, 15, 25, and 35 DAI. Remaining residues were cleaned up from soils, and then oven-dried at 60°C for 2 days. The mass was ground for the N and C content analysis using the Vario EL III CHNOS elemental analyzer. Released N or C was the differences between N or C contents at 0 DAI and at the observation dates per initial N and C contents of the residues (Lawson et al., 2012).

3.2.8. Statistical data analyses

All statistical analyses were performed using R v.3.5.3 (R Core Team, Vienna, Austria) and GraphPad Prism 8.4.2 (GraphPad Software, CA, USA). Data in each experimental year were analyzed separately.

3.2.8.1. Field experiment

Split-plot ANOVA was performed to differentiate the effects of cover crops and fertilizers on soil biochemical properties and DNA quantity using ‘sp.plot’

function of agricolae package. Cover crops, fertilizers, and replicates were the main, split-plot, and random factors, respectively. One-way ANOVA was performed to data of lettuce performance. Two-way ANOVA was performed to data of litter bag with cover crops as variable and time (i.e., DAI) as covariable. To distinguish the significant effects of cover crops, Tukey's HSD at $P < 0.05$ was subsequently performed.

3.2.8.2. Pot experiment

One-way ANOVA was performed to data of lettuce performances at each growth stage. Two-way ANOVA was performed to data of litter bag, soil biochemical properties, and DNA quantities. A Tukey's HSD at $P < 0.05$ of P-value was subsequently performed. Data of DNA quantity were log transformed when normal distribution was not achieved based on the Shapiro-Wilk test. Pearson's correlation analysis was conducted on selected parameters using average data of the 2 experimental years. The t-test at $P < 0.05$ was also necessarily performed.

3.3. Results

A. Field experiment

3.3.1. Climatic conditions during the preparation and experimental periods

Rainfall accumulation was 337 mm in 2017 and 275 mm in 2018 during the preparation periods (April–June). In 2018, however, the rains came more frequently (i.e., almost every day) compared with those in 2017. Rainfall accumulation was 178 mm in 2017 and 63.2 mm in 2018 that was scarce during the experimental periods (July–August).

In the experimental periods, average of soil and air temperatures were 22.0°C in 2017 and 20.2°C in 2018; and solar radiation was 11–16 MJ m^{-2} day^{-1} in 2017 and 10–14 MJ m^{-2} day^{-1} in 2018.

3.3.2. Biomass and N input (N_{in}) of cover crops

HV biomass in HV plot in 2018 was 44.52% declined, reducing the N_{in} over the 2017 (Table 3.2A). Biomass and N_{in} of rye was doubled in 2018 over the 2017. The rye:HV biomass ratio in rye+HV plots varied in 2 experimental years, i.e., 66:34 in

2017 and 96:4 in 2018 in percent per total biomass of rye and HV.

3.3.3. Performances of lettuce plants

Lettuce yield and specific N_{up} from cover crops per area in 2018 were depleted in all treatments over the 2017 (Fig.3.1). However, the trends of the effects of cover crops and fertilizers were the same in both years: (a) HV-0N significantly improved lettuce yields; (b) rye-0N suppressed the yields and specific N_{up} over the control-0N. Lettuce performance in rye+HV-0N was improved only in 2017.

Lettuce yield was significantly enhanced by 2.5N sub-treatment over the 0N sub-treatment in control ($P = 0.0070$) and rye ($P = 0.0078$) based on the t-test. However, lettuce yields in HV and rye+HV with 0N and 2.5N were similar. Lettuce yields were strongly and positively correlated with general N_{up} ($R = 0.97$, $P = 1.47 \times 10^{-6}$) in the two years of experiments.

3.3.4. Changes on soil biochemical properties and microbial abundance

Cover crops affected soil pH and BG activity in 2017. Specifically, HV increased in BG activity (Table 3.3), but decreased in soil pH over the control (data not shown). Cover crops and fertilizer interactively affected SMB in 2017, wherein the combination of HV cover crop and 2.5N fertilizer application rate improved SMB compared with control-2.5N. In 2018, cover crops affected soil inorganic N, which HV showed a higher remained concentration of soil inorganic N than control. Fertilizer affected SMB in 2018, wherein the average of SMB was higher in 6N sub-treatment (5.24 g C kg^{-1} soil) than in 0N sub-treatment (4.02 g C kg^{-1} soil).

Fertilizers affected bacterial and fungal DNA quantities in 2017 (Table 3.4). In detail, average of bacterial and fungal DNA quantities was increased and decreased in 0N sub-treatment (1.22 ng DNA and 0.86 ng DNA) over the 2.5N sub-treatment (0.90 ng DNA and 1.16 ng DNA) in 2017. In addition, rye+HV-2.5N increased fungal DNA quantity compared with the control-2.5N in 2017. In 2018, cover crops and fertilizers interactively affected bacterial DNA quantity without resulting in any specific effects.

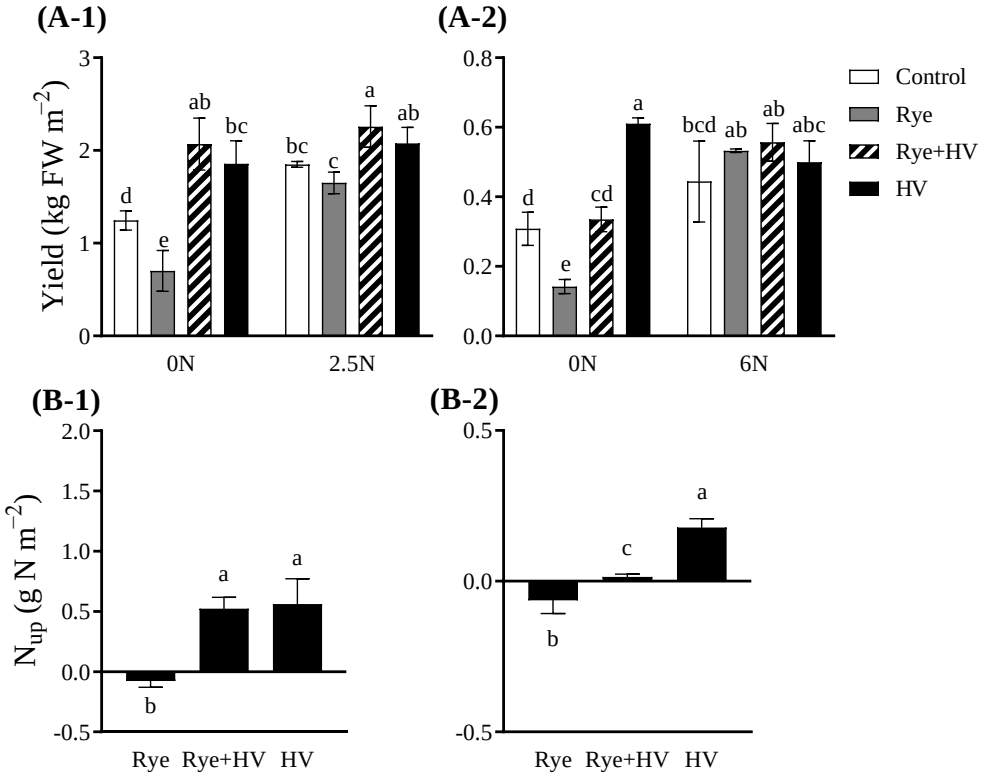


Fig.3.1. (A) Lettuce yield and (B) estimation of specific nitrogen uptake (N_{up}) from cover crops in (1) 2017 and (2) 2018 field experiments. Data were collected at 38 and 31 days after cover crops incorporation in 2017 and 2018, respectively. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Different letters indicate significant difference, based on Tukey's HSD at $P < 0.05$ (n = 3). Vertical bars represent the standard deviation.

Table 3.3. Soil biochemical properties in the fields with different cover crops and fertilizer application rates

Treatment (T)	Fertilizer (F)	2017			2018		
		Inorganic N (mg N kg^{-1} soil)	BG activity (μg ρNP g^{-1} soil h^{-1})	SMB (g C kg^{-1} soil)	Inorganic N (mg N kg^{-1} soil)	BG activity (μg ρNP g^{-1} soil h^{-1})	SMB (g C kg^{-1} soil)
Control	0N	13.8 ± 2.12 d	58.2 ± 46.4 b	3.95 ± 0.44 abc	30.0 ± 2.90 b	226 ± 42.0	3.41 ± 1.10
Rye		15.6 ± 3.95 cd	82.9 ± 42.3 ab	1.47 ± 1.98 bc	12.3 ± 2.92 b	176 ± 143.6	4.75 ± 1.03
Rye+HV		17.4 ± 0.38 cd	175 ± 130 ab	2.77 ± 1.94 abc	20.7 ± 2.29 b	75.2 ± 52.2	3.10 ± 0.51
HV		25.6 ± 7.07 bcd	308 ± 102 a	0.51 ± 0.13 c	56.8 ± 15.5 ab	342 ± 173	4.84 ± 0.82
Control	2.5N, 6N	37.0 ± 16.11 abc	48.1 ± 35.1 b	1.07 ± 0.33 bc	53.0 ± 14.6 ab	77.4 ± 31.3	4.96 ± 0.07
Rye		36.5 ± 2.38 abc	45.3 ± 21.2 b	4.50 ± 1.08 ab	54.1 ± 34.8 ab	218 ± 51.0	5.63 ± 0.70
Rye+HV		40.9 ± 3.49 ab	138 ± 74.4 ab	4.05 ± 0.96 abc	51.7 ± 27.5 ab	148 ± 74.4	5.70 ± 0.37
HV		51.8 ± 4.88 a	161 ± 50.6 ab	5.84 ± 1.12 a	124 ± 43.0 a	330 ± 246	4.68 ± 0.51
ANOVA	T	NS	**	NS	**	NS	**
	F	***	NS	*	**	NS	*
	T × F	NS	NS	**	NS	NS	NS

Data were collected at 38 and 31 days after cover crops incorporation in 2017 and 2018, respectively. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The N, BG, and SMB abbreviate nitrogen, β-glucosidase enzyme, and soil microbial biomass, respectively. The asterisks (*, **, ***) denote 0.05, 0.01, and 0.001 significance, respectively, and NS means nonsignificant, based on the split-plot ANOVA. Each mean ± standard deviation was accompanied by a letter, in which different letters show the significance according to Tukey's HSD at $P < 0.05$ (n = 3).

Table 3.4 Microbial abundance in the fields with different cover crops and fertilizer application rates

Treatment (T)	Fertilizer (F)	2017		2018	
		Bacteria	Fungi	Bacteria	Fungi
Control	0N	0.97 ± 0.05	0.87 ± 0.12 ab	0.89 ± 0.12	1.64 ± 0.61
Rye		1.37 ± 0.30	0.80 ± 0.04 ab	1.86 ± 0.70	2.36 ± 1.47
Rye+HV		1.31 ± 0.29	0.95 ± 0.22 ab	1.24 ± 0.49	2.34 ± 1.31
HV		1.23 ± 0.09	0.81 ± 0.05 ab	1.21 ± 0.37	2.11 ± 1.53
Control	2.5N, 6N	0.88 ± 0.15	0.76 ± 0.30 b	1.32 ± 0.36	1.08 ± 0.09
Rye		0.91 ± 0.12	1.05 ± 0.30 ab	1.08 ± 0.13	1.67 ± 0.34
Rye+HV		1.00 ± 0.04	1.76 ± 0.61 a	1.65 ± 0.09	1.74 ± 0.04
HV		0.82 ± 0.15	1.08 ± 0.19 ab	1.46 ± 0.27	1.45 ± 0.35
ANOVA	T	NS	*	NS	NS
	F	**	*	NS	NS
	T × F	NS	NS	*	NS

Data were collected at 38 and 31 days after cover crops incorporation in 2017 and 2018, respectively. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The asterisks (* and **) denote 0.05 and 0.01 significance, respectively, and NS means nonsignificant, based on the split-plot ANOVA. Each mean ± standard error was accompanied by a letter, in which different letters show the significance according to Tukey's HSD at $P < 0.05$ (n = 3). Log transformed was necessarily performed.

3.3.5. Cover crop residual decomposition, and N and C mineralization

The trends of rates of residual decompositions, released N, and released C of the three cover crops were similar in the two experimental years (Fig.3.2), i.e., HV ≥ rye+HV ≥ rye. The rates were slower in 2018 than in 2017 based on the t-test (Table S3.1A). Additionally, average of weight of the remained residues, percentage of released N, and percentage of released C from the cover crops were 29.5% heavier, 16.0% lower, and 24.6% lower, respectively, in 2018 than in 2017 (Fig.3.2).

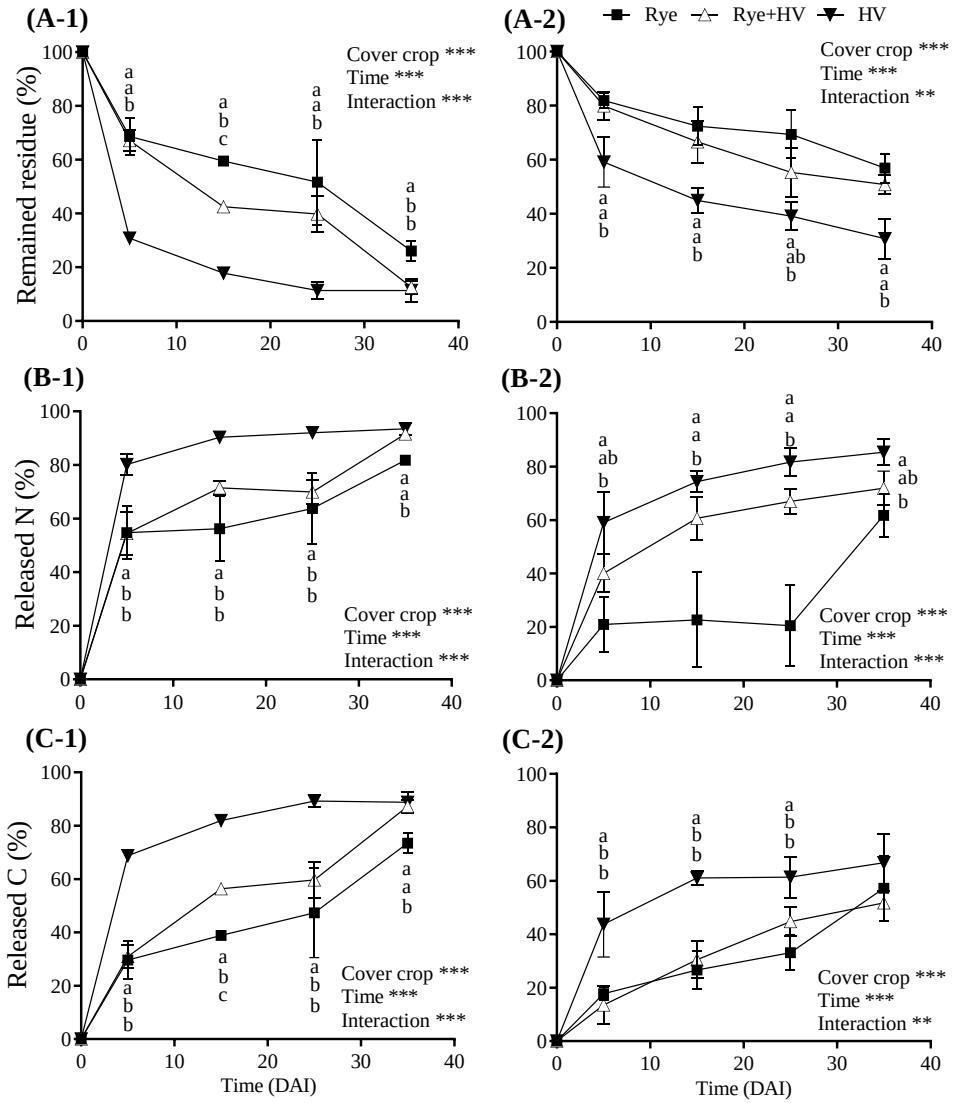


Fig.3.2. Trends of (A) residual decomposition, (B) nitrogen release, and (C) carbon release from cover crops in (1) 2017 and (2) 2018 field experiments. Cover crops were rye, hairy vetch (HV), and mix of rye and HV (rye+HV). The N and C abbreviate nitrogen and carbon, respectively. The asterisks (** and ***) denote significance in 0.01 and 0.001, respectively, according to the two-way ANOVA of cover crop and time, i.e., day after incorporation (DAI) of cover crops. Different letters at the same day clarified the significance among cover crops at the day based on Tukey's HSD at $P < 0.05$ (n = 3). Arcsine root square-transformation was necessarily performed. Vertical bars represent the standard deviations.

B. Pot experiment

3.3.6. Performances of lettuce plants

Trends of lettuce performance per individual plant at the mature stage in the pots (Fig.3.3) were similar with those in 0N split plots of the fields (Fig.3.1A). In 2017 at the mid growth stages, HV suppressed lettuce yield (Fig.3.3A-1) and specific N_{up} (Fig.3.3B-1) over the control. Rye+HV consistently promoted lettuce performances at both plant growth stages and experimental years over the control.

Lettuce yields were strongly and positively correlated with general N_{up} at the mid growth stages ($R = 0.92$, $P = 0.0012$) and mature stages ($R = 0.99$, $P = 1.85 \times 10^{-6}$).

3.3.7. Dynamics of soil biochemical properties and microbial abundance

Concentration of soil inorganic N was improved in HV, followed by rye+HV over the rye that was equal to control during the experiments (Fig.3.4A). Soil pH was unchanged in all treatments during the experiment in 2017. In 2018, soil pH was reduced in HV and rye+HV treatments at 10–20 DAI over the control and rye (data not shown).

The BG activity was increased in HV at 25 and 38 DAI over the rye and control in 2017 (Fig.3.4B-1) and at 10 DAI among other treatments in 2018 (Fig.3.4B-2). Range of BG activity was higher in 2017 than in 2018. The SMB was increased in HV at 25 DAI among other treatments in both years (Fig.3.4C). Moreover, HV and rye increased SMB at 38 DAI in 2017 over the control and rye+HV.

Cover crop and time interactively affected the dynamics of bacterial and fungal DNA quantities (Fig.3.5), with the exception of bacterial DNA quantity in 2017 (Fig.3.5A-1). Specifically, bacterial DNA tended to be higher in all cover crops at 3–10 DAI in 2018 (Fig.3.5A-2). Fungal DNA peaked at 5 DAI in rye and rye+HV in the two experimental years (Fig.3.5B).

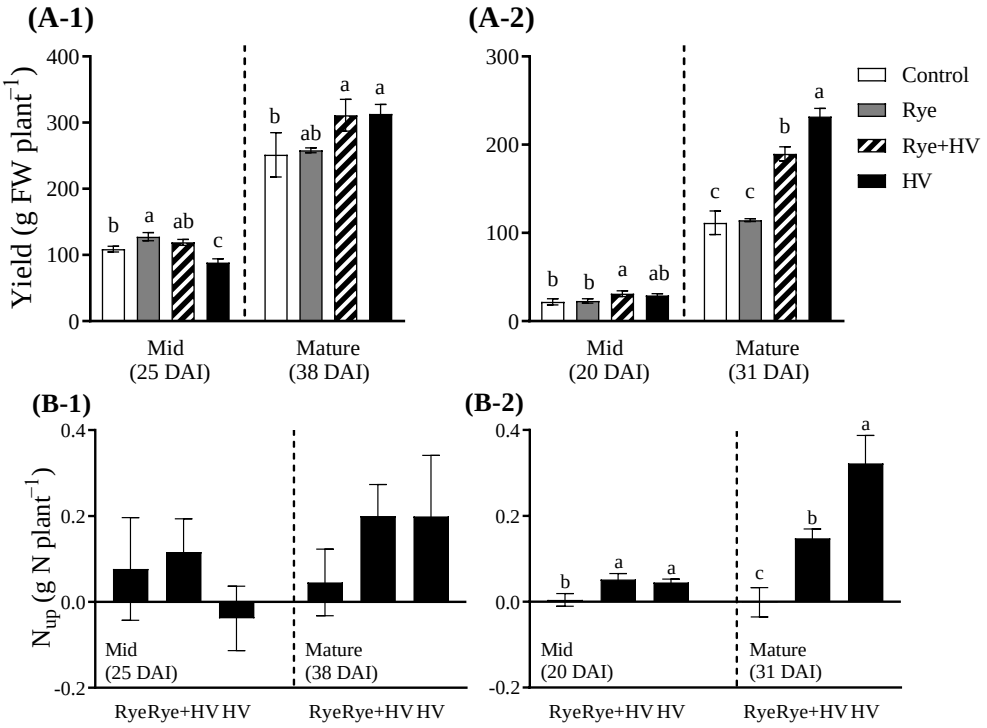


Fig.3.3. (A) Lettuce yield and (B) estimation of specific nitrogen uptake (N_{up}) from cover crops in (1) 2017 and (2) 2018 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Different letters indicate significant difference based on Tukey's HSD at $P < 0.05$ (n = 3). Vertical bars represent the standard deviation. DAI abbreviates days after incorporation of cover crops.

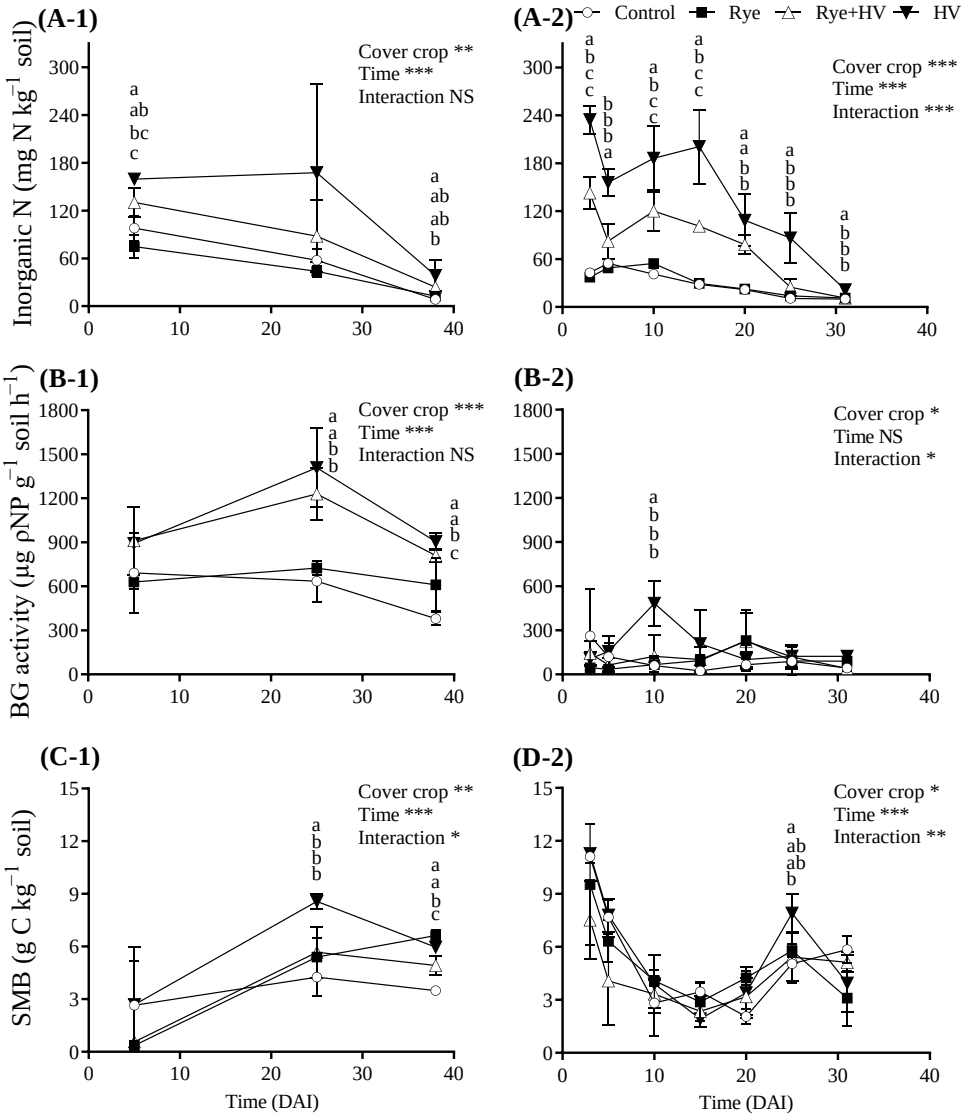


Fig.3.4. Dynamics of (A) soil inorganic nitrogen (N), (B) β-glucosidase enzyme (BG) activity, and (C) soil microbial biomass (SMB) during cover crop decomposition periods in (1) 2017 and (2) 2018 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The asterisks (*, **, ***) denote significance at 0.05, 0.01, and 0.001, respectively, and NS means [continue] nonsignificant based on the two-way ANOVA of cover crop and time, i.e., day after incorporation (DAI) of cover crops. Different letters clarify the significance of cover crops at each time, based on Tukey's HSD at $P < 0.05$ (n = 3). Vertical bars show the standard deviation.

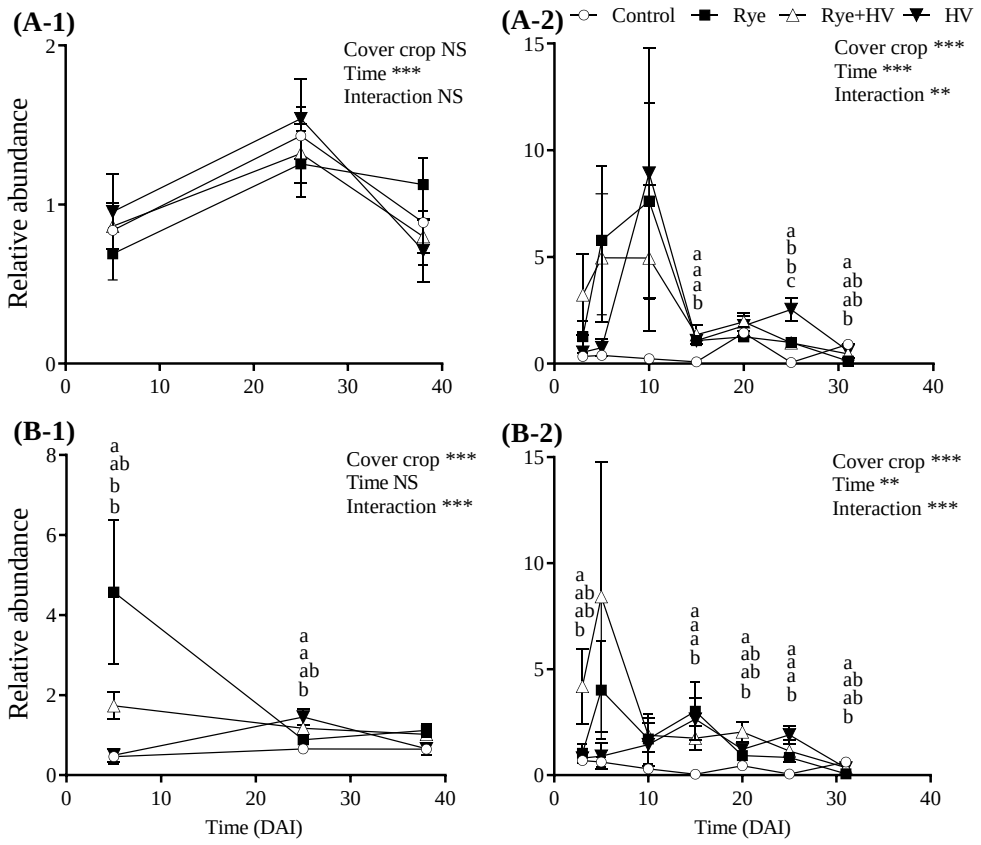


Fig.3.5. Dynamics of (A) bacterial and (B) fungal abundance during cover crop decomposition periods in (1) 2017 and (2) 2018 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The asterisks (** and ***) denote significance at 0.01 and 0.001, respectively, and NS means nonsignificant based on the two-way ANOVA of cover crop and time, i.e., day after incorporation (DAI) of cover crops. Different letters clarify the significance of cover crops at each time based on Tukey's HSD at $P < 0.05$ (n = 3). Log transformed was necessarily performed. Vertical bars show the standard error.

3.3.8. Cover crop residual decomposition, and N and C mineralization

The trends of the rates of residual decompositions, released N, and released C of cover crops in the pots (Fig.3.6) were similar with that in the fields (Fig.3.2) in both years, i.e., HV ≥ rye+HV ≥ rye.

Rates of released N of all cover crops were similar across the years, but rates of residual decomposition and C release varied until 35 DAI (Table S3.1B). Percentage of released N and concentration of soil inorganic N at 5 DAI, when the soil inorganic N had not yet taken up by lettuce plants, were strongly and significantly correlated in both years (Fig.S3.2).

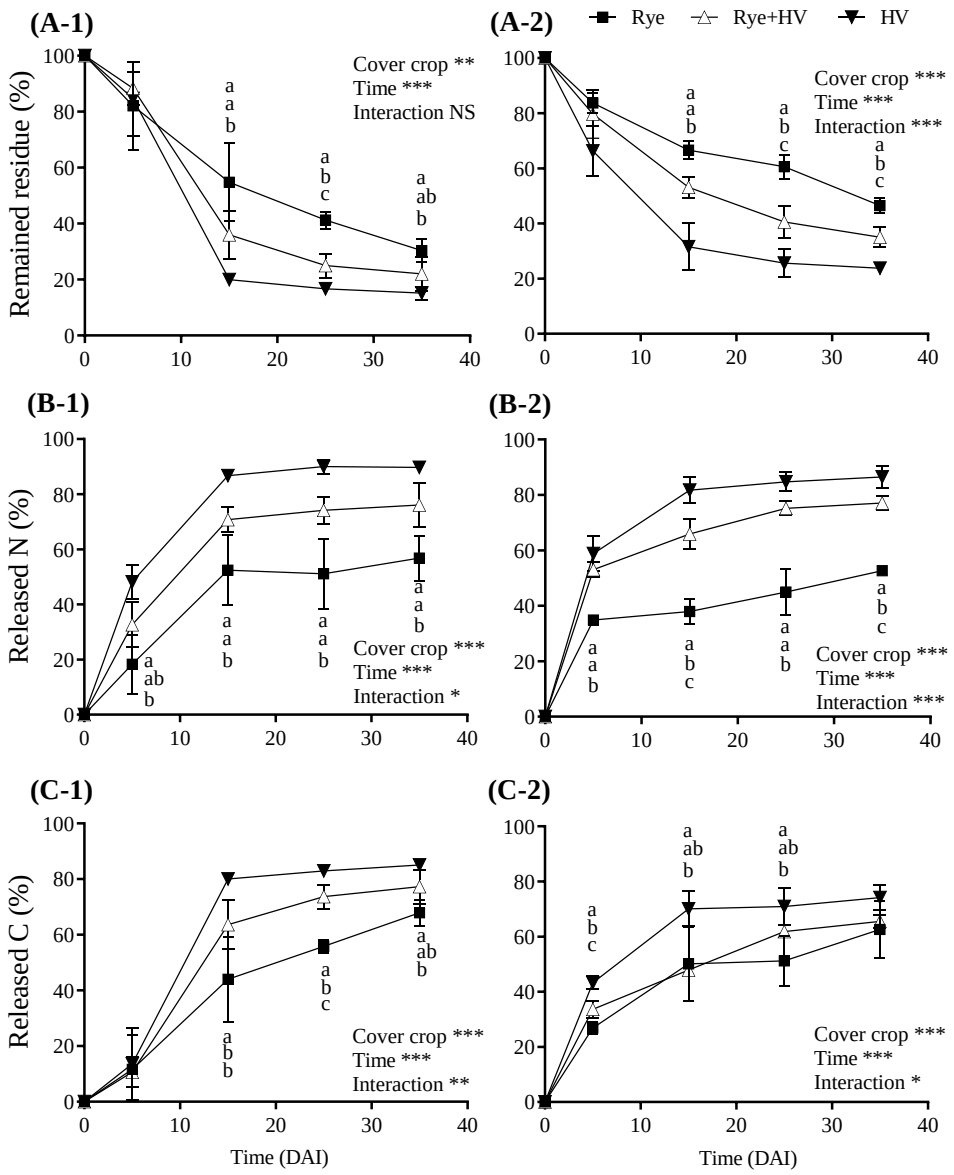


Fig.3.6. Trends of (A) residual decomposition, (B) nitrogen release, and (C) carbon release from cover crops in (1) 2017 and (2) 2018 pot experiments. Cover crops were rye, hairy vetch (HV), and mix of rye and HV (rye+HV). The N and C abbreviate nitrogen and carbon, respectively. The asterisks (*, **, ***) denote significance in 0.05, 0.01, and 0.001, respectively, and NS means nonsignificant effects based on the two-way ANOVA of cover crops and time, i.e., day after incorporation of cover crops (DAI). Different letters at the same day clarified significance among cover crops, based on Tukey's HSD at $P < 0.05$ (n = 3). Arcsine root square-transformed was necessarily performed. Vertical bars represent the standard deviations.

3.4. Discussion

A. Field experiment

3.4.1. Variation on biomass and nitrogen input (N_{in}) of cover crops

The rainfall frequency, but not rainfall accumulation, during the preparation periods could be more responsible on biomass production of cover crops. The high rainfall regularity in 2018 led HV to grow poorly, reducing the biomass production (Table 3.2A). This was in contrast with Fraiser et al. (2017), who have showed that rainfall accumulation as 290 mm over precipitation than the historical average caused a 44% reduction on HV biomass. HV is a succulent plant preferring well drained soils during the growth (Mauro et al., 2013). It is assumed that HV plants in the 2017 fields may get plenty periods to absorb the water under rainy days and maximally utilize the water for the growth during dry days. More frequent rainfall in 2018, contrarily, provided insufficient periods for HV to maximize the growth.

Unlike HV, rye preferred more rainfall frequency to double the biomass in 2018 (Table 3.2A). Additionally, rye has natural initial motive to vigorously growth, compared with HV. It is e.g., (1) a 100% germination of rye seeds, while only 50% germination of HV seeds (Lee et al., 2014); (2) biomass of rye roots are 2.67 times larger than HV roots (Lee et al., 2014), thus rye plants can establish to climates more quickly than legume plants (Kuo and Sainju, 1998). These adaptable characteristics of rye allow the plants to survive and grow well even in days with more regular rainfalls, such as that occurred in 2018.

The low germination ability and adaptability of HV plants resulted in smaller ratio of HV biomass than rye biomass in rye+HV plots, although sowing rates of rye and HV were equals (Lawson et al., 2012) as demonstrated in this study. Additionally, legume (white clover) and non-legume (rye-grass) compete with each other when they are cultivated together (Sorensen and Thorup-Kristensen, 2003). In the present study, rye plants vigorously developed the clumps and occupied the plots, which may lead to the space competition with HV plants. Thus, lower ratio of HV in rye+HV plots was yielded. Based on Kuo and Sainju (1998), the 66:34 biomass ratio of rye:HV in 2017 was assigned as a balance ratio, whereas the 96:4 biomass ratio in 2018 was classified as an imbalance ratio.

The N_{in} of the aboveground of cover crops in the fields depended on the cover crop biomass productions. Vigorous growth of HV in 2017 provided great N_{in} (Table 3.2A), because HV plants highly fixed N from the air (Kuo and Sainju, 1998; Sung et al., 2010; Fraiser et al., 2017). Vigorous growth of rye in 2018 may take up more N that is dissolved in water in the soils (Rosecrane et al., 2000), leading to a double N_{in} of rye residue. Vigorous growth of rye, however, inconsistently indicated a high N_{in} as shown in 2018 fields, when rye biomass in rye+HV plots was 20.4% higher than that in 2017, but the N_{in} in both years was similar (Table 3.2A). Fraiser et al. (2017) demonstrated that 44% more rye biomass in rye+HV plots increase 5.24 g m^{-2} N_{in}. Thus, it is suggested that the poor HV biomass significantly controlled the N_{in} in rye+HV plots in 2018.

3.4.2. Effects of cover crops and synthetic N fertilizer on lettuce performances

The HV and rye+HV cover crops supplied sufficient inorganic N, improving lettuce yield and N_{up}. The high N_{in} from HV residue in the fields (Table 3.2A) was rapidly released (Fig.3.2B), compared with the lower N_{in} from rye and rye+HV. The rapid N release may provide abundant concentration of soil inorganic N during lettuce cultivation periods, which remained in a high concentration at the end of the experiments (Table 3.3). The inorganic N was maximally taken up by lettuce plants (Fig.3.1B), thus promoting lettuce yield (Fig.3.1A). This result conforms the results of study of Benincasa et al. (2011) showing that a high N_{in} promote the plants on maximizing the N_{up} ability. Although rye+HV residue had lower N availability than HV, specific N_{up} of lettuce plants under rye+HV treatment was positive (Fig.3.1B). This indicates that HV- and rye+HV-lettuce rotation could be recommended for the cover cropping system with shorten interval periods. Further, HV and rye+HV benefited even in 0N split plots, these cover crops led to an alternative purpose to N fertilizer applications on lettuce cultivation systems.

The positive effects of rye+HV were especially obtained when the biomass ratio was balance in 2017. Kuo and Sainju (1998) conclude that biomass proportion of HV in rye–HV mixture should not be less than 40% to prevent less N_{in} and N_{up} of the subsequent plants. The N_{in} in the 2017 fields was acceptably qualified as the practical step in cover cropping system in the fields.

On the other hand, lettuce cultivated under rye treatments depended on additional synthetic N fertilizer. It was statistically clarified that large lettuce yield depended on high N_{up} as stated by Hoque et al. (2010) and Sapkota et al. (2019) that lettuce N content depends on N_{in} but not on other nutrients, such as P and K. That was why improvement on lettuce yield and N_{up} was achieved in rye-6N (Fig.3.1).

Inappropriate external factors during the experimental periods interrupted lettuce performances as depletion on lettuce yield and N_{up} in 2018 in comparison with that in 2017 (Fig.3.1). The first factor was the scarce rainfall in 2018 that lacked water demand of lettuce plant for inorganic N transportation from the soil (Bottoms et al., 2012). Daily watering with 1.8 L of water per plant, as done in the pots, is necessarily prepared in the fields. The second factor was the 12% less solar radiation in 2018 over the 2017. It may disturb lettuce growth as described by Marrou et al. (2013) demonstrating that 50% of shadowing treatments reduced photosynthesis process, thus reducing lettuce yields up to 19%. The third factor was the soil pH that was below 5.5 in 2018 fields (Table 3.1) that could not be tolerated by lettuce plants. More inappropriately, 6N fertilizer addition significantly decreased the average of soil pH (5.15) over the 0N (5.30), which may cause the severe potential on the yield lost.

In term of lettuce quality, an evaluation in the 2017 fields showed that synthetic N fertilizer could indirectly decline content of anthocyanin and ascorbic acid. Specifically, application of 2.5N fertilizer rate in all cover crop treatments reduced color value a^* compared with rye-0N treatment (Table S3.2). On the contrary, the low biomass of lettuce plants cultivated in rye-0N plots had more red color. It is suggested that the plants consisted more anthocyanin (Yang et al., 2016). Moreover, color value a^* is positively correlated with ascorbic acid (Mampholo et al., 2016), which can reduce coronary heart disease and cancer (Osganian et al., 2003). Thus, the results in this study showed that rye cover cropping suppressed lettuce performance; but the management could improve lettuce quality to benefit human health. A further study focused on the quality of subsequent plants in cover cropping system would add the recommendation on cover crop applications.

3.4.3. Effects of cover crops and synthetic N fertilizer on soil biochemical properties and microbial abundance

Synthetic N fertilizer variously affected microbial activity, biomass, and relative abundance, in which the effects could be singly or in combination with particular cover crop types. The easily decomposable HV residue promoted BG activity (Table 3.3), confirming the previous result of field experiments by Liang et al. (2014) and Mbuthia et al. (2015). The 2.5N fertilizer did not increase BG activity (Table 3.3), stressing the importance of plant residue for microbial enzymatic activity (Mbuthia et al., 2015).

Combination of the 2.5N fertilizer and HV cover crop resulted in a significant promotion on SMB over the control (Table 3.3), indicating that microorganisms in bulk soil require the easily assimilated C and N (Sugihara et al., 2010; Gonzalez-Quiñones et al., 2011; Li et al., 2018) both from HV residues and 2.5N fertilizer. Previous researches demonstrate a negative or nonsignificant effect of N fertilizers (Gonzalez-Quiñones et al., 2011) in several fertilizer application rates (i.e., 34N, 67N, and 101N, Mbuthia et al., 2015) toward SMB. Based on the current study, low amounts of fertilizers (i.e., 2.5N) sensitively triggered SMB within 38 days.

Bacteria and fungi oppositely responded to the 2.5N application rate. The lower bacterial DNA quantity in 2.5N split plots than in 0N split plots (Table 3.4) was opposite to the results of Fraiser et al. (2016); they showed a promotion of bacterial abundance in rye+40N compared with rye+0N at two months after the cover crops termination. They address that the promotion is due to a high availability of soluble N compounds in the rye+40N treated soil. The 2.5N application rate in this study might be insufficient, leading to nutrient competition among the soil bacteria (Finlay and Thorn, 2019). The competition actions are by poisoning and killing with each other (Deveau et al., 2018), so that the DNA quantity was relatively lower in the fertilized soils than that in the unfertilized soils. This result may show a disadvantage of synthetic N fertilizer on maintaining bacterial total number.

On the other hand, fungal DNA quantity was relatively higher in soil with 2.5N and rye+HV residue (Table 3.4), implying the sufficiency of nutrients for fungal

growth. In relationship with residual C:N ratio, fungi prefer residue with high C:N ratio (Brennan and Acosta-Martinez, 2017; Khan et al., 2020). Therefore, fungal DNA is reduced in plots with rye+HV, over the plots with rye (Fraiser et al., 2016). Based on the results in the current study, however, the intermediate C:N ratio of rye+HV and additional 2.5N fertilizer jointly benefited fungal growth up to 38 days.

3.4.4. Cover crops decomposition and N supply

Trends of residual decomposition and N and C mineralizations are determined by residual C:N ratio (Kuo and Sainju 1998; Kuo et al., 2001; Sainju et al., 2001; Lawson et al., 2012) as clarified in the present study. In detail, the lower residual C:N ratio of the cover crops (Table 3.1), the faster rates of residual decomposition, N release and C release (Fig.3.2). In addition, the C and N of each cover crop were released in similar trends. This points out that soil microorganisms synchronize the utilization of both C and N in the transformation process to regulate inorganic N (McDaniel and Grandy, 2016).

Rates of decomposition and C and N mineralization may relate to the fungal DNA quantity. The fiber components of hemicellulose, cellulose, and lignin are specific contents of C and N in plant residues. Simplicity of HV fiber contents (392 g kg^{-1} residue) compared with rye (457 g kg^{-1} residue, Lawson et al., 2012) may stimulate microorganisms in bulk soils with HV to increase the BG activity and SMB. In the result, HV residue and the N were rapidly decomposed and mineralized. The intermediate fiber contents of (418 g kg^{-1} residue) rye+HV residue in 50:50 proportion (Lawson et al., 2012) may concomitantly stimulate fungal growth, which may lead to the intermediate rates of decomposition and N supply.

Scarce rainfall slowed the rates of residual decomposition of all cover crops in the 2018 fields, over the 2017 fields (Table S3.1A), as heavier remained residue was found in 2018 than in 2017 (Fig.3.3A). In consequence, rates of C and N mineralization were also slowed in 2018 (Table S3.1A). Scarce rainfall did not change the soil temperature ranges (i.e., 20–26°C) from the optimal ranges for bulk soil microorganisms to decompose plant residues (i.e. 10–28°C, Conant et al., 2008). Nevertheless, the required volume of water, such as 91 mm accumulation rainfall

in the experimental periods for microbial actions (Sugihara et al., 2010), was not achieved. Thus, the disturbances on microbial enzymatic activity and biomass enlargement may happen during the experiments, although 2018 showed relatively higher BG activity and SMB than 2017 at the end of field experiments (Table 3.3).

B. Pot experiment

3.4.5. Effects of cover crops on lettuce performances

Lettuce performances (i.e., yield and N_{up}) at the mature stage in the pots clarified the results in 0N split plots of the fields. Specifically, HV and rye+HV promoted lettuce production 1.24–2.08 times higher than control. This was in accordance with Caliskan et al. (2014), who showed that HV resulted in 1.6 times greater lettuce cultivar Marco, over the control. HV supplied high concentration of soil inorganic N supply from the beginning of the experiments (Fig.3.4A), which was maximally taken up by lettuce plants until the end of the experiments (Fig.3.3B). The results in rye+HV treatment with 50:50 cover crop biomass ratio in the pots clarified that balance ratio is crucial to gain a positive effect of mixed cover crops on the plant performances.

Similar to the effects of rye in the fields, the low N_{in} from rye residue in the pots (Table 3.2) was also slowly released (Fig.3.6B), thereby insufficiently supply soil inorganic N during lettuce growth period (Fig.3.4A). In consequence, lettuce N_{up} and yield in rye were as low as that in control (Fig.3.3).

Allelopathic-like effect from HV was suggested to reduce lettuce performance at the mid growth stage of plant. Phenolic substances that cause allelopathic effects on subsequent plants are released during the active residual decomposition of HV in 10 DAI (Sung et al., 2010). The substances harm lettuce plants by suppressing lettuce root growth up to 80% (Soltys et al., 2012), which was weighed smaller in HV (2.18 g $plant^{-1}$) than in control (3.85 g $plant^{-1}$) in the current study.

Toxicity of ammonium caused by a high range of ammonium concentration (45.1–180 mg NH_4^+-N L^{-1} water) in hydroponic medium for lettuce suppressed lettuce shoot development, but do not interfere root growth (Andriolo et al., 2006). In the pots in the current study, range of ammonium concentration in HV-treated

soil was 15.9–16.7 mg NH_4^+-N kg^{-1} soil at 5–25 DAI in 2017. This low range may not toxic lettuce plants as concluded by Savvas et al. (2006); they show that lower range of ammonium concentration (21.1–63.3 mg NH_4^+-N L^{-1} water) in lettuce hydroponic system promoted shoot and root growth. This stresses the assumption that HV allelopathic effect was the causal factor of lettuce root growth suppression in HV-treated soil up to 25 DAI in 2017 pot experiments. Due to the lack of evaluation on allelochemical concentration in the soils, the assumption is marked as HV allelopathic-like effect.

However, the allelopathic-like effect phenomenon from HV was absent at lettuce mid growth stage in the 2018 pots, when HV-treated soil in 2018 had 2.75% lower average of soil TC content than that in 2017 ($P = 0.0090$, Fig.S3.1). Soil TC content is positively correlated with total phenolic released from cover crop residues (Gallet and Keller, 1999), due to more active residual decomposition and C mineralization. In the current study, HV residue and the C was decomposed and mineralized slower in 2018 than in 2017 (Table S3.1B), suggesting to a lower phenolic concentration in the 2018 soil than in 2017 soil with HV residue.

The disadvantage of HV residue disappeared within 25–38 DAI, resulting in promotion of lettuce performance at the final harvest time (Fig.3.3). This was in accordance with Sung et al. (2010), who showed that allelopathic effect ceased after 30 DAI. The classic concern of allelopathic effect phenomenon from HV cover crop has been recognized in several vegetable plants, e.g., common chickweed, redroot pigweed, wild carrot, tomato, corn, and cucumber (Hill et al., 2007). Therefore, the allelopathic-like effects in lettuce plants observed at the mid growth stage in this study may happen in other vegetable plants. However, recovery phenomenon at the mature stage led to the recommendation of shorten the interval periods.

3.4.6. Significance of BG activity and SMB to N supply from cover crops

The microbial BG activity was activated more by the easily decomposable HV rather than rye and rye+HV. The activation by HV consistently occurred at several timings (i.e., 10, 25, and 38 DAI, Fig.3.4B). Neither rye+HV nor rye residues altered the BG activity constantly. However, the enhancements in rye and rye+HV

in 2017 pots indicated potential of those cover crops on maintaining this microbial enzymatic response.

BG activity was in higher range in 2017 than in 2018 (Fig.3.5B), indicating a higher microbial contribution to decomposition and mineralization. Soils with 9.08% of initial TN facilitate N mineralization from clover leaves twice faster than soils with 2.56% of initial TN (Frøseth et al., 2015). A higher initial soil TN and TC contents in 2017 soil (Table 3.1) may cause a higher BG activity range in 2017 over the 2018 (Fig.3.5B). In consequence, higher rates of residual decomposition and C mineralization of all cover crops were found in 2017 than that in 2018 (Table S3.1B). It is also suggested that 2017 soils may include more decomposers that produce BG in comparison with 2018 soils.

Similar to BG activity, SMB was increased by HV compared with control, especially at 25 DAI (Fig.3.4C), when HV residual decomposition and N mineralization achieved in plateau shapes (Fig.3.6B). It is assumed that the living C sink (Sugihara et al., 2010; Gonzalez-Quiñones et al., 2011; Li et al., 2018) in the current study was fully filled after HV residues were nearly completely decomposed. In addition, rye+HV residue potentially maintained microbial biomass by promoting SMB at 38 DAI in 2017 (Fig.3.4C-1).

BG activity and SMB may play role in N supply from cover crop residues, marked as the sequential peaks of BG activity at 10 DAI (Fig.3.4B-2) and SMB at 25 DAI (Fig.3.4C-2) in 2018. Li et al. (2014) found similar sequential peaks at the midterm of wood litter decomposition in the microcosm experiment. The sequential peaks imply that BG-producing microorganisms in bulk soils were in prior actions before they expressed a high SMB, as have been detailed in sub-section 2.2.3.1 and 2.2.3.2 in Chapter 2. Li et al. (2014) also suggest that a highly positive correlation exists between the two microbial activities, as shown in the current study (Fig.S3.2). Further, BG activity and SMB were positively correlated, yet not significant, with soil inorganic N in the two experimental years. This amplify the assumption of microbial roles in N supply from cover crops.

N immobilization as high SMB occurred in rye-treated soil, resulting in low amount of N supply. Increasing in N-based SMB in cover crop-treated soils implies the N immobilization, a condition that microorganisms keep the N for themselves (Nannipieri et al., 1990). The N-based SMB was not evaluated in the current study, but trends of C-based SMB in rye in 2017 were progressive over the time (Fig.3.4C-1), pointing out the N immobilization. Negative correlations between SMB and rates of N and C mineralization at 5 DAI in both experimental years were found (Fig.S3.2), amplifying the assumption of potential of N immobilization in rye-treated soil. This was according to the microbial concomitant demand of C and N in the process of N mineralization (Kuzyakov and Blagodatskaya, 2015; McDaniel and Grandy, 2016). Moreover, net N mineralization from rye residue at 5 DAI in the two experimental years was in negative values (Table S3.3), which clearly show the N immobilization (Rosecrance et al., 2000) and may happen during the decomposition periods in the current study.

3.4.7. Significance of microbial abundance to N supply from cover crops

Bacteria and fungi in bulk soil responded all cover crops input by dynamically expressed their DNA quantity at the early periods after the incorporation. This implies no preference of total microbes on the cover crops with various C:N ratio. The immediate microbial responses were related to the abundant amount of labile nutrients released from the beginning terms after residue introduction (Koranda et al., 2014). Timings of DNA peaks in the current study were similar to that in the study of Drost et al. (2020) demonstrating the peaks of bacteria 16S rRNA copy number and fungi biomass at 3 DAI in HV-treated soil and at 7 or 12 DAI in oat- and vetch-oat-radish mix-treated soils, respectively.

The peaks of bacterial and fungal DNA up to 10 DAI (Fig.3.5), may relate to N supply from cover crop residues. The N was actively released up to 15 DAI, before achieving in plateau shapes (Fig.3.6B). These results may show that bacteria and fungi actively grew (Drost et al., 2020) along with decomposing all cover crop residues and mineralizing the N. Furthermore, SMB was promoted following microbial growth promotion. It may imply that growth of soil microorganisms in total number and biomass was the continuous responses to cover crops input.

Low initial soil TC and TN content and inorganic N concentration in 2018 (Table 3.1) resulted in higher range of bacterial and fungal abundances (0.07–8.42 ng DNA) than the range in 2017 (0.50–4.58 ng DNA, Fig.3.5) in the cover crop-treated soils. This was because microbial niches, such as bulk soils, with growth-limiting resources can promote soil microbes to more effectively utilize available resources (Dini-Andreote and van Elsas, 2019). The over expressed of microbial abundance in 2018 did not lead to faster, but similar, N mineralization rates in 2018 compared with that in 2017 (Table S3.1B).

3.5. Conclusion

The two years of pot and field studies confirmed that HV – leguminous cover crop – without synthetic N fertilizer addition could maintain soil fertility, i.e., adequate concentration of soil inorganic N. Specifically, the pure HV and mixed (rye+HV) application methods with 0N fertilizers resulted in the high and intermediate N supplies, respectively, which improved lettuce yield and N_{up}. Conversely, the meager N traits of rye – gramineous cover crop – may require additional fertilizer more than 6N application rate in lettuce cultivation.

In the inorganic N supply from the cover crops, BG activity, SMB, and microbial abundance may play important roles. Specifically, easily decomposable HV residues sequentially promoted BG activity and SMB, which may be the pathway of the N mineralization. A higher range of BG activity in 2017 soil may relate to the higher rates of residual decomposition in 2017 than that in 2018. Further, all cover crops in the pots and rye+HV in the fields promoted bacterial and fungal DNA quantities, which may be related to the inorganic N supply.

Supplementary Material

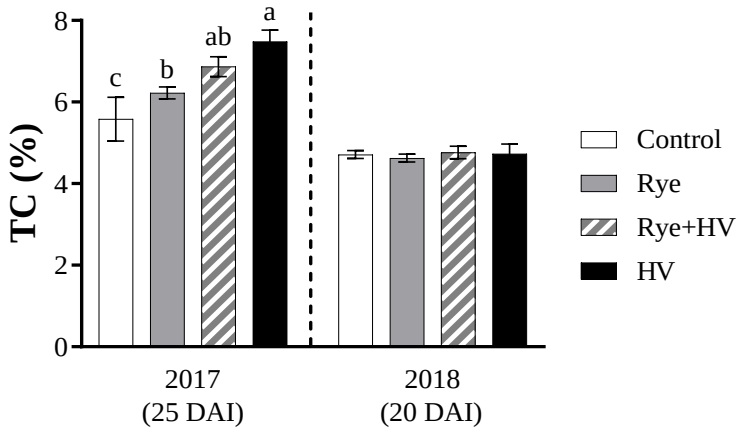


Fig.S3.1. Total carbon (TC) in cover crop-treated soils at lettuce mid growth stage in 2017 and 2018 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Different letters indicate the significance based on Tukey's HSD at $P < 0.05$ (n = 3) in the same year of experiment. Vertical bars show standard deviation. DAI abbreviates days after incorporation of cover crops.

Table S3.1. *P*-value based on t-test of the rates of residual decomposition, nitrogen release, and carbon release between 2017 and 2018 up to 35 days after cover crops incorporation in the (A) field and (B) pot experiments

(A)	*P* -value		
Cover crop	Residual decomposition	N release	C release
Rye	0.0017	0.1700	0.1700
Rye+HV	0.0076	0.0507	0.0039
HV	0.0767	0.0955	0.0900
All	0.0162	0.0289	0.0004

(B)	*P* -value		
Cover crop	Residual decomposition	N release	C release
Rye	0.0248	0.4895	0.5233
Rye+HV	0.0187	0.8505	0.1333
HV	0.0003	0.0994	0.0178
All	2.2×10^{-5}	0.1662	0.0081

The *P*-values at < 0.05 are significant values. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The N and C abbreviate nitrogen and carbon, respectively.

Table S3.2. Redness values of lettuce plants at the mature stage in the fields in 2017

Treatment (T)	Fertilizer (F)	*a**
Control	0N	2.67 ± 1.38 ab
Rye		4.28 ± 1.38 a
Rye+HV		1.87 ± 2.07 ab
HV		0.20 ± 0.35 b
Control	2.5N, 6N	1.96 ± 0.39 ab
Rye		1.29 ± 1.19 b
Rye+HV		0.65 ± 1.13 b
HV		0.40 ± 0.69 b
Anova	T	NS
	F	**
	T × F	NS

Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Sub-treatments were the application rates of synthetic fertilizer at 0 and 2.5 g N kg^{-1} soil. Each mean ± standard deviation is followed by different letter, indicating the significance at $P < 0.05$ based on Tukey's HSD (n = 3) using the split-plot ANOVA.

Table S3.3. Estimation of nitrogen (N) mineralization for different cover crop components, based on the concentration of soil inorganic N at 5 days after cover crops incorporation in the pot experiments

Treatment	Net N mineralization (mg N kg^{-1} soil d^{-1})		Calculated net N mineralization for cover crop component (mg N kg^{-1} soil d^{-1})	
	2017	2018	2017	2018
Control	19.6 ± 3.15 bc	11.0 ± 0.17 b		
Rye	14.9 ± 2.93 c	9.77 ± 0.39 b	−4.68 ± 4.97 b	−1.19 ± 0.56 b
Rye+HV	26.0 ± 3.68 ab	16.5 ± 4.38 b	6.44 ± 1.66 a	5.53 ± 4.33 b
HV	31.9 ± 1.08 a	31.2 ± 3.36 a	12.3 ± 3.11 a	20.2 ± 3.22 a

Calculated net nitrogen (N) mineralization for cover crop component was estimated by subtracting mineralization rate of soil N in control. Negative values indicate N immobilization (Rosecrance et al., 2000). Treatments were soil without any cover crops (control) and soils with rye, hairy vetch (HV), mix of rye and HV (rye+HV) cover crops. Mean ± standard deviation is followed by a letter. Different letters indicate significant difference based on Tukey's HSD at $P < 0.05$ (n = 3).

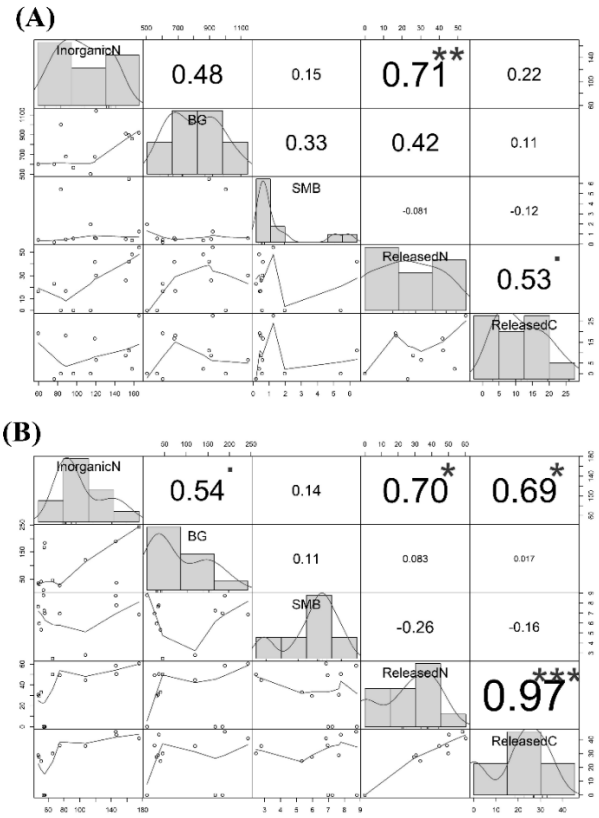


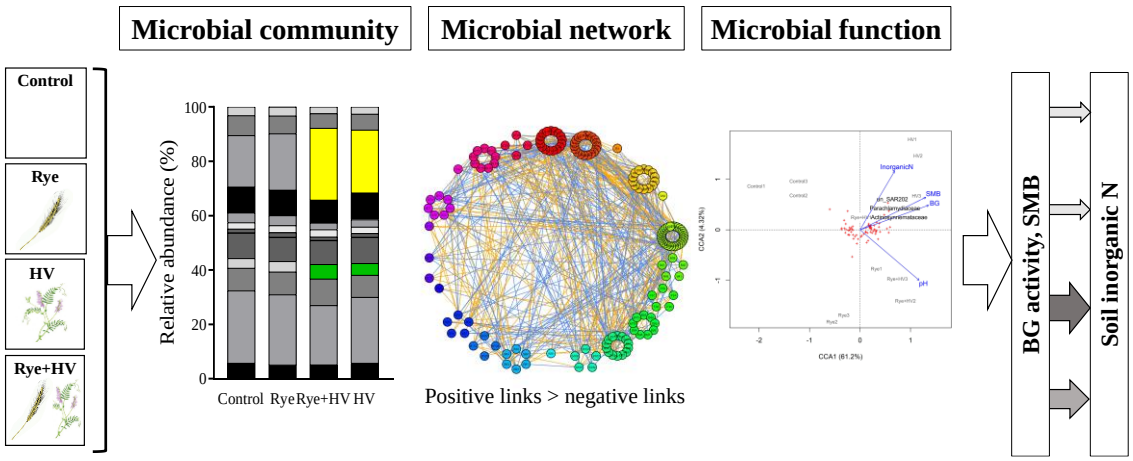
Fig.S3.2. Correlation within soil biochemical properties at 5 days after cover crops incorporation in (A) 2017 and (B) 2018 pot experiments. Data were raw data of 3 replicates of all treatments: control, rye, hairy vetch (HV), and mix of rye and HV (rye+HV). The values are the correlation coefficient (R^2). The asterisks (*, **, ***) denotes the 0.05, 0.01, and 0.001 significance, respectively, based on the Pearson's correlation test.

CHAPTER 4

Properties of bulk soil microorganisms and the roles in nitrogen supply from cover crops during residual the decomposition periods

This chapter is rewritten based on the parts of manuscript that has been submitted to Applied Soil Ecology.

Graphical abstract and highlight



- Hairy vetch (HV) and rye+HV boosted abundance of some bacteria and fungi decomposers.
- All cover crops produced positively-linked microbe–microbe correlation networks.
- Some influenced microbial taxa were correlated with the nitrogen (N) mineralization indicators (activity of β-glucosidase enzyme, soil microbial biomass, soil inorganic N, and soil pH).

4.1. Research gap and potential solution

In Chapter 3, rye, hairy vetch (HV), and mixed (rye+HV) cover crops similarly enhanced bacterial and fungal gene abundance in the early periods after the incorporation. Thus, copiotrophs that are decomposers (Carrera et al., 2007) may be more pronounced in cover crop-treated soil than in control, the untreated soil. However, abundance of microorganism decomposers is possibly increased, decreased, or not changed after leguminous and gramineous cover crop inputs (Maul et al., 2014; Detheridge et al., 2016;

Brennan and Acosta-Martinez, 2017; Zheng et al., 2018; Schmidt et al., 2019). Therefore, it is required to evaluate how rye, HV, and rye+HV cover crops alter microbial community.

Networks within and between bacteria and fungi during decomposition and N mineralization from cover crops are still unknown, whereas the results can unlock one part of understanding about the roles of bulk soil microorganisms in N supply (Deveau et al., 2018). Therefore, it is crucial to evaluate microbial communication types (i.e., positive or negative). A network analysis in basis of correlation test is simple, but the results can emphasize the fast or slow, respectively, decomposition and mineralization rates of cover crops (Zheng et al., 2018; Finlay and Thorn, 2019).

Alongside with general contributions of bacteria and fungi to plant residual decomposition (Langille et al., 2013; Nguyen et al., 2016; Detheridge et al., 2016; Schmidt et al., 2019), specific contributions of them are also essential to be understood. It is especially following the results in Chapter 3 that dynamics of β-glucosidase enzyme (BG) activity and soil microbial biomass (SMB) may relate to the N mineralization. The specific roles can be assessed by correlating relative abundance of influenced microbial taxa and values of BG activity and SMB using the multivariate analysis (Fernandez et al., 2016a; Drost et al., 2020; Khan et al., 2020). Further, some of influenced microbial taxa may also directly contribute to N supply by correlating with soil N (Fernandez et al., 2016a). Therefore, multivariate analysis can be adapted in this study by recruiting the evaluated soil biochemical properties, i.e., BG activity, SMB, soil inorganic N, and soil pH, as the environmental factors.

The general rates of residual decomposition were faster in 2017 soil than in 2018 soil, whereas rates of C release were in opposite result (Chapter 3). These facts lead to the question of whether microbial community composition, microbial networks, and specific microbial roles matter for the functions of decomposition and N supply under different cover crops amendment. The hypotheses were that (1) abundance of decomposers and positive communication types in 2017 were more pronounced in soils with HV than in soils with rye and rye+HV due to the easily decomposable residue of HV; and (2) each cover crop shows each specific microbial function related to the N supply.

4.2. Materials and Methods

4.2.1. Treatments and design

The experiment was performed in pot level in 2017 and 2018 as mentioned detail in sub-section 3.2.1.2 and 3.2.2.2 in Chapter 3. The treatments were soils without any cover crops (control) and soils with HV, rye, and mixed (rye+HV) cover crops. The experiments were designed in randomized block with three replicates.

4.2.2. Soil sampling, DNA extraction, and DNA purification

Bulk soil samples were those collected at the middle of residual decomposition periods: 25 days after incorporation (DAI) in 2017 and 20 DAI in 2018. The timings were also when lettuce plants were harvested for the first time. For detail of soil sampling methods from pots, please refer to sub-section 3.2.4.2 in Chapter 3. Methods of DNA extraction and purification have been described in sub-section 3.2.6.1 in Chapter 3.

4.2.3. Library preparation and sequencing of 16S rRNA and ITS

The same primers targeting bacteria and fungi had been used in the qPCR (sub-section 3.2.6.2 in Chapter 3) were used to amplify soil DNA. The 16S rRNA libraries were prepared by performing the PCR, wherein the reaction was 10 µL AmpliTaq Gold 360 Master Mix (AB Applied Biosystems, CA, USA), 0.4 µL of each primer, 1–3 µL template DNA, and 6.2–8.2 µL nuclease-free water. The first amplification setting was initial denaturation at 95°C for 10 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The amplicons were purified with AMPure XP reagent. The second amplification was done using the same set of primers attached to barcodes and an adaptor in identical procedure with the first amplification with 5 cycles in annealing step.

In ITS library preparation, the PCR reaction was 10 µL AmpliTaq Gold 360 Master Mix, 0.8 µL of each primer, 1–3 µL template DNA, and 5.2–7.4 µL nuclease-free water. The first amplification setting was based on Brown et al. (2013): initial denaturation at 94°C for 4 min; 31 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The amplicons were purified with AMPure XP reagent. The second amplification was done using the

same set of primers attached to barcodes and an adaptor in identical procedure with the first amplification with 10 cycles in annealing step.

Barcoded amplicons were purified with AMPure XP reagent. Concentration of 16S rRNA and ITS libraries was measured using Qubit dsDNA HS Assay Kit and confirmed using Bioanalyzer High Sensitivity DNA kit (Agilent Technologies Inc.). All libraries were diluted with TE buffer to achieve 50 pM concentration and loaded onto an Ion 318 chip (Ion Torrent Life Technologies, CA, USA) using an Ion PGM Hi-Q Chef kit. The chips were then sequenced with the Ion Torrent PGM system.

4.2.4. Processing data of sequence

Sequence data were obtained from the Metagenomics system of Ion Torrent. Sequence reads were depleted of barcodes and primers and filtered through a quality threshold using the Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010). Low-quality reads (quality score < 19) were discarded. The average of the high-quality reads for 16S rRNA and ITS were 92748 and 9361, respectively, in 2017 and 15367 and 6588, respectively, in 2018. Samples of 16S rRNA and ITS were then respectively rarefied based on the lowest number of qualified reads in 2017 (63852 and 4701) and 2018 (10268 and 1675). The qualified and rarefied reads were then systematically aligned for bacterial and fungal community structure by performing the α and β diversity analysis at phylum and family levels using QIIME pipeline. The 16S rRNA alignment was based on the GreenGenes v13_5 (accessed April 3, 2019; ftp://ftp.microbio.me/greengenes_release/). The ITS alignment was based on the UNITE QIIME (accessed April 3, 2019; UNITE Community (2019) version 18.11.2018; https://doi.org/10.15156/BIO/786334). The identified phylum and family were defined by clustering at 97% similarity. Kingdom archaea and unassigned taxa among the bacterial sequences and unassigned taxa among the fungal sequences were excluded before further analysis.

Microbial richness was number of identified bacterial and fungal phyla and families. Microbial diversity was evaluated with the Shannon index at phylum and family levels based on the α-diversity of taxon abundance tables using the following equation:

Shannon index (H) = $-\sum P_i \times \ln P_i$

The proportion of species *i* relative to the total number of species (P_i) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln P_i$). The resulting product is summed across species; and multiplied by −1.

4.2.5. Correlation network analysis

Correlation network was determined for each treatment in each year between bacteria–bacteria (B–B), fungi–fungi (F–F), and bacteria–fungi (B–F) at family level. First, families with average relative abundance higher than 0.1% were selected and subjected to the Spearman's correlation test. Network analysis was performed using 'igraph' package in R. Only negative and positive links were considered based on strength of correlation at values less than or equal to −0.99 or greater than or equal to 0.99 and *P*-value at < 0.05.

4.2.6. Functional analysis

Influenced taxa of bacteria and fungi were selected by subjected all identified families to the one-way ANOVA followed by Tukey's HSD at $P < 0.05$. Based on the statistical difference of soil biochemical properties at the middle of decomposition in each year (Table S4.1), multivariate analyses were focused on BG activity and SMB in 2017 and soil inorganic N and pH in 2018. A canonical correspondence analysis (CCA) was run as the multivariate analysis with influenced taxa as the community factors and soil biochemical properties as the environmental factors. The analysis was done using the 'vegan' package in R (Oksanen et al., 2017).

As an additional, general potential functions of bacteria and fungi were predicted. Identified bacterial families were online analyzed using the PICRUSt software (Langille et al., 2013), in which species functions were aligned with 97% similarity and normalized with the KEGG orthology values at level 3 references. The reads in each sample were compared with total reads to obtain sequence proportions expressed in percent. Bacterial functions categorized under "Metabolism" were selected. The category may relate to residual decomposition by explaining nutrient cycling-related activity (KEGG pathway database; https://www.genome.jp/kegg/pathway.html).

Identified fungal families were online analyzed using the FUNGuild method following the instructions at www.stbates.org/guilds/app.php (Nguyen et al., 2016). Fungal functions (i.e., guilds) of two trophic modes (i.e., saprotroph and pathotroph) that may relate to residual decomposition were selected.

4.2.7. Estimation of N budget

The N budget was estimated based on the simple concept of Biological Input:Output (BIO) budget, in which N input equals to N output (Watson and Atkinson, 1999). The calculation was modified for the pot-based experiments with cover crops input at 25 DAI in 2017, following the calculation method in cover crop-pepper rotation (Lee et al., 2014). Factors included on the estimation are compactly described in Table 4.1.

Table 4.1. The input and output factors used in estimation of nitrogen (N) budget

Inputs and outputs in N balance			Data sources and explanation
Inputs	1) N from cover crop residues	N_{in}	Based on % mineralized N at 25 DAI in 2017 (Fig.3.6B-1, Chapter 3)
Output	2) N uptake by lettuce plant	N_{up}	General N_{up} at the mid growth stage in 2017
Store/Loss	3) Remained/loss N	N_{rmd}/N_{lo}	$N_{lo} = N_{in} - N_{up}$

4.2.8. Quantifying bacterial N cycle-related gene abundance

This assessment was subjective to bulk soils collected from the pots at 25 DAI in 2017, so that the results can be linked to the results of N budget. Methods in preparation of DNA template, standard series, reaction mixture, and gene abundance measurement and calculation have been detail described in sub-section 3.2.6.2 in Chapter 3. Specific primer sets were used to target the specific genes, which thermal cycle condition was adjusted for each primer in the Mx3005P qPCR system (Table 4.2).

The PCR efficiency and correlation coefficient (R^2) of the standard curves were considered: *nifH* (> 53.1% and > 0.995); *chiA* (> 98.4% and > 0.910); AOB 16S rRNA (> 84.2% and > 0.998); *nirS* (> 81.0% and > 0.983).

Table 4.2. Sets of specific primers targeting the bacterial N cycle-related genes

Primer name	Oligonucleotide sequence (5' - 3')	Target gene	Thermal cycle condition	Reference
Po1F Po1R	TGCGAYCCSAARGCBGAC TC ATSGCCATCATYTCRCCGGA	N fixation (*nifH*)	55°C, 43 cycles	Poly et al., 2001
chiA-F chiA-R	GATATCGACTGGGAGTTCCC CATAGAAGTCGTAGGTCATC	Decomposition (*chiA*)	58°C, 38 cycles	Ramaiah et al., 2000
AOB1149f AOB1295r	CTTTA(A/G)TGAGACTGCCGGTGA GCGCTTTCTGAGATTAGCTCC	Nitrification (AOB 16S rRNA)	60°C, 35 cycles	Layton et al., 2005
nirS1F nirS3R	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T GCCGCCGTC(A/G)TG(A/C/G)AGGAA	Denitrification (*nirS*)	72°C, 45 cycles	Braker et al., 1998

The primer set includes the forward (F or f) and reverse (R or r).

The families with *nifH*, *chiA*, AOB 16S rRNA, and *nirS* genes was selected from the identified bacteria families produced from the sub-section 4.2.4. The selection was based on the ARB database format (Heller et al., 2014) for *nifH* gene and EMBL nucleotide sequence database (https://www.ebi.ac.uk/ena/browser/) for *chiA*, AOB 16S rRNA, and *nirS* genes. They are listed in Table S4.2. Relative abundances of the families in the same phylum or class were sum and presented.

4.2.9. Statistical data analyses

All statistical analyses were performed in R and GraphPad Prism. One-way ANOVA followed by Tukey's HSD test at $P < 0.05$ were used to differentiate the cover crop effects on microbial richness, Shannon index, relative abundances of bacteria and fungi at phylum and family levels, and sum of relative abundance of microbial taxa with specific genes. The arcsine transformation was necessarily applied to bacterial and fungal relative abundance before the ANOVA, when normal distribution was not achieved based on the Shapiro-Wilk test. Similarity of microbial communities under different treatments (β-diversity) was tested with NMDS based on Bray-Curtis distances in permutational multivariate ANOVA (perMANOVA) using 'adonis' function in 'vegan' package in R (Oksanen et al., 2017). The t-test at $P < 0.05$ was necessarily performed.

4.3. Results

4.3.1. Microbial richness and diversity

Bacterial richness and diversity were similar among the treatments at both phylum and family levels in both years (Table 4.3A). Fungal richness at phylum level and fungal diversity at family level in 2018 was promoted in rye+HV compared with rye and control, respectively (Table 4.3B).

4.3.2. Microbial community structures and the influenced taxa

Bacterial and fungal community structures in 2017 and 2018, respectively, were dissimilar among the treatments at phylum and family levels according to the perMANOVA (Table 4.3). The dissimilarities in bacterial community in 2017 were shown as the significantly increase in relative abundance of phylum Bacteroidetes in HV (3.99%) and rye+HV (4.25%), over the control (2.63%). Relative abundance of family *Chitophagaceae*, belonging to Bacteroidetes, was increased in HV and rye+HV, over the control in 2017 (Fig.4.1A-1). Further, relative abundance of class Gammaproteobacteria was increased in HV (5.64%) and rye+HV (6.19%), over the control (3.96%) in 2017. Relative abundance of family *Sinobacteraceae*, belonging to Gammaproteobacteria, was promoted in HV and rye+HV, over the control (Fig.4.1A-1).

The dissimilarities in the fungal community in 2018 were shown as the significantly increase in relative abundance of phylum Basidiomycota in HV (18.4%) and rye+HV (27.1%), over the control (4.16%). Moreover, relative abundance of Basidiomycota was higher in rye+HV than in rye (4.17%). Relative abundance of family *Leucosporidiaceae*, belonging to Basidiomycota, was increased in HV, over the control in 2018 (Fig.4.1B-2).

The 2017 fungal and 2018 bacterial community structures at two taxonomic levels were similar among the treatments (Table 4.3). However, relative abundances of some families were statistically influenced (Fig.4.1A-2 and Fig.4.1B-1).

Table 4.3. Microbial richness, Shannon index, and differentiation of community structures of (A) bacteria and (B) fungi at phylum and family levels in bulk soils of the pot experiments.

(A)	Treatment	Richness		Shannon index		PerMANOVA	
		Phylum	Family	Phylum	Family	Phylum	Family
2017	Control	41.0 ± 2.00	391 ± 12.2	2.15 ± 0.00	4.33 ± 0.05	0.005	0.001
	Rye	39.7 ± 0.67	389 ± 7.75	2.13 ± 0.01	4.41 ± 0.03		
	Rye+HV	42.0 ± 0.58	384 ± 1.86	2.14 ± 0.01	4.41 ± 0.01		
	HV	42.3 ± 1.86	408 ± 22.1	2.12 ± 0.01	4.40 ± 0.07		
2018	Control	30.7 ± 1.76	275 ± 7.37	2.71 ± 0.02	4.38 ± 0.06	0.461	0.432
	Rye	35.0 ± 4.51	293 ± 36.9	2.86 ± 0.09	4.42 ± 0.12		
	Rye+HV	31.3 ± 1.86	273 ± 14.5	2.79 ± 0.07	4.36 ± 0.06		
	HV	35.7 ± 2.19	294 ± 17.5	2.81 ± 0.10	4.45 ± 0.05		

(B)	Treatment	Richness		Shannon index		PerMANOVA	
		Phylum	Family	Phylum	Family	Phylum	Family
2017	Control	6.67 ± 0.67	45.0 ± 5.69	0.64 ± 0.06	2.08 ± 0.36	0.582	0.371
	Rye	5.00 ± 1.00	30.3 ± 3.71	0.66 ± 0.10	1.63 ± 0.10		
	Rye+HV	5.00 ± 0.58	27.3 ± 0.67	0.57 ± 0.15	1.52 ± 0.19		
	HV	4.67 ± 0.67	44.3 ± 15.2	0.69 ± 0.04	1.88 ± 0.34		
2018	Control	4.33 ± 1.20 ab	18.3 ± 7.88	0.64 ± 0.23	1.28 ± 0.18 b	0.022	0.006
	Rye	3.33 ± 0.33 b	11.3 ± 2.85	0.50 ± 0.15	1.48 ± 0.29 ab		
	Rye+HV	7.67 ± 0.88 a	45.3 ± 13.3	1.02 ± 0.15	2.38 ± 0.19 a		
	HV	4.33 ± 0.33 ab	13.3 ± 2.33	1.08 ± 0.06	2.00 ± 0.26 ab		

Treatments were soil without any cover crops (control) and soils with rye, mix of rye and hairy vetch (rye+HV), and hairy vetch (HV) cover crops. Data were obtained at 25 and 20 days after cover crop incorporation in 2017 and 2018, each. The mean ± standard error in each parameter is followed by different letters showing significance among the treatments based on Tukey's HSD at $P < 0.05$ (n = 3). The *P*-value of permutational ANOVA (perMANOVA) at < 0.05 means dissimilarity of microbial community structure among the treatments.

Fig.4.1. Heatmaps showing relative abundance of influenced taxa of (A) bacteria and (B) fungi in bulk soils at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops.

4.3.3. Correlation networks within and between bacteria and fungi

Overall, trends of link number in B–B, F–F, and B–F networks in all treatments were positive links > negative links in both years (Fig.4.2A–C). The range of link percentage per total links was more fluctuated in F–F networks, which was 1.6%–11% in 2017 and 0.5%–21% in 2018.

Total links in 2017 and 2018 were 4889 and 5081, respectively (Fig.4.2D). Negative links in F–F and B–F networks in soil with rye+HV residue in 2018 were over-expressed compared with that in 2017.

4.3.6. Correlation between specific influenced taxa and soil biochemical properties

BG activity and SMB in HV were significantly and positively correlated with the unidentified bacteria of class SAR202 and family *Parachlamydiaceae* in 2017, based on the CCA1 axis (Fig.4.3A-1). Average of relative abundance of *Parachlamydiaceae* and unidentified SAR202 was less than 0.01% across the treatments. Fig.4.3B-1 shows that none of influenced fungal taxa in 2017 were significantly correlated with BG activity either with SMB (). In 2018, families *Alteromonadaceae* in HV and rye+HV (Fig.4.3A-2) and *Leucosporidiaceae* in HV (Fig.4.3B-2) were positively correlated with soil inorganic N, whereas negatively correlated with soil pH.

As the additional results, general functions of bacteria and fungi were shown as Table S4.5. Bacterial function of “Metabolism of other amino acids” was significantly higher in rye and rye+HV compared with that in control in 2017. In addition, bacterial function of “Metabolism” was higher in rye+HV than in rye. In 2018, plant pathogen guild was increased in HV, over the other treatments. Contrarily, plant saprotroph guild was decreased in cover crop-treated soils, over the control.

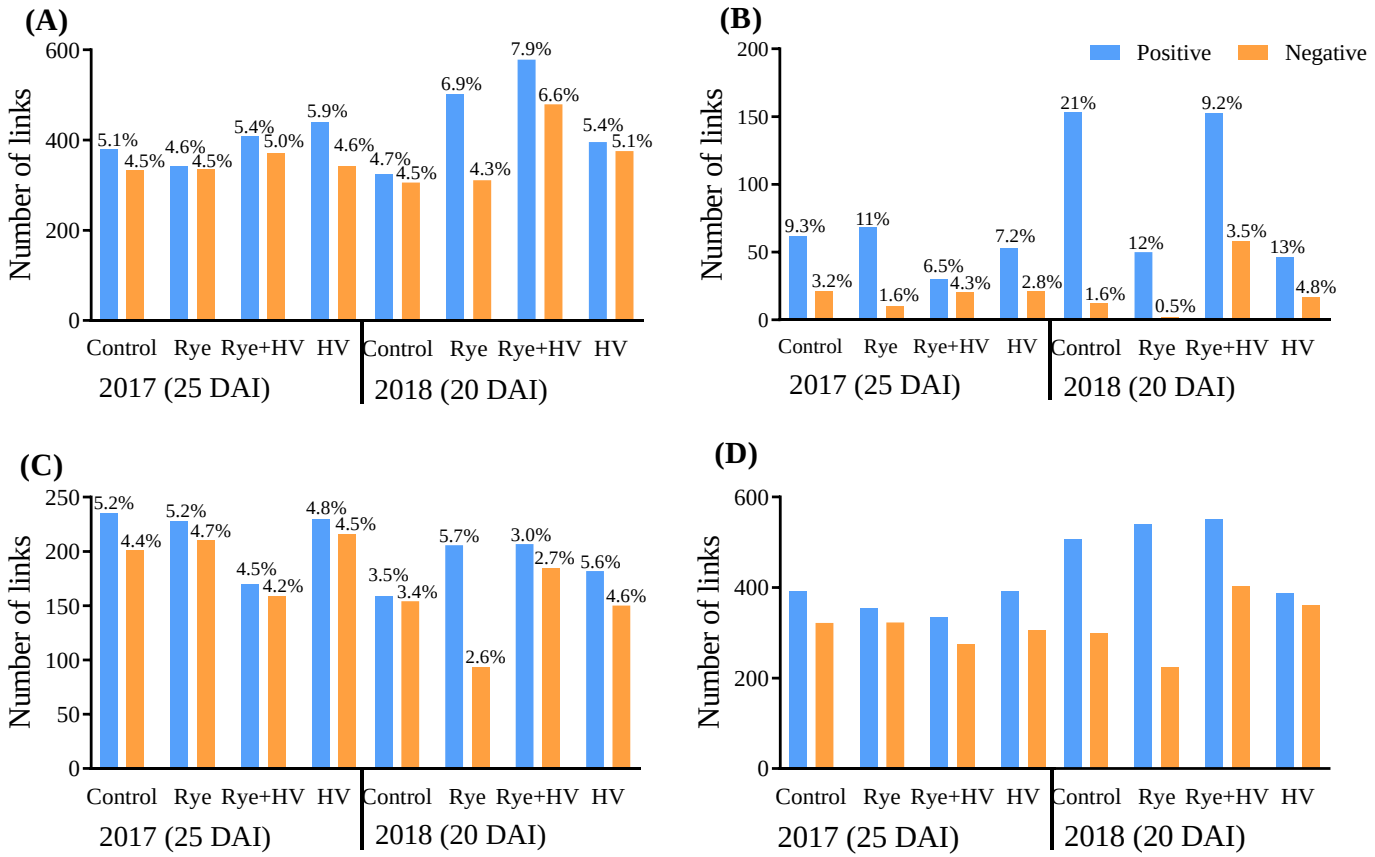


Fig.4.2. Number of links in the network (A) within bacteria (B–B), (B) within fungi (F–F), (C) between bacteria and fungi (B–F), and (D) total significant positive and negative links in bulk soils in 2017 and 2018 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Percent in each bar shows proportion of link number per total links in each treatment. DAI abbreviates days after incorporation.

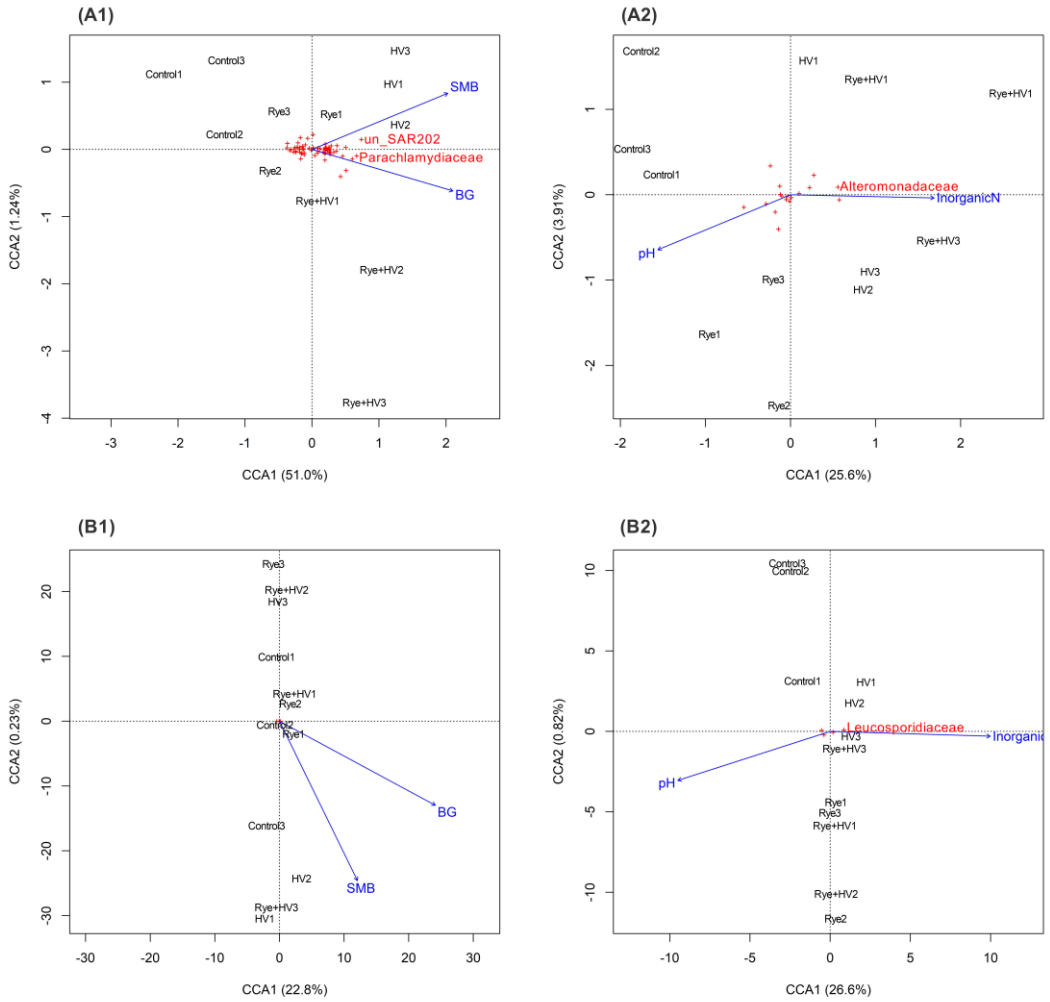


Fig.4.3. Canonical correspondence analysis (CCA) showing the correlations between influenced taxa of (A) bacteria and (B) fungi and soil biochemical properties at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively. Treatments were soils without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Numbers following treatments names represent the replicate. Soil biochemical properties were soil inorganic nitrogen (InorganicN), pH, β-glucosidase enzyme (BG) activity, and soil microbial biomass (SMB). Taxonomic names are only shown for taxa that had significant correlations, while all others appear as plus signs.

4.3.7. N budget and abundance of bacteria with N cycle-related genes

The zero input in control soil and 0.14 g pot^{-1} N_{in} in rye-treated soil resulted in negative values of N_{rmd}/N_{lo} due to the 0.17 and 0.25 g N_{up} per pot in control and rye, respectively (Fig.4.4). HV-treated soil had the highest N_{rmd}/N_{lo} due to the high N_{in} but low N_{up}. Soil with rye+HV residue showed N balance between N_{up} and N_{rmd}/N_{lo}.

Significant difference was shown only in gene abundance of *nirS*, in which HV superiorly promoted the gene abundance, over the rye and rye+HV (Fig.4.5).

Sum of relative abundances of bacteria with specific N cycle-related genes in their phylum or class was different among the treatments (Fig.4.6). Specifically, relative abundance of Proteobacteria with *nifH* (Fig.4.6A) and *nirS* genes (Fig.4.6D) was higher in rye+HV than in control. Relative abundance of Bacteroidetes with *chiA* (Fig.4.6B) and *nirS* (Fig.4.6D) genes was higher in HV than control and rye. In addition, HV increased relative abundance of Betaproteobacteria with AOB 16S rRNA gene (Fig.4.6C).

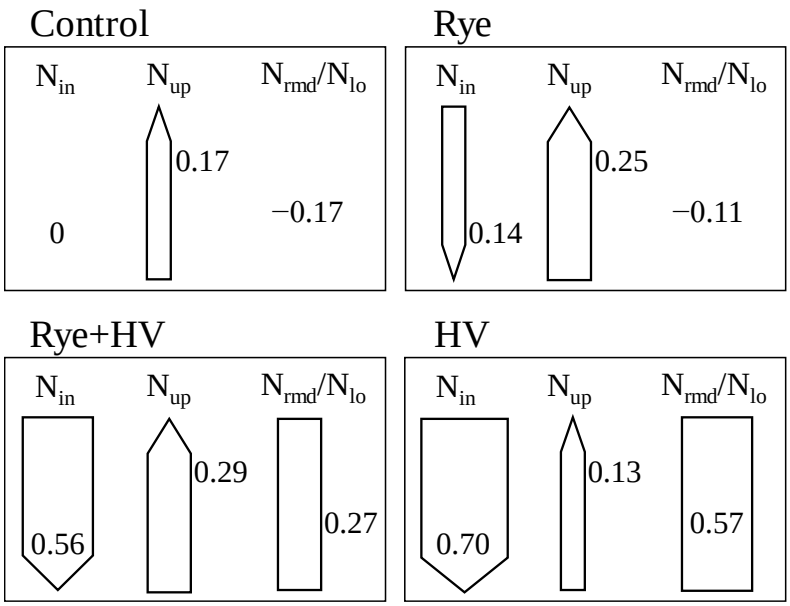


Fig.4.4. Nitrogen (N) budget in g N pot^{-1} soil unit in bulk soil at 25 days after cover crops incorporation in 2017 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Detail calculation has been described in the Table 4.1 in sub-section 4.2.7.

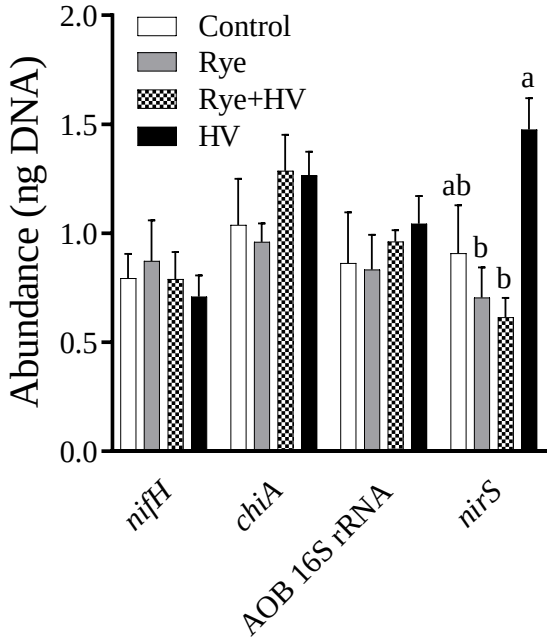


Fig.4.5. Abundance of bacterial specific genes in bulk soils at 25 days after cover crops incorporation in 2017 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Different letters in each bar indicate significant difference at $P < 0.05$ (n = 3) based on Tukey's HSD. The vertical bars show the standard deviation.

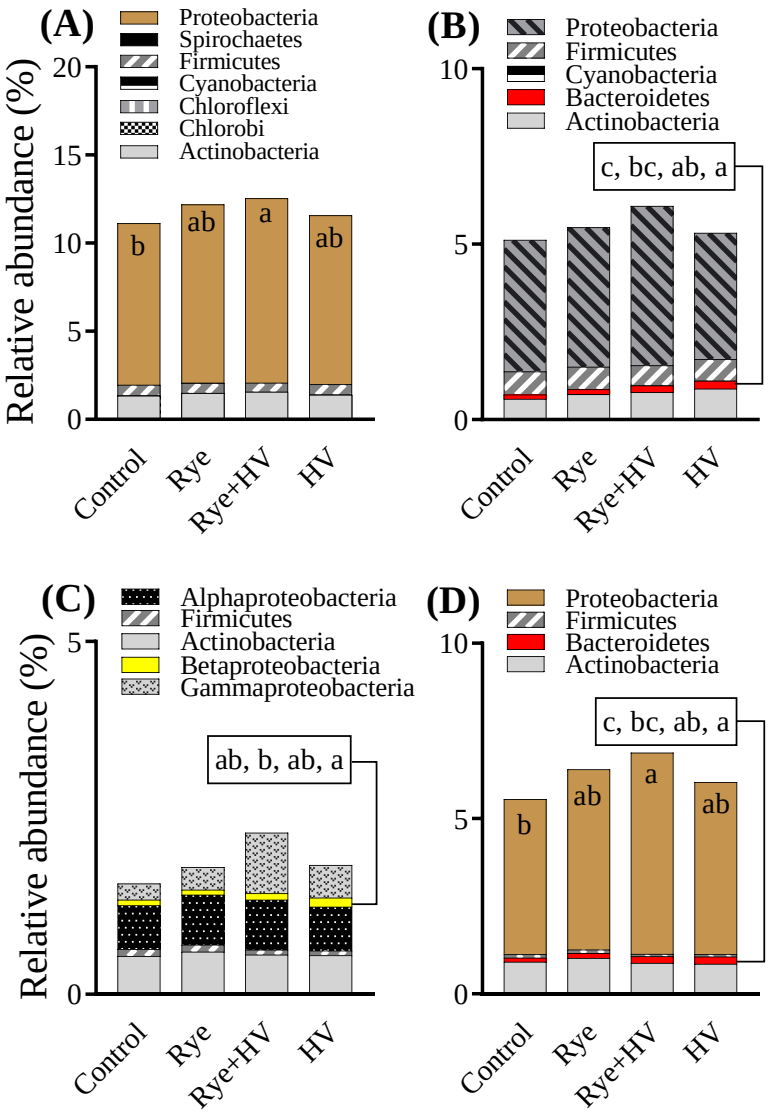


Fig.4.6. Sum of relative abundance of bacteria with (A) *nifH*, (B) *chiA*, (C) AOB 16S rRNA, and (D) *nirS* genes in bulk soil at 25 days after cover crops incorporation in 2017 pot experiments. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The bacteria families with the specific genes are listed in Table S4.2. Colored bars are applied to the data that were statistically different among the treatments at $P < 0.05$ (n = 3) based on Tukey's HSD.

4.4. Discussion

4.4.1. Effects of cover crops on microbial community

Bacterial richness and diversity in bulk soil were established even in the very short-term, i.e., 25 and 20 DAI, shown as the unchanged values following the application of different cover crops (Table 4.3A). This result was in accordance with previous researches: a field study using HV and rye for 6 years (Fernandez et al., 2016b) and a field study using legume or non-legume for 5 years (Nivelle et al., 2016), and a meta-analysis of 60 cover cropping systems showing unchanged Shannon diversity due to cover crop type and soil order (Kim et al., 2020).

Nevertheless, bacterial community structure was changed following inputs of various cover crops by promoting relative abundance of plant decomposers. In specific, HV increased relative abundance of Bacteroidetes and Proteobacteria, the copiotrophs (Fierer et al., 2007). This result was similar to that reported by Toda and Uchida (2017) using HV for 9 years. At family level, relative abundance of *Cytophagaceae*, the decomposer of complex C content (McBride et al., 2014), was increased by rye+HV input (Fig.4.1A). Additionally, relative abundances of families *Sinobacteraceae* and *Alteromonadacea*, the decomposers of straw and legume–rye residues (Banerjee et al., 2016; Brennan and Acosta-Martinez, 2017), were increased following HV input. These increases in the abundances of the bacterial copiotrophs may support decomposition and N release from the easily and intermediately decomposable residues, i.e., HV and rye+HV, respectively.

Fungal richness, diversity, and abundance were promoted by rye+HV, thus changing fungal community structure. It has been expected due to a broader range of soil microbial groups utilizing two or more different types than a single type of cover crop residue (Drost et al., 2020). In specific, relative abundance of Basidiomycota, a phylum covers a wide range of plant saprotrophs (Finlay and Thorn, 2019), was promoted in soil with rye+HV in 2018. This was accordance with results of Schmidt et al. (2019) in the field with legume and non-legume mixtures. Moreover, soil with rye+HV had more unique fungal families than soils with other cover crops (i.e., 52 unshared families in Venn diagram, Fig.S4.1), in which half of them were members of Basidiomycota. We assumed that combination of rye and HV could allow more species to colonize *sensu* Brahmaprakash et al. (2017).

Moreover, *Leucosporidiaceae*, a member of Basidiomycota, was positively responding to HV residue in 2018 (Fig.4.1B-2). The results imply that fungal decomposers may support the decomposition and N release from rye+HV and HV residues, especially in 2018 soil with low initial TC and TN contents and inorganic N concentration.

4.4.2. Microbe–microbe networks under different cover crops incorporation

The positive links > negative links trends found in control soils in both years (Fig.4.2) indicated that initial microbe–microbe competitions occur in few. This was a contrast to the results of experiment of Banerjee et al. (2016) in soil with initial 1.3% TC. Soils used in the current study had > 4% initial TC. Thus, it is assumed that initial C demand of soil microorganisms for their initial interactions was fulfilled.

Introduction of cover crops kept the positive links > negative links trends (Fig.4.2), pointing out that a high synergistic microbe–microbe associations occurred. The synergistic association means that bacteria and fungi cooperated with each other to decompose all the type of cover crop residue, declining the first hypothesis in this chapter.

The 2018 soil consisted of low initial TC content and N status, indicating a low active organic material pool (Doran and Smith, 1991). In consequence, bulk soil microorganisms have been stimulated to vigorously mine the active organic materials. As the result, 192 more total links were found in 2018 soils compared with that in 2017 soils (Fig.4.2D). Higher total links in 2018 than that in 2017 may correspond to the wider ranges of bacterial and fungal relative DNA quantities in 2018 than in 2017 (Fig.3.5, sub-section 3.4.7 of Chapter 3). Higher total links in 2018 than in 2017, however, did not lead to superior impact on N supply, but similar rates of N mineralization in both experimental years (Table S4.3).

Slower decomposition rates of rye and rye+HV residues in 2018 than in 2017 (Table S4.3) may not relate to the phenomenon of total links, due to the positive links > negative links pattern. However, low initial soil TC and TN contents in 2018 caused low range of BG activity during the decomposition, thus declining decomposition rates of rye and rye+HV residues in 2018 (Fig.3.4C, Chapter 3). This was corresponding with Frøseth and Bleken (2015), who conclude that a low microbial activity based on initial soil TC

limitation slow residual decomposition rates.

Inclusion of rye+HV residue led to more negative interactions between fungi and bacteria in 2018 that may slow the rates of C release. The high fluctuation of fungal network in this study indicates the active communication by soil fungi during the decomposition. The communications were described as follow. Richer fungal communities increase total links within fungi (Zheng et al., 2018), as shown in 2018 soil with rye+HV (Table 4.1B) that had 45 more links in the F–F network over the control in 2018 (Fig.4.2). Those 45 links were negative links, suggesting competition within the fungi on nutrients (de Boer et al., 2005) consisted in rye+HV residues. Additionally, fungi competed with bacteria resulted in more negative links in B–F network in soil with rye+HV in 2018 compared with that in 2017. Those over-expressed negative links in F–F and B–F networks reflected a high amount of antagonistic interactions between bacteria and fungi (Deveau et al., 2018) that are toxic to saprotrophic fungi, thereby declining relative abundances of plant decomposers, e.g., unidentified CCU21, unidentified PK29, and unidentified 32-20 that are members of Acidobacteria (Fierer et al., 2007), *Bacillaceae* (Zang et al., 2018), *Mycosphaerellaceae* (Yang et al., 2019) and *Lasiosphaeriaceae* (Nguyen et al., 2016), compared with that in control or other cover crop-treated soils (Fig.4.1B-2). As the consequence, nutrient cycling was slowing down (Finlay and Thorn, 2019), particularly the C release rates from rye+HV residue within 38 DAI in 2018. Regarding to the implications of the coinciding C and N demands of soil microbes (Kuzyakov and Blagodatskaya, 2015) for N supply, further evaluation of microbial networks in a longer period than 38 DAI in soils with rye+HV is needed.

4.4.3. Potential roles of influenced taxa in N availability from cover crops

Minor taxa of bacteria, i.e., *Parachlamydiaceae* and unidentified SAR202, may play roles in N supply from HV in 2017. It was marked as correlations between those bacterial minor taxa and BG activity and SMB under HV treatment (Fig.4.3A-1). Thus, we agreed with Nevins et al. (2018), who demonstrated that influenced bacterial taxa in HV play roles in BG activity, even though they are extremely rare during residual decomposition with a relative abundance much less than 0.1%. Conversely, *Sinobacteraceae* whose relative abundance greater than 0.1% (Fig.4.1A-1) and that is the BG-producing bacteria

(Zhou et al., 2014) did not play role in the BG activity and SMB promotion by HV (Fig.4.3A-1). This clarified the statement that that bacterial functions can be independent from bacterial abundance (Banerjee et al., 2016).

Bacterial contribution on N supply from cover crops was also observed as the direct roles. Specifically, *Alteromonadaceae*, the decomposer of polysaccharides (López-Pérez and Rodriquez-Valera, 2014), was responsible to the enhancement of soil inorganic N concentration in HV and rye+HV in 2018 (Fig.4.3A-2).

The promotion of specific bacterial functions in soils with rye+HV residues was correspondence with the general function of bacterial metabolism that was increased by input of rye+HV in 2017 (Table S4.4A). The function includes metabolism of carbon and fatty acid and degradation of aromatic compound (KEGG pathway database; https://www.genome.jp/kegg/pathway.html), which are included in rye+HV residue.

Fungal functions are not easily altered by cover crops (Schmidt et al., 2019), which was shown by none of the influenced fungal taxa being likely to correlate with the N mineralization indicators in 2017 (Fig4.3B-1). On the other hand, 2018 soil with low initial TC content and N status yielded a significant fungal role in N supply from the cover crops. *Leucosporidiaceae*, a decomposer known from compost that supplies nutrients to the cultivated button mushroom (Pandin et al., 2018), was directly correlated with the promotion of soil inorganic N concentration in HV (Fig.4.3B-2).

In correspondence with fungal general functions, relative abundance of plant pathotroph guild was higher in HV than in rye and rye+HV in 2018 (Table S4.4B). Plant pathotroph guild includes fungi that gain nutrients by harming host cells (Nguyen et al., 2016) and was positively correlated with soil nitrate (Detheridge et al., 2016). The improvement of the guild in HV-treated soil may support N supply from HV residue.

4.4.4. N cycle-associated bacteria in cover crop-treated soils

The bacterially-N fixation may not support N supply during cover crop decomposition. A high sum of relative abundance of Proteobacteria with *nifH* gene in cover crop-treated soils was accordance to Ren et al. (2018). However, the promotion may not function to N

supply, due to the unchanged *nifH* gene abundance in all treatments (Fig.4.5) that may lead to a non-active N fixation pathway (Ren et al., 2018). Furthermore, correlations between *nifH* gene abundance, sum of relative abundance of Proteobacteria, and concentration of ammonium [NH_4^+-N, i.e., the product of N fixation (Levy-Both et al., 2014; Ren et al., 2018)] at 25 DAI in 2017 were negative, weak, and not significant (TableS4.5A).

Members of Bacteroidetes with *chiA*, but not the *chiA* gene, may indirectly contribute to N supply. This was based on the intermediate correlation between Bacteroidetes and ΔN_{in}, i.e., difference of N_{in} at 0 and 25 DAI based on the percentage of released N (Table S4.5B). Specifically, Bacteroidetes was positively and strongly correlated with the potential N_{rmd}/N_{lo} at 25 DAI, although the correlation with ammonium concentration was weak (Table S4.5B). It indicates that Bacteroidetes were more responding to the sum of ammonium and nitrate, rather than ammonium alone. This result clarified our general comparison of bacterial community structure in sub-section 4.4.1 revealed that Bacteroidetes was the copiotroph bacteria and the members may play role in cover crop decomposition and N supply from the residue.

Nitrification during decomposition period was potentially influenced by *Nitrosomonadaceae*, the only member of Betaproteobacteria with AOB 16S rRNA gene (Table S4.2C). Growth rate of bacteria with AOB 16S rRNA gene and copy number of AOB 16S rRNA gene determine nitrification rate (Hayatsu et al., 2008; Levy-Booth et al., 2014). The AOB 16S rRNA gene abundance was similar in all treatments (Fig.4.5), but the relative abundance of *Nitrosomonadaceae* was promoted in HV over the rye (Fig.4.6C). This may show that the specific bacterial abundance more active in regulating the function of AOB 16S rRNA gene in nitrification. Moreover, *Nitrosomonadaceae* was strongly correlated with concentration of nitrate [NO_3^--N, i.e., the product of nitrification (Lindsay et al., 2010; Ren et al., 2018)] (Table S4.5C), underlining roles of the bacteria on the core of N cycling in cover crop-treated soils. These results also clarified that members of Proteobacteria greatly contributed on N supply from cover crops, as discussed in sub-sections 4.4.1, 4.4.3, 4.4.4, and 4.4.5.

The NO_3^--N is the bound N form to soil particles and mostly consisted in total concentration of soil inorganic N. For this reason, a significant correlation between the potential N_{rmd}/N_{lo} and *Nitrosomonadaceae* was resulted (Table S4.5C) and yielded the high N_{rmd}/N_{lo} budget in HV-treated soil (Fig.4.4).

A high N concentration that remained in soils can be related to N retention, avoiding potentially N loss in cover crop-soybean rotation system using wheat, oat, oat+vetch, and vetch cover crops (Landriscini et al., 2019). In the current study, N_{rmd}/N_{lo} was jointly estimated (Fig.4.4) because we did not calculate N leaching and gas emission. The N_{rmd}/N_{lo} values may point out that N loss greatly occurred in HV-treated soil, wherein *nirS* gene and members of Bacteroidetes with *nirS* gene played roles in the denitrification. The assumption was according to some evidences: (1) promotion of *nirS* gene abundance (Fig.4.5), (2) a high sum of relative abundance of Bacteroidetes members with *nirS* gene in HV-treated soil (Fig.4.6D), and (3) significant correlation between Bacteroidetes and N_{rmd}/N_{lo} value (Table S4.5D),

In fact, denitrification occurs in many ways rather than a singular process in the small areas (hot spot, e.g., pots) and brief periods (hot moments, e.g., during decomposition and N mineralization) (Groningen et al., 2015). Other denitrification genes (e.g., *nirK*, *nosZ*, *narG*, Ren et al., 2018) may also involve and relate to the N loss in HV-treated soil. To reach a deeper understanding about N loss potential, evaluating N leaching (Widdison and Burt, 2010), gaseous N, and denitrifiers during decomposition period of cover crops are the potential ideas for further study.

In addition to N retention from cover crops, it is important as the N availability for the following plants (Landriscini et al., 2019). Therefore, a high N_{rmd} in soils with HV and rye+HV residues at 25 DAI (Fig.4.4) was important to improve lettuce N_{up} and yield until 38 DAI, the final harvest timing of lettuce plants (Chapter 3). In facts, plant N_{up} under HV treatment was low at the 25 DAI in 2017 (Fig.4.4), due to the assumption of allelopathic-like effect from HV residue (Chapter 3). To specifically discuss the lettuce N_{up} phenomena at 25 DAI, a research has been carried out and the results are discussed in Chapter 5.

4.5. Conclusion

Incorporation of HV and rye+HV residues promoted relative abundance of decomposers that were correlated with the enhancement of BG activity and SMB in 2017 and soil inorganic N in 2018. The correlations indicate specific roles of microorganisms in bulk soil in N supply from the cover crops.

In microbe–microbe networks, more numbers of positive links than negative links were found not only in HV treatment, but also in other treatments in the two experimental years. This indicates that bacteria and fungi disregarded the cover crop type and cooperated with each other to decompose the residue and mineralize the N during decomposition period. In comparison between 2017 and 2018 soils, total links was higher in 2018 than that in 2017. There was no superiority consequence, but the rates of N release from all cover crop residues in 2017 and 2018 were similar.

Supplementary Material

Table S4.1. Soil biochemical properties at 25 and 20 days after cover crop incorporation in 2017 and 2018 pot experiments, respectively (Chapter 3)

	Treatment	Inorganic N (mg N kg^{-1} soil)	soil pH	BG activity (μg ρNP g^{-1} soil h^{-1})	SMB (g C kg^{-1} soil)
2017	Control	57.6 ± 8.32	6.22 ± 0.11	635 ± 80.5 b	4.25 ± 0.63 b
	Rye	43.7 ± 3.86	6.63 ± 0.04	724 ± 27.6 b	5.40 ± 0.64 b
	Rye+HV	87.9 ± 26.1	6.64 ± 0.10	1229 ± 104 a	5.67 ± 0.85 b
	HV	168 ± 64.7	6.58 ± 0.14	1407 ± 155 a	8.57 ± 0.24 a
2018	Control	21.9 ± 1.82 b	6.47 ± 0.06 a	65.2 ± 24.9	2.06 ± 0.25
	Rye	22.4 ± 1.92 b	6.52 ± 0.04 a	230 ± 120	4.25 ± 0.23
	Rye+HV	78.1 ± 6.96 a	6.24 ± 0.01 b	226 ± 111	3.18 ± 0.61
	HV	108 ± 18.7 a	6.17 ± 0.06 b	101 ± 27.8	3.40 ± 0.83

Treatments were soil without any cover crops (control) and soil with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Mean ± standard deviation is followed by a letter. Different letters among treatments in each experimental year indicate significant difference based on Tukey's HSD at $P < 0.05$ (n = 3). The N, BG, and SMB abbreviate nitrogen, β-glucosidase enzyme, and soil microbial biomass, respectively.

Table S4.2. List of bacteria families with specific genes: (A) *nifH*, (B) *chiA*, (C) AOB 16S rRNA, and (D) *nirS*

(A)

Phylum	Class	Family	Genus or species
Actinobacteria		*Microbacteriaceae*	*Microbacterium* sp. ORS 1472
		Micrococcaceae	*Arthrobacter* sp. 61k
		Corynebacteriaceae	*Corynebacterium* sp. 12a
		Frankiaceae	*Frankia* sp. MgI5
		Micromonosporaceae	*Micromonospora lupini* str. Lupac 0
Chlorobi		*Chlorobiaceae*	*Chlorobaculum tepidum*
Chloroflexi		*Oscillochloridaceae*	*Oscillochloris trichoide*
Cyanobacteria		*Oscillatoriophycideae*	*Katagnymene spiralis*
		Nostocaceae	*Cylindrospermopsis raciborskii* LD-I
		Microchaetaceae	*Tolypothrix distorta* var. *symplocoides* UTEX 'B 424
		Scytonemataceae	*Scytonema* sp. FGP-7A
		Rivulariaceae	*Calothrix* sp. MCC-3A
Firmicutes		*Paenibacillaceae*	*Paenibacillus durus*
		Bacillaceae	*Bacillus megaterium*
		Heliobacteriaceae	*Heliorestis daurensis*
		Clostridiaceae	*Clostridium pasteurianum*
		Eubacteriaceae	*Acetobacterium woodii* DSM 103
		Peptococcaceae	*Desulfotomaculum nigrificans* DSM 57
		Ruminococcaceae	*[Clostridium] cellulolyticum* H1
Spirochaetes		*Spirochaetaceae*	*Spirochaeta aurantia*
Proteobacteria	Alphaproteobacteria	*Phyllobacteriaceae*	*Mesorhizobium* sp. LMG 11892
		Rhizobiaceae	*Rhizobium* sp. NGR181
		Methylobacteriaceae	*Microvirga lupini*
		Xanthobacteraceae	*Starkeya* sp. ORS 1474
		Acetobacteraceae	*Gluconacetobacter diazotrophicus*
		Methylocystaceae	*Pleomorphomonas oryzae*
		Rhodobacteraceae	*Rhodobacter sphaeroides*
		Rhodospirillaceae	*Phaeospirillum fulvum*
		Bradyrhizobiaceae	*Rhodopseudomonas oryzae*
		Sphingomonadaceae	*Sphingomonas* sp. Y49
		Brucellaceae	*Ochrobactrum cytisi*
		Acetobacteraceae	*Gluconacetobacter azotocaptans*

[continue]

Alphaproteobacteria	*Hyphomicrobiaceae*	*Devosia neptuniae*
	Aurantimonadaceae	*Aurantimonas* sp. CCNWGS0021-2
	Beijerinckiaceae	*Methylocapsa aurea*
Betaproteobacteria	*Comamonadaceae*	*Pelomonas puraquae*
	Rhodocyclaceae	*Azonexus hydrophilus*
	Burkholderiaceae	*Burkholderia* sp. TS2
	Alcaligenaceae	*Alcaligenes faecalis*
Deltaproteobacteria	*Desulfovibrionaceae*	*Desulfovibrio dechloracetivoran*
	Geobacteraceae	*Geoalkalibacter ferrihydriticu*
	Desulfomicrobiaceae	*Desulfomicrobium baculatu*
	Desulfobacteraceae	*Desulfobacter latu*
	Syntrophaceae	*Desulfomonile tiedje*
Epsilonproteobacteria	*Campylobacteraceae*	*Arcobacter nitrofigilis* DSM 729
Gammaproteobacteria	*Xanthomonadaceae*	*Stenotrophomonas maltophilia*
	Halomonadaceae	*Halomonas maura*
	Enterobacteriaceae	*Enterobacter* sp. Y61
	Vibrionaceae	*Vibrio* sp. HN011
	Pseudomonadaceae	*Azomonas macrocytogenes*
	Aeromonadaceae	*Oceanimonas* sp. 5a
	Ectothiorhodospiraceae	*Thiorhodospira sibirica* ATCC 70058
	Methylococcaceae	*Methylomonas methanica*
	Thioalkalispiraceae	*Thioalkalispira microaerophila*
	Chromatiaceae	*Thermochromatium tepidum*
	Moraxellaceae	*Acinetobacter* sp. Z21

(B)

Phylum	Class	Family	Species	Accesion no.
Actinobacteria		*Streptomycetaceae*	*Streptomyces coelicolor; Streptomyces aurofaciens*	AB017008; AB106648
		Cellulomonadaceae	*Cellulomonas uda*	AY008839
		Promicromonosporaceae	*Isoptericola jiangsuensis*	GU459224
		Nocardiopsaceae	*Nocardiopsis prasina*	AB086831
		Pseudonocardiaceae	*Amycolatopsis methanolica*	U31277
Bacteroidetes		*Flavobacteriaceae*	*Aquimarina* sp.	MK570955
		Rhodothermaceae	*Rhodothermus marinus*	AY706992
Cyanobacteria		*Microcystaceae*	*Microcystis aeruginosa*	KY067445

[continue]

Firmicutes		*Bacillaceae*	*Bacillus thuringiensis; Exiguobacterium* sp.	KJ508093; MH753122
		Clostridiaceae	*Clostridium paraputrificum*	AB012764
		Streptococcaceae	*Lactococcus garvieae; Streptococcus criceti*	EF450030; DQ017055
		Paenibacillaceae	*Brevibacillus laterosporus; Paenibacillus* sp.	MG725829; AB683959
		Staphylococcaceae	*Staphylococcus* sp.	MH753146
Proteobacteria	Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas* sp.	MH730655
		Morganellaceae	*Proteus mirabilis*	DQ322594
		Methylobacteriaceae	*Methylobacterium* sp.	MH752549
	Betaproteobacteria	*Burkholderiaceae*	*Ralstonia* sp.; *Burkholderia gladioli*	AB443938; AB038997
	Gammaproteobacteria	*Aeromonadaceae*	*Aeromonas hydrophila*	AF251793
		Alteromonadaceae	*Microbulbifer hydrolyticus*	AY646086
		Yersiniaceae	*Serratia* sp.	EF151930
		Pseudoalteromonadaceae	*Pseudoalteromonas tunicata*	AY751752
		Pseudomonadaceae	*Pseudomonas* sp.	GU931689
		Xanthomonadaceae	*Lysobacter enzymogenes; Stenotrophomonas maltophilia; Strenotrophomonas maltophilia; Xanthomonas* sp.	AY667480; AF014950; KX087372;
		Enterobacteriaceae	*Enterobacter* sp.; *Plesiomonas shigelloides; Escherichia coli*	DQ013365; AF059496; DQ322593
		Pasteurellaceae	*Haemophilus ducreyi*	AF187006
		Vibrionaceae	*Aliivibrio* sp.; *Enterovibrio* sp.; *Listonella anguillarum; Vibrio chloerae*	MK570947; MK675637; EU177073; AF097314
		Francisellaceae	*Francisella tularensis*	KY563318
		Rhizobiaceae	*Agrobacterium* sp.	MH753125
		Moraxellaceae	*Acinetobacter* sp.	MH753322

(C)

Phylum	Class	Family	Species	Accesion no.
Actinobacteria		*Micrococcaceae*	*Micrococcus* sp.	MG812628
		Nocardiaceae	*Rhodococcus* sp.	MG812524

[continue]

Firmicutes		*Bacillaceae*	*Bacillus* sp.	KP308382
Proteobacteria	Alphaproteobacteria	*Bradyrhizobiaceae*	*Nitrobacter hamburgensis*	KC769063
	Betaproteobacteria	*Nitrosomonadaceae*	*Nitrosomonas europaeae*	KU848180
		Nitrosomonadaceae	*Nitrosospira multiformis*	MF618258
	Gammaproteobacteria	*Enterobacteriaceae*	*Cronobacter* sp.	KR069104
		Moraxellaceae	*Acinetobacter* sp.	KR069103
		Chromatiaceae	uncultured *Nitrosococcus* sp.	KC769050
		Pseudomonadaceae	*Pseudomonas* sp.	MN987560

(D)

Phylum	Class	Family	Species	Accesion no.
Actinobacteria		*Micrococcaceae*	*Micrococcus endophyticus; Arthrobacter* sp.; *Kocuria flava*	MH025535; AF335922; MH025504
		Corynebacteriaceae	*Corynebacterium* sp.	EU035284
		Microbacteriaceae	*Microbacterium aquimaris*	MH025507
		Intrasporangiaceae	*Janibester melonis*	MH025503
Bacteroidetes		*Flavobacteriaceae*	*Zunongwangia profunda; Sufflavibacter maritimus; Flavobacterium* sp.	MH025513;; MH025514; AJ440497
Firmicutes		*Bacillaceae*	*Anaerobacillus alkalilacustris; Bacillus* sp.	MH025530; AF335924
		Staphylococcaceae	*Staphylococcus* sp.	AF335923
Proteobacteria	Alphaproteobacteria	*Rhodobacteraceae*	*Paracoccus* sp.; *Roseobacter denitrificans*	AM230902; AJ224911
		Rhodospirillaceae	uncultured *Magnetospirillum* sp.; *Azospirillum brasilense; Thalassospira tepidiphila*	MH432523; AJ224912; MH025520
		Bradyrhizobiaceae	*Bradyrhizobium* sp.	AB542304
	Betaproteobacteria	*Zoogloeaceae*	*Thauera* sp.; *Azoarcus toluvorans* ; *Zoogloea* sp.	AM230892; AY078270; JQ582713
		Azonexaceae	*Dechloromonas* sp.; uncultured *Dechloromonas* sp.	AM230913; MH432480
		Burkholderiaceae	*Cupriavidus necator; Burkholderia* sp.	AM230890; AB545715

[continue]

Betaproteobacteria	*Comamonadaceae*	*Alicycliphilus* sp.; *Comamonas* sp.; uncultured *Brachymonas* sp.; *Brachymonas denitrificans; Ideonella* sp.	AM230896; AM230897; AB808879; DQ865925; AB937707
	Alcaligenaceae	*Alcaligenes faecalis; Achromobacter* sp.	AJ224913; GU122964
	Thiobacillacaeae	uncultured *Thiobacillus* sp.	MH432530
	Gallionellaceae	uncultured *Sideroxydans* sp.	MH432538
	Hydrogenophilaceae	uncultured *Sulfuricella* sp.	MH432541
	Chromobacteriaceae	*Pseudogulbenkiania* sp.	KU175426
	Neisseriaceae	*Vogesella* sp.	JQ582711
	Rhodocyclaceae	*Azospira* sp.	JQ582708
	Oxalobacteraceae	*Herbaspirillum* sp.	AB542309
Gammaproteobacteria	*Rhodanobacteraceae*	uncultured *Rhodanobacter* sp.; *Rhodanobacter* sp.	KC962222; AB480490
	Halomonadaceae	*Chromohalobacter isralensis; Salinicola salarius; Halomonas halodenitrificans*	MH025515; MH025505; FJ686155
	Vibrionaceae	*Vibrio caribbeanicus; Grimontia marina*	MH025516; MH025527
	Shewanellaceae	*Shewanella corallii*	MH025526
	Moraxellaceae	*Psychrobacter maritimus; Acinetobacter haemolyticus*	MH025498; MK944076
	Idiomarinaceae	*Idiomarina zobellii*	MH025506
	Alteromonadaceae	*Alteromonas macleodii; Marinobacter hydrocarbonoclasticus*	MH025496; FJ686157
	Enterobacteriaceae	*Kosakonia cowanii* [current name - *Enterobacter cowanii*]	MH025499
	Oceanospirillaceae	*Marinomonas communis*	MH025517
	Xanthomonadaceae	uncultured *Arenimonas* sp.	MH432369
	Pseudomonadaceae	*Pseudomonas* sp. [also JX826514 - *Pseudomonas stutzeri* ; MH421847 - *P. putida*]	AM230904
	Thiotrichaceae	*Thiothrix unzii*	KC855767

Table S4.3. *P*-value based on t-test of rates of residual decomposition, nitrogen (N) release, and carbon (C) release between 2017 and 2018 experimental years up to 25 days after cover crops incorporation in the pot experiments

	P -value		
Cover crop	Residual decomposition	N release	C release
Rye	0.0188	0.4321	0.3473
Rye+HV	0.0417	0.7456	0.0342
HV	0.1261	0.1604	0.0882
All	0.0002	0.1025	0.0018

Values that are < 0.05 show the significance based on the t-test.

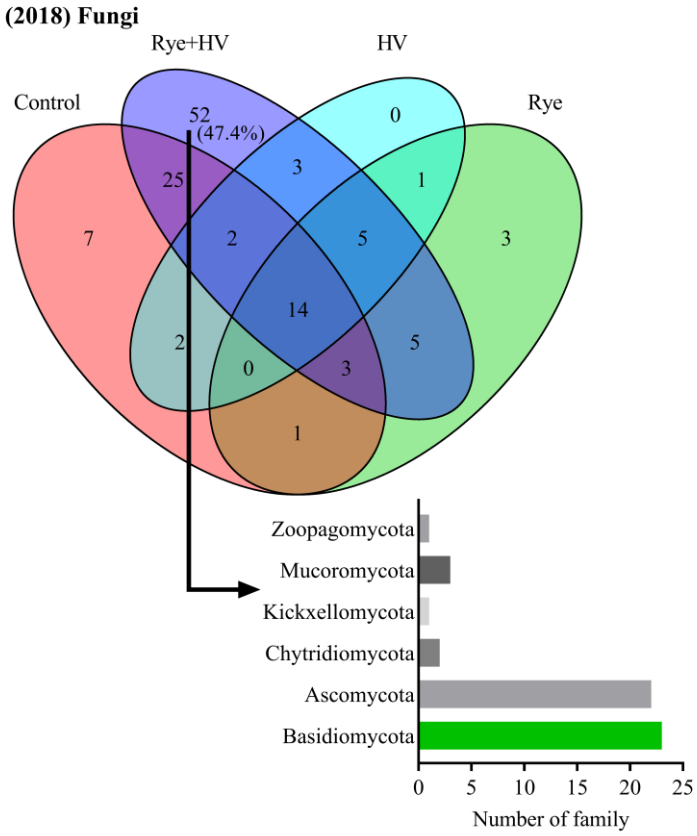


Fig.S4.1. Venn diagram of fungal family in bulk soils in 2018 pot experiments. Treatments were soil without any cover crops (control), and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Number of unique fungi in rye+HV treatment was followed by percentage of them per total identified families in the community.

Table S4.4. General functions of (A) bacteria and (B) fungi in bulk soils at 25 and 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively

(A)			Sequence proportion (%)	
		Category	Metabolism	
	Treatment	Function	Metabolism of other amino acids	Metabolism
2017	Control		1.72 ± 0.01 b	2.59 ± 0.02 ab
	Rye		1.76 ± 0.01 a	2.57 ± 0.01 b
	Rye+HV		1.75 ± 0.00 a	2.60 ± 0.01 a
	HV		1.74 ± 0.01 ab	2.59 ± 0.02 ab
2018	Control		1.76 ± 0.03	2.57 ± 0.02
	Rye		1.69 ± 0.05	2.64 ± 0.01
	Rye+HV		1.72 ± 0.06	2.67 ± 0.02
	HV		1.72 ± 0.06	2.61 ± 0.00

(B)			Relative abundance (%)	
		Mode	Pathotroph	Saprotroph
	Treatment	Guild	Plant pathogen	Plant saprotroph
2017	Control		54.3 ± 9.95	3.81 ± 0.66
	Rye		52.5 ± 4.41	8.18 ± 6.53
	Rye+HV		60.0 ± 8.42	2.79 ± 1.45
	HV		39.1 ± 7.20	2.05 ± 1.14
2018	Control		14.4 ± 2.62 b	0.80 ± 0.11 a
	Rye		14.4 ± 1.60 b	0.00 ± 0.00 c
	Rye+HV		9.63 ± 5.54 b	0.00 ± 0.00 c
	HV		56.1 ± 12.6 a	0.34 ± 0.34 b

Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Mean ± standard deviation is followed by a letter. Different letters among treatments in each year indicate significant difference based on Tukey's HSD at $P < 0.05$ (n = 3). The N, BG, and SMB abbreviate nitrogen, β-glucosidase enzyme, and soil microbial biomass, respectively.

Table S4.5. Correlations between specific gene abundance, sum of relative abundance of bacteria with specific genes, and nitrogen (N) variables at 25 days after cover crops incorporation in 2017 pot experiments

(A) N fixation

	N_{in}	NH_4^+-N_{rmd}	*nifH*
Proteobacteria	0.35	−0.26	0.29
nifH	−0.07	−0.19	1.00

(B) Decomposition

	N_{in}	N_{rmd}	*chiA*
Bacteroidetes	0.87***	0.87***	0.73**
chiA	0.50•	0.56•	1.00

(C) Nitrification

	N_{in}	N_{rmd}	NO_3^--N_{rmd}	AOB 16S rRNA
Betaproteobacteria	0.69*	0.67*	0.53•	0.25
AOB 16S rRNA	0.15	0.25	0.48	1.00

(D) Denitrification

	N_{lo}	*nirS*
Bacteroidetes	−0.75**	0.27
Proteobacteria	−0.34	−0.43
nirS	−0.56•	1.00

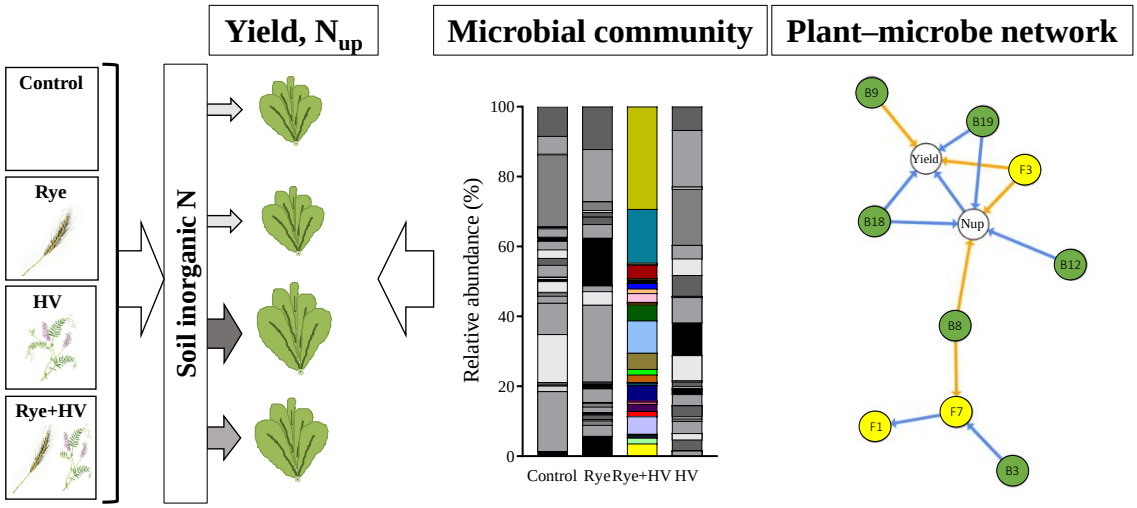
Values show correlation coefficient (R^2). The asterisks (**, ***) denote the significance at 0.01, and 0.001, respectively, based on the Spearman's correlation test. Specific N variables are selected to explain the potential functions of the specific genes as shown in Fig.4.6. Data of DNA quantity of the specific genes (i.e., *nifH*, *chiA*, AOB 16S rRNA, *nirS*) are shown in Fig.4.7. Data of sum of relative abundance of bacteria with specific genes in the same phylum or class are shown in Fig.4.8. Detail of the N variables, sum of relative abundance of bacteria with specific genes, and gene abundance are mentioned in sub-section 4.2.7 and sub-section 4.2.8.

CHAPTER 5

Properties of microorganisms in lettuce rhizosphere soil
and the roles in plant nitrogen utilization at the mid growth stage

This chapter is written based on the constructed manuscript.

Graphical abstract and highlight



- Fungal richness and diversity and relative abundance of some influenced microbial taxa in HV treatment of lettuce rhizosphere soil may indicate plant growth.
- Plant–microbe interactions in lettuce rhizosphere soils in HV treatment showed more negative or positive links related to plant suppression or promotion, respectively.

5.1. Research gap and potential solution

Rhizosphere soils of plant are the hot spot for microorganisms to intimately associate with the plant, in which the association is a reciprocal interaction for the wealth of both rhizosphere microbiomes and plant. Therefore, there are influences from the properties of rhizosphere microbiomes (i.e., microbial richness, diversity, relative abundance, and function) to the poor or vigorous plant performances [i.e., yield and N uptake (N_{up})] or *vice versa*.

Properties of rhizosphere microbiomes can be dependent or independent from cover crops input in bulk soils (Maul et al., 2014; Li et al., 2016; Fernandez et al., 2016a; Zhang

et al., 2017). The dependency and independency imply the effects of cover crops and rhizosphere, respectively. Rhizosphere effects are recognized that healthier plant roots may attract more rhizosphere microbiomes (Brahmaprakash et al., 2017), leading to higher microbial richness. Nonetheless, rhizosphere microbiomes can be both the good (beneficial) and the bad (harmful) taxa (Mendes et al., 2013; Schirawski and Perlin, 2018). Thus, further analyses are needed to examine the roles of rhizosphere microbiomes: beneficial or harmful to the associated plants (Samad et al., 2019).

There are primary roles of rhizosphere microbiomes that are determined through microbe–microbe networks (Bonfante and Anca, 2009; Nihorimbere et al., 2011; Haldar and Sengupta, 2015; de Vries and Wallenstein, 2017). However, the primary roles cannot be simply emphasized as beneficial or harmful by evaluating positive or negative links, due to the complex interactions in the hot spot. Therefore, evaluation of plant–microbe interactions is required (Haldar and Sengupta, 2015; van der Heijden and Hartmann, 2016; de Vries and Wallenstein, 2017; Schirawski and Perlin, 2018). One of the methods is directed network analysis, which is performed by correlating relative abundance of specific microbial taxa, e.g., influenced taxa, with values of plant productivity's indicators, e.g., plant N_{up} and yield (Poudel et al., 2016).

Lettuce plant N_{up} and yield under hairy vetch (HV) treatment in 2017 were suppressed, while those in 2018 were vigorous at its mid growth stage (Chapter 3, Chinta et al., 2020). Plant–microbe interactions are more interactive when plant actively absorb soil nutrients (Wattenburger et al., 2019) or actively grow (Oberholster et al., 2018). Therefore, it will be highly valuable to evaluate plant–microbe interaction at mid growth stage of lettuce plant, especially to address microbial roles behind the contrast plant responses under HV treatment in two experimental years.

The hypotheses were (1) microbial community structure in rhizosphere soil is related to lettuce plant growth and (2) plant–microbe interactions under HV treatment in 2017 show more negative links than that in 2018.

5.2. Materials and Methods

5.2.1. Treatments and design

The experiment was performed in pot level in 2017 and 2018 as mentioned detail in sub-section 3.2.1.2 and 3.2.2.2 in Chapter 3. The treatments were soil without any cover crops (control) and soils with rye, HV, and mixed (rye+HV) cover crops. The experiments were designed in randomized block with three replicates.

5.2.2. Sampling of lettuce rhizosphere soil

The sampling was done to harvested lettuce plants at the mid growth stage: 25 days after incorporation (DAI) of cover crops in 2017 and at 20 DAI in 2018. At the sampling timings, three from five lettuce plants in each replicate of each treatment were uprooted and the roots were gently shaken for 15–20 times to obtain only the adhering soil to the root system (Gobran and Clegg, 1996). Rhizosphere soils were collected using a clean tweezer. Collected rhizosphere soils from same replicate of each treatment were homogenized and kept at –20°C until the molecular analysis.

5.2.3. DNA extraction and purification, and quantification of microbial abundance

Methods of DNA extraction from soils and purification have been described in sub-section 3.2.6.1 in Chapter 3. Methods of the qPCR for quantifying the total DNA amounts of bacteria and fungi have been described in sub-section 3.2.6.2 in Chapter 3.

5.2.4. Libraries preparation, sequencing, and processing data of sequence

Methods of preparation of the 16S rRNA (bacteria) and ITS (fungi) libraries and methods of the sequencing have been detailed described in sub-section 4.2.3 and 4.2.4 in Chapter 4. Microbial richness and diversity as the Shannon index in rhizosphere soils were evaluated using the same methods used for those in bulk soils.

The processing data of sequence for rhizosphere microbiomes was done in the similar analysis procedures of those for the bulk soil microbiomes described in sub-section 4.2.3 and 4.2.4 in Chapter 4. The average of the high-quality reads for 16S rRNA and ITS were 72578 and 9406, respectively, in 2017 and 15303 and 9922, respectively, in 2018. The high-qualified reads of 16S rRNA and ITS were then rarefied in 40933 and 1788,

respectively, in 2017 and in 10448 and 2914, respectively, in 2018. The qualified and rarefied reads were then systematically aligned for bacterial and fungal community structure by performing the α- and β-diversity analysis at phylum and family levels using the QIIME pipeline.

5.2.5. The beneficial and harmful rhizosphere microbiomes

Several bacterial and fungal families selected to represent the beneficial and harmful rhizosphere microbes are listed in Table S5.1. The IAA producing bacteria (Table S5.1A) and fungi (Table S5.1B) were selected based on Duca et al. (2014) and Fu et al. (2015), respectively. The plant growth promotor (Table S5.1C) and plant pathogen (Table S5.1D) were selected based on various references. Relative abundance of the selected families was presented.

5.2.6. Directed network analysis

Firstly, specific microbial taxa as the influenced taxa were selected using the same method described in detail in sub-section 4.2.6 of Chapter 4. Secondly, directed network graph was built to illustrate the potential roles of rhizosphere microbiome in plant performance. Relative abundance of the influenced taxa of bacteria and fungi was statistically correlated with values of lettuce yield and N_{up} at the mid growth stage of plant (Table S5.2) based on the Spearman's correlation test using the 'Hmisc' package in R. Only strong and significant correlations were considered, based on correlation strength at values less than or equal to −0.90 or greater than or equal to 0.90 and *P*-value at < 0.05. The plant–microbe links were illustrated with direction from vertices of influenced taxa to vertices of plant yield and N_{up} using 'igraph' package (Csárdi and Nepusz, 2006) in R.

5.2.7. Statistical data analyses

All statistical analyses were performed in R and GraphPad Prism. One-way ANOVA followed by Tukey's HSD test at $P < 0.05$ were used to differentiate cover crop effects on microbial DNA quantity, microbial richness, Shannon index, and relative abundances of bacteria and fungi. The logarithm and arcsine transformations were necessarily applied to the DNA quantity and relative abundance, respectively, before performing the one-way

ANOVA, when normal distribution was not achieved according to the Shapiro-Wilk test. Similarity of microbial communities (β-diversity) under different treatments and habitats was tested with NMDS based on Bray-Curtis distances in permutational multivariate ANOVA (perMANOVA) using 'adonis' function in 'vegan' package in R (Oksanen et al., 2017). The t-test at $P < 0.05$ and Spearman's correlation test were necessarily performed.

5.3. Results

5.3.1. Bacterial and fungal abundance in lettuce rhizosphere soil

Microbial DNA quantity in lettuce rhizosphere soils varied among the treatments (Table 5.1), except for bacterial DNA quantity in 2017 (Table 5.1A). The non-significant correlations were resulted between bacterial and fungal DNA and plant yield ($P = 0.63$ and $P = 0.34$ in 2017 and $P = 0.34$ and $P = 0.38$ in 2018) and between bacterial and fungal DNA and N_{up} ($P = 0.86$ and $P = 0.98$ in 2017 and $P = 0.42$ and $P = 0.34$ in 2018).

At the same observation day (i.e., 25 DAI in 2017 and 20 DAI in 2018), the average of bacterial and fungal abundances in lettuce rhizosphere soils (6.37 ng DNA and 3.28 ng DNA) were higher ($P = 0.0002$ and $P = 4.17 \times 10^{-8}$) than those in bulk soils (1.50 ng DNA and 1.10 ng DNA).

5.3.2. Community structures of rhizosphere microbiomes

Bacterial richness was similar in all treatments in the two experimental years (Table 5.1A). Fungal richness and Shannon index at family level in HV was significantly decreased in 2017 over the control and rye (Table 5.1B). In contrary, fungal Shannon index at family level in HV was equal to that in control and higher than that in rye in 2018.

There were no interaction effects of treatments (i.e., control, rye, rye+HV, and HV) and microbial habitats (i.e., bulk and rhizosphere soils) on microbial community structures. However, bacterial and fungal community structures in lettuce rhizosphere soils were different from those in bulk soils in the two experimental years ($P < 0.05$ and $P < 0.01$). Treatments significantly affected community structures of bacteria in 2017 ($P = 0.001$) and fungi in 2018 ($P = 0.001$).

Bacterial community structures in both taxonomical levels and experimental years were different among the treatments (Table 5.1A). In specific, relative abundances of phyla Bacteroidetes and Proteobacteria in rye+HV (5.37% and 26.4%) were higher than that in control (3.58% and 18.9%) in 2017. Moreover, members of Bacteroidetes and Proteobacteria: families *Cytophagaceae*, *Erythrobacteriaceae*, and *Piscirickettsiaceae*, were significantly increased in rye+HV over the control in 2017 (Fig.5.1A-1). In 2018, relative abundance of phylum Proteobacteria (34.6%) and its families, *Flavobacteriaceae* and *Pseudomonadaceae* (Fig.5.1A-2), in HV were increased over the control (26.8%). In addition, relative abundance of *Burkholderiaceae* and *Rhizobiaceae* were increased in HV and rye+HV, respectively, compared with other treatments in 2018 (Fig.5.1A-2).

Fungal community structures were different among the treatments in both taxonomic levels in 2017 (Table 5.1B). Specifically, relative abundance of phylum Basidiomycota was higher in rye (17.0%) than HV (2.61%) in 2017. Contrarily, relative abundance of phylum Ascomycota was higher in HV (93.5%) than rye (73.8%). At family level, relative abundance of *Mrakiaceae* was increased in rye+HV compared with other treatments in 2017 (Fig.5.1B-1). All cover crops decreased relative abundances of family *Hypocreaceae*, over the control in 2017. In 2018, fungal community structures were similar among the treatments, both in phylum and family levels (Table 5.1B), leading to none of the phylum or family that was significantly influenced.

None of specific beneficial or harmful families were affected by different treatments (Fig.S5.1; Fig.S5.2). When the relative abundances were sum, all cover crop-treated soils accumulated abundance of beneficial fungi (70.3% in rye, 72.4% in rye+HV, and 86.0% in HV) over the control (67.9%) in 2017 (Fig.S5.1B-1). In 2018, lettuce rhizosphere soil under HV treatment had the highest accumulated relative abundance of beneficial bacteria (17.7%) and fungi (14.2%) (Fig.S5.1A-2). Rye showed high relative abundance of family *Ceratobasidiaceae* in the group of the selected harmful rhizosphere microbiome in both years (Fig.S5.2). When the relative abundances of all harmful microbes were sum, rye consistently showed the highest percentage in 2017 (2.26%) and 2018 (15.4%) among the treatments. In addition, sum of relative abundance of plant pathogen in lettuce rhizosphere soil under HV (0.20%) was lower than that under control (1.88%) in 2017 (Fig.S5.2A-1).

Table 5.1. Microbial abundance, richness, diversity, and differentiation of (A) bacterial and (B) fungal community structures in lettuce rhizosphere soils at the mid growth stage of plant in the pot experiments

(A)	Treatment	Abundance	Richness		Shannon index		PerMANOVA	
		(ng DNA)	Phylum	Family	Phylum	Family	Phylum	Family
2017	Control	2.62 ± 0.76	38.0 ± 1.53	344 ± 2.31	2.13 ± 0.02	4.26 ± 0.05	0.049	0.017
	Rye	2.51 ± 0.18	41.7 ± 2.60	392 ± 29.4	2.14 ± 0.01	6.82 ± 2.50		
	Rye+HV	2.61 ± 0.30	38.7 ± 0.88	357 ± 10.3	2.08 ± 0.01	4.36 ± 0.04		
	HV	2.69 ± 0.16	41.0 ± 1.15	376 ± 13.6	2.10 ± 0.01	4.32 ± 0.03		
2018	Control	0.80 ± 0.13 b	30.0 ± 2.89	245 ± 6.44	2.09 ± 0.01	4.21 ± 0.05	0.025	0.012
	Rye	13.0 ± 1.36 a	35.7 ± 2.03	276 ± 19.7	2.09 ± 0.01	7.14 ± 2.78		
	Rye+HV	11.4 ± 5.53 a	33.0 ± 1.00	269 ± 10.8	2.04 ± 0.00	4.35 ± 0.05		
	HV	15.3 ± 9.74 a	34.7 ± 0.88	259 ± 4.04	2.06 ± 0.01	4.33 ± 0.02		

(B)	Treatment	Abundance	Richness		Shannon index		PerMANOVA	
		(ng DNA)	Phylum	Family	Phylum	Family	Phylum	Family
2017	Control	1.95 ± 0.51 b	5.67 ± 0.33	39.3 ± 3.84 a	0.60 ± 0.11 ab	1.93 ± 0.08 a	0.022	0.005
	Rye	2.34 ± 0.41 b	4.67 ± 0.67	31.0 ± 2.31 a	0.75 ± 0.04 a	1.76 ± 0.16 a		
	Rye+HV	2.49 ± 0.49 b	5.33 ± 0.33	23.7 ± 3.48 ab	0.57 ± 0.13 ab	1.37 ± 0.02 ab		
	HV	4.06 ± 0.53 a	4.00 ± 0.00	11.3 ± 4.37 b	0.28 ± 0.08 b	0.69 ± 0.37 b		
2018	Control	0.71 ± 0.28 b	4.67 ± 0.33	25.7 ± 9.21	0.79 ± 0.18	2.20 ± 0.13 a	0.834	0.285
	Rye	2.10 ± 0.55 ab	3.00 ± 0.00	9.67 ± 1.86	0.44 ± 0.22	1.13 ± 0.18 b		
	Rye+HV	3.02 ± 0.84 a	5.33 ± 1.33	27.0 ± 13.3	0.87 ± 0.18	2.10 ± 0.24 ab		
	HV	1.58 ± 0.84 ab	3.67 ± 0.33	21.3 ± 9.13	0.91 ± 0.02	2.16 ± 0.42 a		

Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. [continue]

The mid growth stage was at 25 and 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively. The mean ± standard deviation is followed by a letter. Different letters in each microbial property and each year indicate significance different based on Tukey's HSD at $P < 0.05$ (n = 3). The *P*-value of permutational ANOVA (perMANOVA) at < 0.05 means dissimilarity of microbial community structure among the treatments.

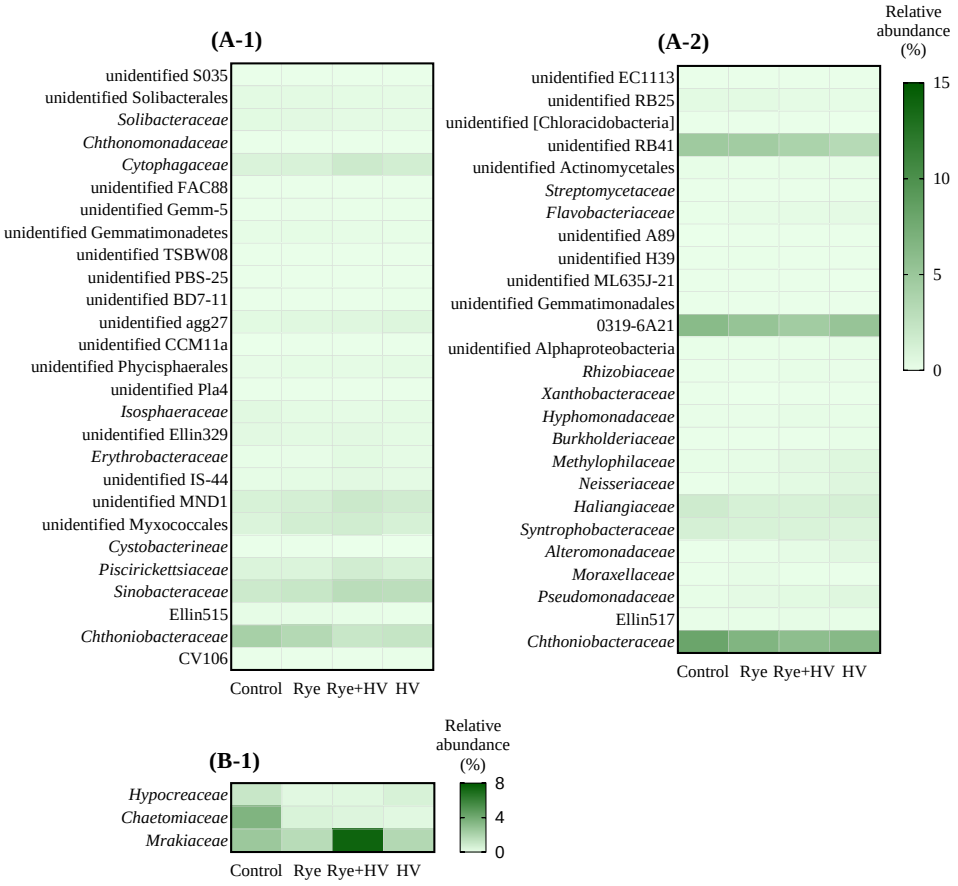


Fig.5.1. Heatmaps showing relative abundance of influenced taxa of (A) bacteria and (B) fungi in family level in lettuce rhizosphere soil at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops.

5.3.3. The directed network

Yield vertices in control in 2017 was not connected with any microbial vertices (Fig.5.2A-1). Few numbers of microbial vertices (i.e., 2–3 vertices) were connected to yield and N_{up} vertices in control in both years (Fig.5.2A). In rye treatment in 2017, unidentified bacteria of class Gemm-5 was negatively correlated with lettuce N_{up} and yield and influenced taxa that were positively correlated with lettuce performance's indicators (Fig.5.2B-1). A successive positive correlation was observed from family *Hypocreaceae* to the unidentified bacteria of order CCM11a to lettuce yield in rye+HV treatment in 2017 (Fig.5.2C-1). A successive positive correlation was also observed in rye+HV treatment in 2018: from family *Rhizobiaceae* to the unidentified bacteria of order RB41 to family Chthoniobacteraceae to lettue yield (Fig.5.2C-2). In HV treatment in both years, influenced taxa that had negative correlations with lettuce N_{up} and yield were negatively correlated with other influenced taxa that had positive correlations with lettuce performance's indicators (Fig.5.2D). Furthermore, there were more positive correlations among the influenced taxa in HV in 2018 compared with that in HV in 2018.

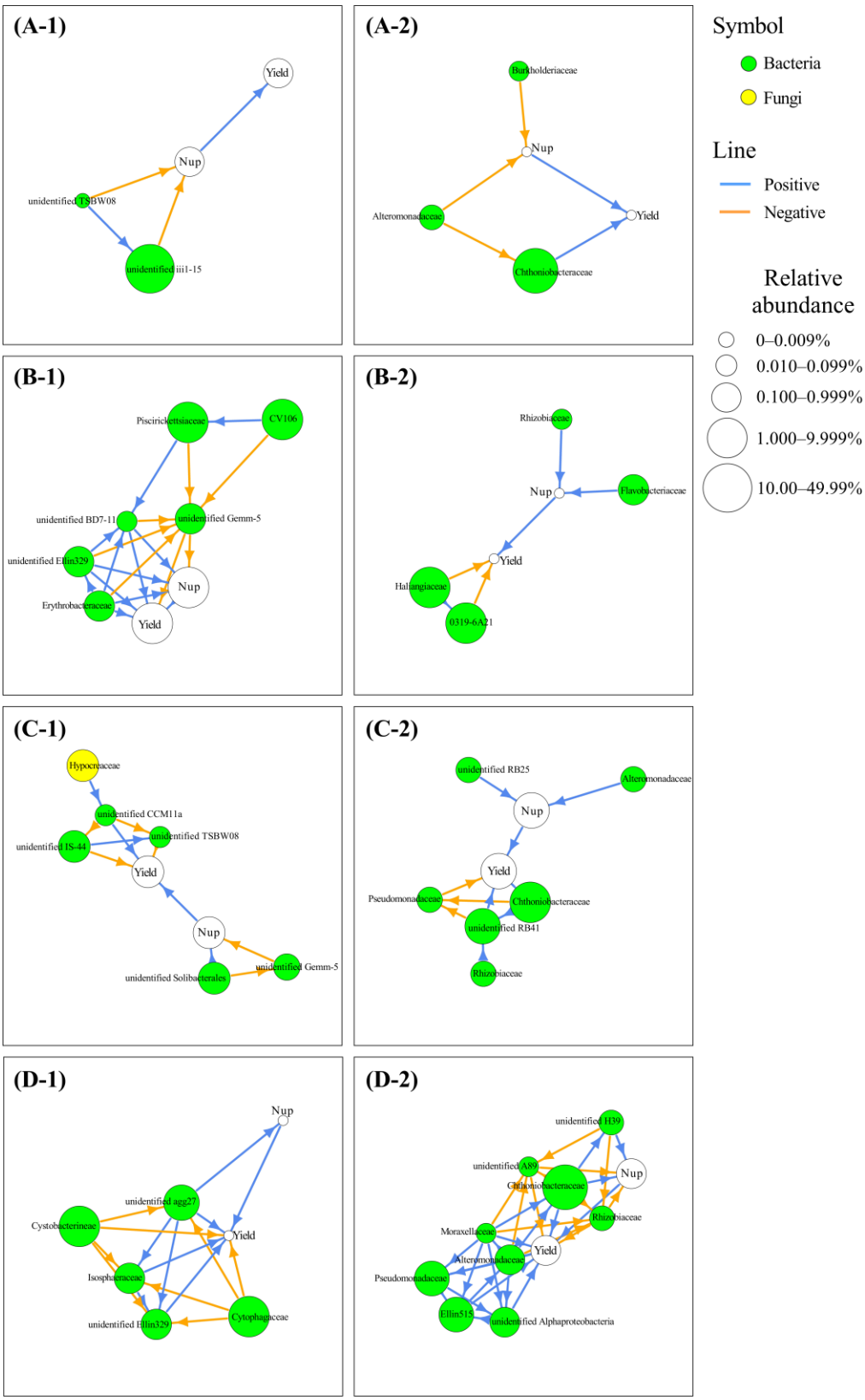


Fig.5.2. Directed networks of influenced microbial taxa and lettuce yield (Yield) and nitrogen uptake (Nup) in (A) control soil and soils with (B) rye, (C) mixed [continue]

(rye+HV), and (D) hairy vetch (HV) cover crops at (1) 25 and (2) 20 days after the incorporation in 2017 and 2018 pot experiments, respectively.

5.4. Discussion

5.4.1. Effects of cover crops on richness and diversity of rhizosphere microbiomes

Rhizosphere soils of lettuce plants as the hot spot of microorganisms attracted more numbers of bacteria and fungi. It was shown as double microbial DNA quantity in rhizosphere soil in comparison with bulk soils (Fig.S5.1). The hot spot includes a high concentration of nutrient sources (Maloney et al., 1997; Smalla et al., 2001; Buyer et al., 2010; Ai et al., 2012). Microbial gene expression in plant rhizosphere soils can predict microbial existence and function (Mendes et al., 2013). Nonetheless, the microbial abundance could not explain microbial function to the plant performances based on the non-significant correlations between microbial DNA quantity and plant indicator values in this study.

Microbial richness and diversity in lettuce rhizosphere soils represented the growth of lettuce plant at the mid growth stage. Poor growth of lettuce plant under HV treatment in 2017 (Table S5.2) evidentially led to a low fungal richness and diversity (Table 5.1B). On the contrary, vigorous lettuce growth under HV treatment in 2018 resulted in a high fungal richness and diversity, specifically over the rye treatment (Table 5.1B). This result was accordance with the result of Buyer et al. (2010); they demonstrated that mulch using HV increased tomato marketable yield with higher PLFA concentrations of fungi and AM fungi in tomato rhizosphere soils compared with mulch using rye. Additionally, all results clarified the statement of Brahmaprakash et al. (2017) that a healthier plant may excrete more abundant root exudates, attracting more number and diverse rhizosphere microbiomes. Furthermore, abundant organic C and N consisted in the root exudates are positively correlated with rhizosphere microbial growth (Maloney et al., 1997; Buyer et al., 2010; Ai et al., 2012).

In term of microbial diversity, generally, application of different cover crops into the bulk soils unchanged microbial diversity in the subsequent plant's rhizosphere soils. This is reported by Buyer and Kaufman (1997) who compared legume cover crops in corn cultivation, Tian et al. (2013) who compared sweet corn, common bean, Garland

chrysanthemum, and edible amaranth cover crops in cucumber cultivation, and Manici et al. (2018) who compared barley and HV cover crops in zucchini and tomato cultivations. Conversely, various Shannon index were observed in lettuce rhizosphere soil under HV, rye, and rye+HV cover crops (Table 5.1). This shows that not only rhizosphere effect (Samad et al., 2019), but also management using different cover crops in bulk soils involved the influence on microbial diversity (Schmidt et al., 2019).

5.4.2. Effects of cover crops on microbial community structures in lettuce rhizosphere soil

Effects of rhizosphere and soil management also drove microbial community in plant rhizosphere as stated by Schmidt et al. (2019). Specifically, the microbial community structures in lettuce rhizosphere soils were different from those in bulk soils as the different β-diversity separately in bulk soil and rhizosphere soil (Table 4.3; Table 5.1). Furthermore, different structures of influenced microbial taxa were found under various cover crop treatments (Fig.4.1; Fig.5.1).

Among the influenced microbial taxa, for instance, *Cytophagaceae*, the decomposer of C (McBridge et al., 2014), whose the relative abundance was promoted by rye+HV in bulk soil (Fig.4.1A-1, Chapter 4) and lettuce rhizosphere soil (Fig.5.1A-1) in 2017. This promotion may show the concomitant effects of rye+HV in both microbial habitats. Specifically, *Cytophagaceae* may degrade rye+HV residue in bulk soil and its high relative abundance may be allowed to inhabit the lettuce rhizosphere, which the plants vigorously grew under rye+HV treatment in 2017 (Table S5.2). The contribution of *Cytophagaceae* in lettuce rhizosphere soil may be degrading the complex organic material of root exudates, so that functioning on nutrient mineralization (Wattenburger et al., 2019).

The significance of plant–microbe *vice versa* effects were also clarified through several changes on relative abundance of bacterial taxa. *Erythrobacteriaceae*, *Piscirickettsiaceae*, *Sinobacteraceae*, and *Flavobacteriaceae* were the C degrader (Zhou et al., 2014; Zang et al., 2018). Their relative abundances in lettuce rhizosphere soils were promoted under particular cover crop treatment in particular experimental year (Fig.5.1), when lettuce plants were vigorously grown. Moreover, increase in relative abundance of *Pseudomonadaceae*, *Burkholderiaceae*, and *Rhizobiaceae* (Fig.5.1A-2), the popular plant

growth promotor (Table S5.1A; Table S5.1C), was found in vigorous plant growth in HV and rye+HV in 2018.

Relative abundance of *Hypocreaceae*, the biological control agent (Brahmaprakash et al., 2017), was decreased by all cover crops (Fig.5.1B). However, the reduction may minorly affected lettuce growth due to a high sum of relative abundance of selected beneficial microbes in all cover crop treatments over the control in 2017 (Fig.S5.1A).

Poor lettuce performance under HV treatment in 2017 may not be related to either individual or sum of relative abundance of the harmful microbes in lettuce rhizosphere soils (Fig.S5.2A). This was due to inconsistent presence of plant pathogen in lettuce rhizosphere soil. Specifically, rye treatment kept showing high relative abundance of plant pathogen when lettuce plant grew either vigorously or poorly. The poor lettuce growth under HV treatment may be related to the reduction of relative abundance families *Solibacteraceae* (Fig.5.1A-1) and *Mrakiaceae* (Fig.5.1B-1). Mendes et al. (2018) and Bian et al. (2020) have respectively reported that *Solibacteraceae* are more abundant in rhizosphere soils of common bean that is resistant to *Fusarium* pathogen and *Mrakiaceae* are more abundant in rhizosphere soils of healthy ginseng root. Therefore, it is assumed that the low abundance of *Solibacteraceae* and *Mrakiaceae* in HV-treated lettuce roots reduced the roles in promoting plant N_{up} and yield.

5.4.3. Microbe–microbe interactions in lettuce rhizosphere soil

The microbe–microbe relationships in lettuce rhizosphere soils could not clearly determine the primary function of rhizosphere microbiome to plant performance. Overall, rhizosphere microbiomes had positive links > negative links trends in microbe–microbe networks under all treatments (Table S5.3). These results reflected the trends of microbe–microbe networks in bulk soil (Fig.4.2, Chapter 4), which was corresponding with the conclusion of Haldar and Sengupta (2015). However, the trends were not consistent with lettuce plant performances, which varied under different cover crop treatments.

Specific results of microbial networks neither could easily emphasize microbial roles in plant performance. Positive interactions among rhizosphere microbiomes could be either commensalism or cooperation (Groβkopf and Soyer, 2014), which will benefit for

plants if the interactions were formed within beneficial rhizosphere microbiomes (Poudel et al., 2016). However, positive links were also formed within the harmful microorganisms. For instance, interactions from and to *Ceratobasidiaceae*, the pathogen of lettuce plant (Bohn, 1953; Raid et al., 2004) that may be related to the low plant N_{up} and yield were low in rye in 2018 (Table S5.2).

Negative links can support plant growth if the interactions are competitions and predations (Groβkopf and Soyer, 2014) by biocontrol agent to plant pathogen (Poudel et al., 2016). HV and rye+HV produced 4–13 more negative links than positive links in B–B networks (Table S5.3), when lettuce growth was promoted in HV and rye+HV (Table S5.2). Those over expressed negative links might be the supportive effects to the vigorous treated plants in HV and rye+HV.

5.4.4. Plant–microbe interactions in lettuce rhizosphere soil under different cover crop treatments

Influenced microbial taxa may directly and directly play roles in plant productivity. The roles were beneficial (positive links) and harmful (negative links) and related to microbial relative abundance. Lettuce cultivated in control soil had few plant–microbe interactions (Fig.5.2A) or none of specific microbial taxa were correlated with lettuce yield vertices in control in 2017 (Fig.5.2A-1), although lettuce yield in control was as high as that in rye+HV in 2017 (Table S5.2). This indicates a low dependency of plant–microbe interactions in control soil. On the contrary, cover crops stimulated communications between plant and rhizosphere microbiomes.

In HV in 2017, the suppressed lettuce plants (Table S5.2) may produce low amount of root exudates, which may be insufficient for demand of *Cytophagaceae* and *Cystobacterineae*, the C decomposers (McBridge et al., 2014). In consequence, they competed with other influenced bacteria involved in the plant performance (Fig.5.2D-1). The results pronounce that a single taxon can greatly signify its function to the associated plant, when the relative abundance is high (Poudel et al., 2016).

In HV in 2018, conversely, vigorous lettuce plant attracted more positive interactions from C decomposers, i.e., *Chthoniobacteraceae* (Janssen, 2015) and *Alteromonadaceae*

(López-Pérez and Rodriquez-Valera, 2014), whose the relative abundances were increased along with the increase in plant performance. Moreover, their action to mineralize C in rhizosphere soil may not disturb plant growth (Nihorimbere et al., 2011). The *Pseudomonadaceae* and unidentified Alphaproteobacteria, the IAA-producing bacteria (Duca et al., 2014), synergistically interacted with other beneficial rhizosphere bacteria (Fig.5.2D-2) to support plant performance. *Rhizobiaceae* cover nitrifier (Heller et al., 2014). *Rhizobiaceae* may not adapt well in rhizosphere soil of vigorous lettuce plants, such as that in HV in 2018 (Fig.5.2D-2), compared with rhizosphere soil of poor lettuce plants in rye in 2018 (Fig.5.2B-2).

In rye in 2017, vigorous lettuce growth recruited more beneficial taxa, such as *Erythrobacteraceae* and unidentified Ellin329, members of Alphaproteobacteria that covers many IAA-producing bacteria (Duca et al., 2014). The results in rye in 2017 fulfill the general prediction that beneficial correlations are positively linear to plant health (Nihorimbere et al., 2011; Mendes et al., 2013; Haldar and Sengupta, 2015). In 2018, the low lettuce yield was caused by low soil inorganic N supply (Chapter 3) that may be directly related to negative correlation from 0319-6A21, the bacteria nitrifier (Wang et al., 2017) in the rhizosphere soil.

It is unique that the successive positive correlations only occur in rye+HV treatment, wherein the IAA-producing bacteria, i.e., family *Rhizobiaceae* (Duca et al., 2014) and IAA-producing fungi, i.e., family *Hypocreaceae* (Fu et al., 2015) were parts of the actors to elongate lettuce roots and improve the plant N_{up} (Etesami et al., 2015). This result implies the benefit of complexity of rye+HV residue on attracting more species (Brahmaprakash et al., 2017).

5.5. Conclusion

Properties of rhizosphere microbiomes indicated productivity of lettuce plants. Specifically, poor and vigorous growth of lettuce plants under HV treatment showed low and high fungal diversity, respectively. Moreover, relative abundance of some influenced microbial taxa was decreased or increased following the suppression or promotions of plant performances, respectively.

Lettuce plant growth under cover crop treatments were depending on the roles of rhizosphere microbiomes marked in plant–microbe interaction. There were negative correlations from two influenced bacterial taxa to plant N_{up} and yield and other influenced taxa in plant–microbe interaction in the poor lettuce growth in HV. On the contrary, vigorous lettuce growth resulted in more positive correlations in plant–microbe networks, including those under rye, HV, and rye+HV treatments.

Supplementary Material

Table S5.1. Lists of rhizosphere microbiomes with specific functions related to plant growth: (A) IAA-producing bacteria, (B) IAA-producing fungi, (C) plant growth promotor, and (D) plant pathogen

(A)

Phylum	Class	Family	Species
Actinobacteria		*Nocardiaceae*	*Rhodococcus* sp.; *R. fascians; R. ruber; R. equi; R. globerulus; R. erythropolis*
			Nocardia asteroides
		Streptomycetaceae	*Streptomyces* sp.; *S. exfoliatusstreptomyces violaceus; S. scabies*
		Micrococcaceae	*Kocuria varinus*
		Brevibacteriaceae	*Brevibacterium butanicum*
		Corynebacteriaceae	*Corynebacterium* sp.
Firmicutes		*Bacillaceae*	*Bacillus subtilis; B. amyloliquefaaciens*
		Paenibacillaceae	*Paenibacillus polymyxa*
Proteobacteria	Alphaproteobacteria	*Rhodospirillaceae*	*Azospirillum brasilense; A. lipoferum*
		Acetobacteraceae	*Gluconacetobacter diazotrophicus; G. azotocaptans*
		Rhizobiaceae	*Agrobacterium tumefaciens*
			Rhizobium sp.; R. meliloti; R. fredii; R. loti; R. leguminosarum; R. rhodochrous
		Methylobacteriaceae	*Methylobacterium extorquens*
		Bradyrhizobiaceae	*Bradyrhizobium japonicum*
	Betaproteobacteria	*Burkholderiaceae*	*Ralstonia solanacearum*
			Burkholderia cenocepacia
		Alcaligenaceae	*Alcaligenes* sp.; *A. faecalis*
	Gammaproteobacteria	*Erwiniaceae*	*Pantoea agglomerans*
			Erwinia herbicola; E. crysanthemi
		Enterobacteriaceae	*Klebsiella aerogenes*
			Enterobacter cloaceae; E. agglomerans
		Pseudomonadaceae	*Pseudomonas stutzeri; P. putida; P. fluorescens; P. savastanoi; P. syringae*
		Vibrionaceae	*Vibrio* sp.

[continue]

(B)

Phylum	Family	Species
Ascomycota	*Glomerellaceae*	*Colletotrichum gleosporioides*
	Nectriaceae	*Fusarium delphinoides*
		Nectria pterospermi
	Saccharomycetaceae	*Saccharomyces cerevisiae*
	Debaryomycetaceae	*Candida tropicalis*
	Phaffomycetaceae	*Williopsis saturnus*
	Hypocreaceae	*Trichoderma virens; T. atroviride*
	Trichocomaceae	*Penicillium* sp.
	Sporocadaceae	*Pestalotiopsis* aff. *neglecta*
Basidiomycota	*Pleurotaceae*	*Pleurotus ostreatus*
	Bulleribasidiaceae	*Hannaella coprosmaensis*

(C)

Phylum	Class	Family	Action	Reference
Actinobacteria		*Micromonosporaceae*	Relate to organic material degradation and produce secondary metabolites and enzymes	Trujillo et al., 2014
		Microbacteriaceae	Stimulate nutrient uptake	Bhattacharyya and Jha, 2012
		Streptomycetaceae	Induce root formation and development; BCA by nutrient and niche competition; ISR; Produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012
		Pseudonocardiaceae	Produce antibacterial substances	Platas et al., 1998
Firmicutes		Bacillales *incertae sedis*	BCA by antagonistic, production of lytic enzymes, siderophore, hydrogen cyanide, and ammonia	Verma et al., 2016
		Bacillaceae	Solubilizer of P; promote cytokins concentration in lettuce shoots and roots; BCA by antibiotically poisoning or killing pathogens; ISR	Brahmaprakash et al., 2017
			Induce root formation and development; stimulate nutrient uptake	Bhattacharyya and Jha, 2012
		Paenibacellaceae	Promote plant rooting and root growth; BCA by nutrient and niche competition; ISR; produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012

[continue]

Proteobacteria	Alphaproteobacteria	*Rhodospirillaceae*	Produce giberellin to promote root hair density and shoot elongation	Brahmaprakash et al., 2017
			Reduce production of ethylene; BCA by nutrient and niche competition; ISR; produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012
		Sphingomonadaceae	Antagonist to plant pathogens	Glaeser and Kämpfer, 2014
		Rhizobiaceae	Solubilizer of P	Brahmaprakash et al., 2017
			Biofertilizer to promote root growth	Bhattacharyya and Jha, 2012
		Xanthobacteraceae	Biofertilizer	Bhattacharyya and Jha, 2012
		Phyllobacteraceae	Biofertilizer	Bhattacharyya and Jha, 2012
	Betaproteobacteria	*Oxalobacteraceae*	Produce IAA, ACC, siderophore, and enzymes (chitinase, peptidase, nuclease, phosphatase)	Baldani et al., 2014
		Comamonadaceae	Produce plant growth regulator (e.g., IAA, gibberellin, cytokinins)	Bhattacharyya and Jha, 2012
		Alcaligenaceae	Reduce production of ethylene; induce root formation and development	Bhattacharyya and Jha, 2012
		Burkholderiaceae	BCA by antibiotically poisoning or killing pathogens	Brahmaprakash et al., 2017
			Biofertilizer	Bhattacharyya and Jha, 2012
	Deltaproteobacteria	*Bdellovibrionaceae*	Predator of pathogens	Oyedara et al., 2016
	Gammaproteobacteria	*Pseudomonadaceae*	Solubilizer of P; BCA by competition and producing lytic enzyme to hydrolize pathogen cells; SAR and ISR	Brahmaprakash et al., 2017
			Produce plant growth regulator (e.g., IAA, gibberellin, cytokinins); reduce production of ethylene; induce root formation and development; BCA by nutrient & niche competition; ISR; produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012
		Enterobacteriaceae	Reduce production of ethylene;BCA by nutrient & niche competition; ISR; produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012
		Xanthomonadaceae	BCA by antibiotically poisoning or killing pathogens	Brahmaprakash et al., 2017

[continue]

	Gammaproteobacteria	*Erwiniaceae*	BCA by antibiotically poisoning or killing pathogens	Brahmaprakash et al., 2017
Planctomycetes		*Pirellulaceae*	Promote plant health	Zhang et al., 2017
Ascomycota		*Trichocomaceae*	Solubilizer of P	Brahmaprakash et al., 2017
		Bionectriaceae	BCA as parasite to R. solani and Pythium sp.	Brahmaprakash et al., 2017
		Hypocreaceae	BCA as mycoparasite to plant pathogen	Brahmaprakash et al., 2017
Ascomycota		*Sclerotiniaceae*	Antagonist to fungal pathogen	Brahmaprakash et al., 2017
		Leptospaeriaceae	Antagonist to fungal pathogen	Brahmaprakash et al., 2017
		Phaeosphaeriaceae	BCA as mycoparasite to pathogen of powdery mildew	Brahmaprakash et al., 2017
		Helotiaceae	Support plant absorption ability on water and nutrient from the soils	Cannon and Kirk, 2007
Glomeromycota (Mucoromycota)		*Glomeraceae*	BCA by nutrient and niche competition; ISR; produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012
		Gigasporaceae	BCA by nutrient and niche competition; ISR; produce anti-bacterial/anti-fungal substances	Bhattacharyya and Jha, 2012
Mortierellomycota		*Mortierellaceae*	Assist crops and microbes on P acquisition	Nguyen et al., 2019

(D)

Phylum	Class	Family	Disease	Reference
Proteobacteria	Gammaproteobacteria	*Enterobacteriaceae*	Lettuce root rot (*Erwinia carotovora*)	Bohn, 1953
Ascomycota		*Nectriaceae*	Lettuce root rot (*Fusarium oxysporum* f.sp. *lactucae)*	Matuo and Motohashi, 1967
		Sclerotiniaceae	Lettuce drop (*Sclerotinia sclerotiorum* ; *S. minor*)	Bohn, 1953; Raid et al., 2004
			Gray mould (*Botrytis cinérea*)	Bohn, 1953; Raid et al., 2004
		Hyponectriaceae	Lettuce anthracnose (*Microdochium panattonianum*)	Raid et al., 2004
		Mycosphaerellaceae	Lettuce cercospora leaf spot (*Cercospora* sp.)	Raid et al., 2004
Basidiomycota		*Ceratobasidiaceae*	Lettuce bottom rot (*Rhizoctonia solani*)	Bohn, 1953; Raid et al., 2004
		Atheliaceae	Lettuce southern blight (*Sclerotium rolfsii* Sacc.)	Raid et al., 2004

Table S5.2. Lettuce yield and nitrogen uptake (N_{up}) at the mid growth stage of plant in the pot experiments (Chapter 3)

	Treatment	Yield (g FW plant^{-1})	N_{up} (g N plant^{-1})
2017	Control	109 ± 4.38 b	0.17 ± 0.06 ab
	Rye	128 ± 6.16 a	0.25 ± 0.06 ab
	Rye+HV	119 ± 4.57 ab	0.29 ± 0.03 a
	HV	88.6 ± 5.45 c	0.13 ± 0.02 b
2018	Control	21.8 ± 3.47 b	0.05 ± 0.02 b
	Rye	22.8 ± 2.50 b	0.05 ± 0.00 b
	Rye+HV	31.1 ± 3.21 a	0.10 ± 0.01 a
	HV	29.1 ± 2.04 ab	0.09 ± 0.01 a

Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. The mid growth stage was at 25 and 20 days after cover crops incorporation in 2017 and 2018, respectively. FW and N abbreviate fresh weight and nitrogen, respectively. Mean ± standard deviation is followed by different letters denoting the significance in each year based on Tukey's HSD at $P < 0.05$ (n = 3).

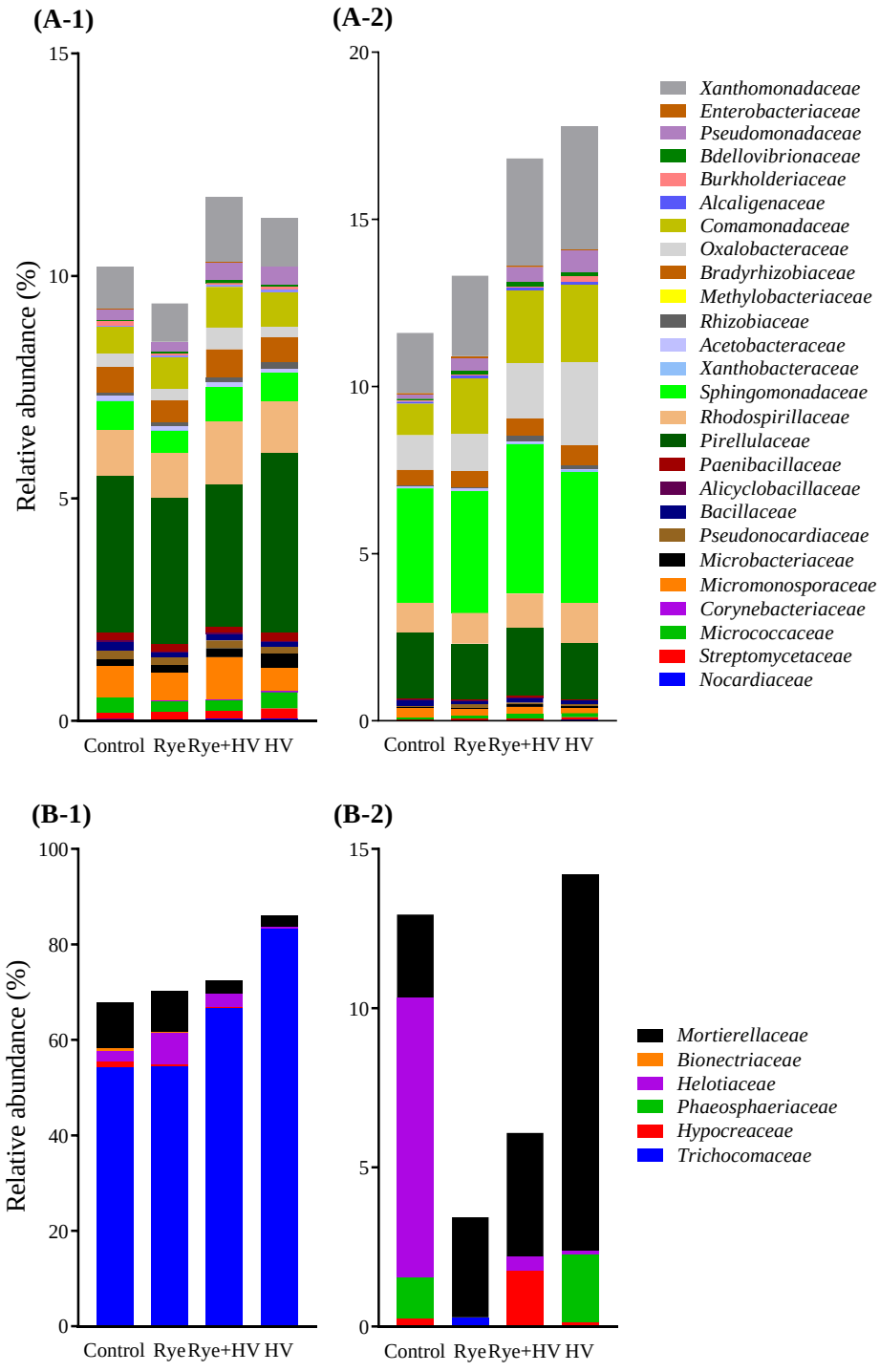


Fig.S5.1. Relative abundance of selected beneficial (A) bacteria and (B) fungi in lettuce rhizosphere soils in family level at (1) 25 and (2) 20 days after cover crops incorporation in 2017 and 2018 pot experiments, respectively. Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops.

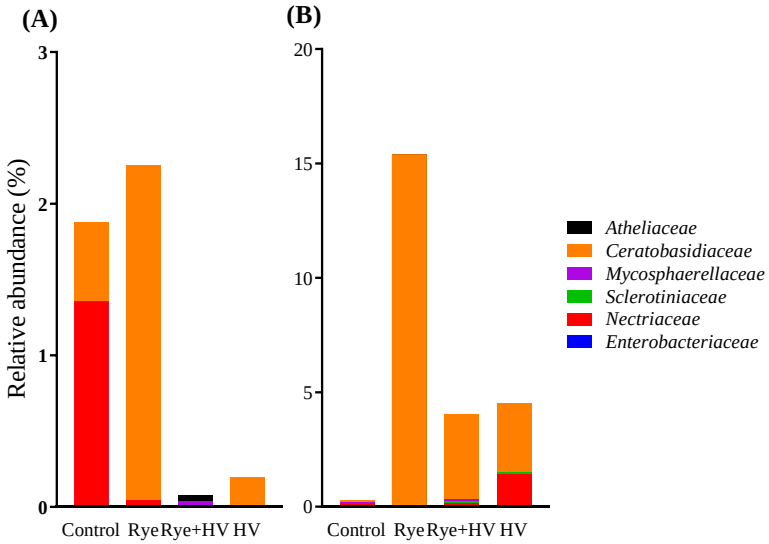


Fig.S5.2. Relative abundance of selected plant pathogen of lettuce rhizosphere soils at (A) 25 and (B) 20 days after cover crop incorporation in 2017 and 2018 pot experiments, respectively. Treatments were soil without any cover crops (control) and soils with rye, mix (rye+HV), and hairy vetch (HV) cover crops.

Table S5.3. Number of links in the microbe–microbe correlation networks in lettuce rhizosphere soils

	Treatment	B–B		F–F		B–F	
		Positive	Negative	Positive	Negative	Positive	Negative
2017	Control	364	341	26	10	175	142
	Rye	287	332	35	14	138	133
	Rye+HV	346	359	14	17	166	150
	HV	319	324	74	13	105	64
2018	Control	326	316	159	17	266	170
	Rye	286	254	23	0	81	91
	Rye+HV	275	268	141	11	238	129
	HV	269	273	78	1	195	127

Treatments were soil without any cover crops (control) and soils with rye, mixed (rye+HV), and hairy vetch (HV) cover crops. Data were collected at 25 and 20 days after cover crop incorporation in 2017 and 2018 pot experiments, respectively. The B–B, F–F, and B–F denote correlations within bacteria, within fungi, and between bacteria and fungi, respectively. The positive and negative links were the strong ($R^2 \leq -0.99$ or $R^2 \geq 0.99$) and significant ($P < 0.05$) correlations.

CHAPTER 6

General discussion

6.1. Supply of soil inorganic N from cover crops mediated by microorganisms existing in bulk soils

In this study, the residual quality-based effects of hairy vetch (HV), rye, and mix of rye and HV (rye+HV) cover crops on the inorganic nitrogen (N) supply were clarified during the decomposition phase of the residues (i.e., within 38 days, Chapter 3). The clarifications were based on several previous studies that have conducted in longer periods (Kuo and Sainju 1998; Kuo et al., 2001; Sainju et al., 2001; Lawson et al., 2012). Therefore, the results highlighted the targeted functions of cover crops, specifically the functions in supplying N in the early periods post incorporation of the residues.

Furthermore, application of HV and rye+HV in the fields could alternate application of synthetic N fertilizer (Chapter 3), leading to an environmentally friendly vegetable cultivation system (Robačer et al., 2016; Chahal and Eerd, 2019; Brust, 2019).

The effects of cover crop residues on stimulating microbial properties in bulk soils were also clarified that were in accordance with several previous researches (Sugihara et al., 2010; Li et al., 2014; Liang et al., 2014; Maul et al., 2014; Mbuthia et al., 2015; Detheridge et al., 2016; Brennan and Acosta-Martinez, 2017; Zheng et al., 2018; Schmidt et al., 2019). Specifically, the β-glucosidase enzyme (BG) activity and soil microbial biomass (SMB) were sequentially promoted in bulk soils following HV cover crop introduction (Chapter 3). The sequential peaks agreed to Li et al. (2014) and were interpreted as the pathways of bulk soil microorganisms in mineralizing N from the cover crop residues to the soils as concluded by other researchers (Nannipieri et al., 1990; Rosecrance et al., 2000; Sugihara et al., 2010; Kuzyakov and Blagodatskaya, 2015; McDaniel and Grandy, 2016).

The microbiome data in Chapter 4 provide the evidences that some bacterial and

fungal taxa were correlated with BG activity, SMB, soil pH, and soil inorganic N in each cover crop-treated soil. The significant correlations indicate microbial roles on N mineralization from cover crop residues (Fernandez et al., 2016a; Drost et al., 2020; Khan et al., 2020). Moreover, the roles were fluctuated (Banerjee et al., 2016; Detheridge et al., 2016; Nevins et al., 2018; Schmidt et al., 2019), regarding to the correlations that were either dependent or independent from their relative abundances (Chapter 4). The final results of the inorganic N supply from the cover crops, however, were consistent every year.

Although the results of this study could not calculate the required cost on application of cover crops (Daryanto et al., 2019), the results could be used as the microbiome-based cover crop selection for cover crop-vegetable rotation systems.

Monoculture system of horticulture has potentially declined plant productivity. The risk may be absent in cover crop-vegetable rotation. It is due to the benefits of cover crops to improve soil fertility, defining as the improvements on nutrient supply and beneficial microbial properties, that has demonstrated in this study.

6.2. Effects of cover crops on lettuce productivity assisted by microorganisms inhabiting lettuce rhizosphere soils

The superior benefits of HV cover crop to the production and N utilization of the subsequent plants during the decomposition periods in comparison with rye cover crop were confirmed. The results were corresponding with several previous studies in the longer periods that demonstrate that HV cover crop is a tool for achieving the sustainable agriculture systems (Benincasa et al., 2011; Bottoms et al., 2012; Caliskan et al., 2014; Maul et al., 2014; Li et al., 2016; Fernandez et al., 2016a; Muchanga et al., 2017; Zhang et al., 2017).

Uniquely, the effects of HV was soil carbon dependent (Gallet and Keller, 1999) in the current study, wherein lettuce yield and N uptake (N_{up}) at the mid growth stage of plant were inhibited and promoted in soils with 6.89% and 4.74% initial soil carbon, respectively (Chapter 3). Trough detail observation of microbial properties in lettuce rhizosphere soils, fungal richness and diversity of the poorly

grown lettuce plants were declined (Chapter 5). Moreover, functions of the rhizosphere bacteria and fungi were disturbed as the significantly negative correlations with lettuce N_{up} and yield. The results lead to the importance of plant–microbe communications in the plant rhizosphere soils during the plant growth (Maloney et al., 1997; Smalla et al., 2001; Buyer et al., 2010; Ai et al., 2012; Brahmaprakash et al., 2017; Samad et al., 2019).

In fact, lettuce plants have recovered between the mid growth stage and the mature stage, resulting in the promotion on lettuce N_{up} and yield at the end of the experiments under HV treatment (Chapter 3). According to the results at the final harvest time, i.e., at the lettuce mature stages in this study, the strategy to shorten the interval from 2 weeks to 5 days and cultivate lettuce plants was acceptable in order to maximize the utilization of inorganic N supplied from cover crops.

Interestingly, rye+HV resulted in the consistent promotion on lettuce N_{up} and yield in both plant growth stages in the two experimental years (Chapter 3). The promotions in rye+HV treatment were not followed by either the richer or more diverse rhizosphere microbiome (Chapter 5), which was in contrast with the results of several previous studies (Buyer and Kaufman, 1997; Buyer et al., 2010; Tian et al., 2013; Brahmaprakash et al., 2017; Manici et al., 2018). However, pathways of rhizosphere microbiome in the promotions were found as their successive beneficial roles shown in the plant–microbe networks under rye+HV treatment (Chapter 5). These results highlighted the significance of how plant–microbe interactions characterize plant health and productivity (Bonfante and Anca, 2009; Nihorimbere et al., 2011; Haldar and Sengupta, 2015; Poudel et al., 2016; de Vries and Wallenstein, 2017; Wallenstein, 2017; Samad et al., 2019).

In the final, rye+HV could be the most recommended cover crop among the three different cover crops for the purpose of N supply and N utilization during the decomposition periods studied currently. It is regarding to the several benefits of rye+HV found in this study:

1) The inorganic N supplied from rye+HV residue was efficiently utilized by lettuce plants in both plant growth stages in the two experimental years in the

fields and pots (Chapter 3). Moreover, this effect of rye+HV ignored status of the initial content of soil carbon, showing the applicability of the cover crop in broad soil properties.

2) The N utilization efficiency under rye+HV treatment resulting in a low potential of N lost (Chapter 4). This may control N pollution to the environment.
3) Inclusion of rye+HV promoted fungal richness, diversity, and functions in bulk soils, which played roles in the N supply (Chapter 4). The promotions may also become the indicators of an increase in soil fertility.
4) The rye+HV consistently stimulated the subsequent plants to recruit and depend on the beneficial effects of rhizosphere microbiomes on its N utilization (Chapter 5). The consistent promotions may impact to the sustainable plant production in cover cropping system.

It is a challenging, however, to consistently produce the balance ratio of rye:HV biomass (Kuo and Sainju, 1998), e.g., 66:34 in the fields, every year. Therefore, additional strategies are required during the preparation periods, e.g., increase the seeding ratio of HV and arrange the seeding timings (Lawson et al., 2015) in the fields.

CHAPTER 7

Limitation and recommendation for further research

7.1. Application of cover crops in other soil types

The current study was conducted in Calcaric, Eutric Fluvisol light clay as similar with the previous studies (Araki et al., 2009; Sugihara et al., 2016; Muchanga et al., 2017; Muchanga et al., 2019). It has been known that cover crops are practiced and studied worldwide in broad types of soil, such as those listed in Table 7.1.

The effects of rye, HV, and rye+HV cover crops on the N availability and vegetable production might be similar in broad types of soil due to the cover crop quality (Kuo et al., 2001; Sainju et al., 2001; Araki et al., 2009; Brainard et al., 2012; Lee et al., 2014; Fraiser et al., 2017; Muchanga et al., 2017; Muchanga et al., 2019). However, the process of residual decomposition, N mineralization, and N utilization by the subsequent plants may relate to different specific microorganisms. It is due to the various initial soil properties that vary compositions of the indigenous soil microorganisms.

Therefore, it is recommended to do studies with similar focus in different soil types. The results would add the knowledge about the relationship between cover crops and soil microbial functions during decomposition and N mineralization periods of cover crops.

Table 7.1. List of soil types used for the applications of cover crops

No.	Soil type	Location	Reference
1	Sultan silt loam (fine-silt, mixed, mesic Aquic Xerofluent)	Puyallup, Washington	Kuo et al., 2001
2	Silt clay loam	National Academy of Agricultural Science Experimental Farm, Suwon, South Korea	Lee et al., 2014
3	Lexington silt loam (fine-silty, mixed, thermic, Ultic Hapludalf)	West Tennessee Research and Education Center (WTREC), Tennessee	Mbuthia et al., 2015
4	Silt loam Haplustalfs	Weibei Dryland Experimental Station of Northwest A&F University	Zheng et al., 2018
5	Greenville fine sandy loam (fine-loamy, kaolinitic, thermic, Rhodic Kandiudults)	Fort Valley, Georgia	Sainju et al., 2001
6	Sandy loam	INTA Experimental Station at Anguil, La Pampa, Argentina	Fraiser et al., 2017
7	Spinks loamy sand soil (sandy, mixed, mesic Lamellic Hapludalf)	Michigan State University Horticulture Teaching and Research Center	Brainard et al., 2012
8	Fine-loamy mixed thermic	Central Italy	Manici et al., 2018
9	Gleyic, Mollic, Umbric Andosol clay loam	Field Science Center, Ibaraki University College of Agriculture, Japan	Nakamoto et al., 2012
10	Panoche clay loam (fine-loamy, mixed superactive, thermic Typic Haplocambids)	University of California's West Side Research and Extension Center	Schmidt et al., 2019
11	Haplaquent with clay loam	Gyeongsang National University Experimental Farm, Jinju, South Korea	Khan et al., 2020

7.2. Incubation and isotopic experiments

The current study was established in the cover crop-lettuce rotation system to understand the direct relationships between cover crops, soil microorganisms, and the subsequent plant. The N availability from the cover crops and N utilization by the subsequent plant were well estimated. However, the process of N mineralization and the assistance of N_{up} from the soil to the plant is found to be a complex structure

under many environmental factors such as soil physicochemical properties, temperature, and soil moisture related to the rainfall as shown in the field and pot experiments in the current study. Therefore, incubation experiments are recommended to obtain clearer relationships.

A deeper understanding of the N budget in cover crop-treated bulk soils during the crucial periods of N release can also be obtained through an isotopic method as recommended by Asagi and Ueno (2009a) and Sugihara et al. (2016). The method using stable isotopic of ^{15}N directly is expected to estimate the N mobilization from cover crop residues to the bulk soils and subsequent plants.

The current study also demonstrated the potential of microbial roles in the N supply and N_{up}. The potential roles can be confirmed as the actual roles by quantifying the actual abundance and identifying the active microbial taxa with the functions, which were not performed in this study. One of the methods is stable isotope probing applied to the soil microorganisms (Kroeger and Nüsslein, 2019). Specifically, stable isotopes of ^{12}C or ^{13}C and ^{15}N are added to the soils, which are then incubated. The labelled DNA or RNA were extracted and molecularly analyzed through the qPCR and sequencing. The other method is spike-in sequences (Smets et al., 2016; Tkacz et al., 2018; Jiang et al., 2019). In detail, internal standard or synthetic spikes are inserted during the DNA extraction steps. At the bioinformatic steps, calculation of the absolute abundance of amplicon families of the 16S rRNA and ITS per unit mass of sample is allowed. Hence, the absolute microbial roles would be identified.

7.3. Different application methods of cover crops

Incorporation of fresh cover crop residues was practiced in the current study to quickly regulate the inorganic N into soils, due to the short cultivation periods in Hokkaido regions. In other regions, cover crop residues are also practiced in other techniques. For instance, Buyer et al. (2010), Zheng et al. (2018), and Manici et al. (2018), who left the cover crop biomass on the soil surface as a mulch. Miura and Watanabe (2002), Asagi and Ueno (2009b), and Warren et al. (2015), who used cover crops as living mulch in sweet corn, rice, and broccoli fields, respectively.

Kuo and Sainju (1998), Asagi and Ueno (2009a), Ito et al. (2014), and Frøseth et al. (2014), who incorporated dried biomass of cover crops into the soil.

Soil microorganisms in bulk may variously respond to the different residual location (i.e., inside or surface of soils) and condition (i.e., wet or dry). A slow regulation rates of inorganic N can occur in cover crop mulch-treated soil, but a high soil moisture can be kept for long periods (Buyer et al., 2010; Zheng et al., 2018; Manici et al., 2018) that maintain soil microbial abundance and function. Dry biomass of cover crops also results in a slow N regulation, due to the decrease in water content of the residue (Lawson et al., 2012; Nevins et al., 2018). In some areas in Japan with higher air temperature, however, application methods using either surface mulching or dry biomass are suitable (Asagi and Ueno, 2009a; Ito et al., 2014). In term of the living mulch of cover crops, the technique is a challenging for microbiologist and agronomist due to the more complex relationships between the belowground and aboveground in the cultivation system.

Regarding to the various application methods of cover crops in order to adjust the service to the various environments, it is recommended to evaluate the roles of soil microorganisms in the N supply and N utilization by the subsequent plant under different application techniques of cover crops.

List of references

1. Adetunji, A.T., Lewu, F.B., Mulidzi, R., Ncube, B. 2017. The biological activities of β-glucosidase, phosphatase and urease as soil quality indicators: a review. Journal of Soil Science and Plant Nutrition 17:794–807. http://dx.doi.org/10.4067/S0718-95162017000300018
2. Ai, C., Liang, G., Sun, J., Wang, X., Zhou, W. 2012. Responses of extracellular enzyme activities and microbial community in both the rhizosphere and bulk soil to long-term fertilization practices in a fluvo-aquic soil. Geoderma 173–174:330–338. https://doi.org/10.1016/j.geoderma.2011.07.020
3. Andriolo, J.L., Godoi, R.d.S., Cogo, C.M., Bortolotto, O.C., Luz, G.L.d., Madaloz, J.C. 2006. Growth and development of lettuce plants at high NH_4^+:NO_3^- ratios in the nutrient solution. Horticultura Brasileira 24:352–355. http://doi.org/10.1590/S0102-05362006000300016
4. Araki, H., Hane, S., Hoshino, Y., Hirata, T. 2009. Cover crop use in tomato production in plastic high tunnel. Horticulture, Environment, and Biotechnology 50:324–328.
5. Asagi, N., Ueno, H. 2009a. Nitrogen dynamics in paddy soil applied with various ^{15}N-labelled green manures. Plant Soil 322:251–262. http://doi.org/10.1007/s11104-009-9913-4
6. Asagi, N., Ueno, H. 2009b. Effects of legume incorporation of living mulch on rice growth, yield and soil inorganic nitrogen in Southwest Japan. Japanese Journal of Farm Work Research 44:127–136. https://doi.org/10.4035/jsfwr.44.127
7. Ashworth, A.J., DeBruyn, J.M., Allen, F.L., Radosevich, M., Owens, P.R. 2017. Microbial community structure is affected by cropping sequences and poultry litter under long-term no-tillage. Soil Biology and Biochemistry 114:210–219. https://doi.org/10.1016/j.soilbio.2017.07.019
8. Bailey, V.L., Smith, J.L., Bolton Jr, H. 2002. Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. Soil Biology and Biochemistry 34:997–1007. https://doi.org/10.1016/S0038-0717(02)00033-0.
9. Banerjee, S., Kirkby, C.A., Schmutter, D., Bissett, A., Kirkegaard, J.A., Richardson, A.E. 2016. Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. Soil Biology and Biochemistry 97:188–198. http://dx.doi.org/10.1016/j.soilbio.2016.03.017

10. Barel, J.M., Kuyper, T.W., Paul, J., de Boer, W., Cornelissen, J.H.C., Deyn, G.B.D. 2019. Winter cover crop legacy effects on litter decomposition act through litter quality and microbial community changes. Journal of Applied Ecology 56:132–143. https://doi.org/10.1111/1365-2664.13261.
11. Battacharyya, P.N., Jha, D.K. 2012. Plant growth-promoting rhizosbacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology 28:1327–1350. https://doi.org/10.1007/s11274-011-0979-9
12. Benincasa, P., Guiducci, M., Tei, F. 2011. The nitrogen use efficiency: meaning and sources of variation-case studies on three vegetable crops in central Italy. HortTechnology 21:266–273. https://doi.org/10.21273/HORTTECH.21.3.266.
13. Bian, X., Xiao, S., Zhao, Y., Xu, Y., Yang, H., Zhang, L. 2020. Comparative analysis of rhizosphere soil physiochemical characteristics and microbial communities between rusty and healthy ginseng root. Scientific Reports 10:15756. https://doi.org/10.1038/s41598-020-71024-8
14. Bohn, G.W. The important diseases of lettuce. U.S. Department of Agriculture Yearbook 2452: 417–425. https://naldc.nal.usda.gov/download/IND43894356/PDF
15. Bonfante, P., Anca, I.-A. 2009. Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annual Review of Microbiology 63:363–83. https://doi.org/10.1146/annurev.micro.091208.073504
16. Borowik, A., Wyszkowska. 2016. Soil moisture as a factor affecting the microbiological and biochemical activity of soil. Plant Soil Environment 62:250–255. https://doi.org/10.17221/158/2016-PSE.
17. Bottoms, T.G., Smith, R.F., Cahn, M.D., Hartz, T.K. 2012. Nitrogen requirements and N status determination of lettuce. HortScience (2012) 47:1768–1774. https://doi.org/10.21273/HORTSCI.47.12.1768.
18. Bradford, M.A., Tordoff, G.M., Eggers, T., Jones, T.H., Newington, J.E. 2002. Microbiota, fauna and mesh size interactions in litter decomposition. OIKOS 99:317–323. https://doi.org/10.1034/j.1600-0706.2002.990212.x
19. Brahmaprakash, G.P., Sahu, P.K., Lavanya, G., Nair, S.S., Gangaraddi, V.K., Gupta, A. 2017. Microbial functions of the rhizosphere. In: Singh, D.P., Singh, H.B., Ratna, P. (eds.) Plant-Microbe Interactions in Agro-Ecological Perspective. Springer Nature Singapore Pte Ltd., Singapore pp. 117–209. https://doi.org/10.1007/978-981-10-5813-4_10
20. Braker, G., Fesefeldt, A., Witzel, K.-P. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying

bacteria in environmental samples. Applied and Environmental Microbiology 64:3769–3775. https://doi.org/10.1128/AEM.64.10.3769-3775.1998

21. Brennan, E.R., Acosta-Martinez, V. 2017. Cover cropping frequency is the main driver of soil microbial changes during six years of organic vegetable production. Soil Biology and Biochemistry 109:188–204. http://dx.doi.org/10.1016/j.soilbio.2017.01.014
22. Brown, S.P., Callaham Jr, M.A., Oliver, A.K., Jumpponen, A. 2013. Deep ion torrent sequencing identifies soil fungal community shifts after frequent prescribed fires in a southeastern US forest ecosystem. FEMS Microbiol Ecology 86:557–566. https://doi.org/10.1111/1574-6941.12181
23. Brust, G.E. 2019. Chapter 9 - Management strategies for organic vegetable fertility. In: Biswas, D., Micallef, S.A. (eds) Safety and Practice for Organic Food (2019) pp. 193–212. https://doi.org/10.1016/B978-0-12-812060-6.00009-X
24. Buyer, J.S., Kaufman, D.D. 1996. Microbial diversity in the rhizosphere of corn grown under conventional and low-input systems. Applied Soil Ecology 5:21–27. https://doi.org/10.1016/S0929-1393(96)00127-8
25. Buyer, J.S., Teasdale, J.R., Roberts, D.P., Zasada, I.A., Maul, J.E. 2010. Factors affecting soil microbial community structure in tomato cropping systems. Soil Biology and Biochemistry 42:831–841. http://doi.org/10.1016/j.soilbio.2010.01.020
26. Cai, L., Ye, L., Tong, A.H.Y., Lok, S., Zhang, T. 2013. Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. PLoS ONE 8:e53649. https://doi.org/10.1371/journal.pone.0053649
27. Caliskan, S., Yetisir, H., Karanlik, S. 2014. Combined use of green manure and farmyard manure allows better nutrition of organic lettuce. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 42:248–254. https://doi.org/10.15835/nbha4219328.
28. Cannon, P.F., Kirk, P.M. 2007. Fungal Families of the World. CAB International, Cambridge.
29. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303
30. Carrera, L.M., Buyer, J.S., Vinyard, B., Abdul-Baki, A.A., Sikora, L.J., Teasdale,

J.R. 2007. Effects of cover crops, compost, and manure amendments on soil microbial community structure in tomato production systems. Applied Soil Ecology 37:247–255. https://doi.org/10.1016/j.apsoil.2007.08.003

31. Chahal, I., Eerd, L.L.V. 2019. Quantifying soil quality in a horticultural-cover cropping system. Geoderma 352:38–48. https://doi.org/10.1016/j.geoderma.2019.05.039
32. Chen, B., Liu, E., Tian, Q., Yan, C., Zhang, Y. 2014. Soil nitrogen dynamics and crop residues. A review. Agronomy for Sustainable Development 34:429–442. https://doi.org/10.1007/s13593-014-0207-8
33. Conant, R.T., Drijber, R.A., Haddix, M.L., Parton, W.J., Paul, E.A., Plante, A.F., Six, J., Steinweg, J.M. 2008. Sensitivity of organic matter decomposition to warming varies with its quality. Global Change Biology 14:868–877. https://doi.org/10.1111/j.1365-2486.2008.01541.x
34. Csárdi, G., Nepusz, T., 2006. The igraph software package for complex network research. Inter. J. Complex Sys. 1695:1–9. http://igraph.org
35. Daryanto, S., Jacinthe, P.-A., Fu, B., Zhao, W., Wang, L. 2019. Valuing the ecosystem services of cover crops: barriers and pathways forward. Agriculture, Ecosystem and Environment 270–271:76–78. https://doi.org/10.1016/j.agee.2018.10.021
36. de Almeida, R.F., Naves, E.R., da Mota, R.P. 2015. Soil quality: enzymatic activity of soil β-glucosidase. Global Journal of Agricultura Research and Reviews 3:146–150. http://www.globalscienceresearchjournals.org/
37. de Boer, W., Folman, L.B., Summerbell, R.C. Boddy, L. 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews 29:795–811. https://doi.org/10.1016/j.femsre.2004.11.005
38. Detheridge, A.P., Brand, G., Fychan, R., Crotty, F.V., Sanderson, R., Griffith, G.W., Marley, C.L. 2016. The legacy effect of cover crops on soil fungal populations in a cereal rotation. Agriculture, Ecosystem, and Environment 228:49–61. http://dx.doi.org/10.1016/j.agee.2016.04.022
39. Deveau, A., Bonito, G., Uehling, J., Paoletti, M., Becker, M., Bindschedler, S., Hacquard, S., Hervé, V., Labbé, J., Lastovetsky, O.A., Mieszkin, S., Millet, L.J., Vajna, B., Junier, P., Bonfante, P., Krom, B.P., Olsson, S., van Elsas, J.D., Wick, L.Y. 2018. Bacterial–fungal interactions: ecology, mechanisms and challenges. FEMS Microbiology Reviews 42:335–352. https://doi.org/10.1093/femsre/fuy008
40. de Vries, F.T., Wallenstein, M.D. 2017. Below-ground connections underlying above-ground food production: a framework for optimizing ecological connections

in the rhizosphere. Journal of Ecology 105:913–920. https://doi.org/10.1111/1365-2745.12783.

41. Diacono, M., Montemurro, F. 2010. Long-term effects of organic amendments on soil fertility. A review. Agronomy for Sustainable Development 30:401–422. http://doi.org/10.1051/agro/2009040
42. Dini-Andreote, F. 2019. Soil microbiome data analysis. In: van Elsas, J.D., Trevors, J.T., Rosado, A.S., Nannipieri, P. (eds) Modern Soil Microbiology. CRC Press, Boca Raton, FL. pp. 215–226.
43. Dini-Andreote, F., van Elsas, J.D. 2019. The soil microbiome – An overview. In: van Elsas, J.D., Trevors, J.T., Rosado, A.S., Nannipieri, P. (eds) Modern Soil Microbiology. CRC Press, Boca Raton, FL. pp.37–48.
44. Doran, J.W., Smith, M.S. 1991. Role of cover crops in nitrogen cycling. In: Hargrove, W.L. (eds.), Cover Crops for Clean Water. SWCS, Ankeny, IA. pp. 85–90.
45. Doyle, A., Weintraub, M.N., Schimel, J.P. 2004. Persulfate digestion and simultaneous colorimetric analysis of carbon and nitrogen in soil extracts. Soil Science Society of American Journal. 68:669–676. https://doi.org/10.2136/sssaj2004.0669.
46. Drost, S.M., Rutgers, M., Wouterse, M., de Boer, W., Bodelier, P.L.E. 2020. Decomposition of mixtures of cover crop residues increases microbial functional diversity. Geoderma 361:114060. https://doi.org/10.1016/j.geoderma.2019.114060
47. Drury, C.F., Stone, J.A., Findlay, W.I. 1991. Influence of cover crops on denitrification and nitrogen mineralization. In: Hargrove, W.L. (eds) Cover Crops for Clean Water. SWCS, Ankeny, IA. pp: 94–96.
48. Duca, D., Lorv, J., Patten, C.L., Rose, D., Glick, B.R. 2014. Indole-3-acetic acid in plant–microbe interactions. Antonnie van Leeuwenhoek 106:85–125. https://doi.org/10.1007/s10482-013-0095-y
49. Eivazi, F., Tabatabai, M.A. 1990. Factors affecting glucosidase and galactosidase activities in soils. Soil Biology and Biochemistry 22:891–897. https://doi.org/10.1016/0038-0717(90)90126-K
50. Ercoli, L., Masoni, A., Pampana, S., Arduini, I. 2007. Allelopathic effects of rye, brown mustard and hairy vetch on redroot pigweed, common lambsquarter and knotweed. Allelopathy Journal 19:249–256.
51. Etesami, H., Alikhani, H.A., Hosseini, H.M. 2015. Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent

bacteria for rice growth promoting agents. MethodsX 2:72–78. http://dx.doi.org/10.1016/j.mex.2015.02.008

52. Farrar, K., Bryant, D., Cope-Selby, N. 2014. Understanding and engineering beneficial plant–microbe interactions: plant growth promotion in energy crops. Plant Biotechnology Journal 12:1193–1206. https://doi.org/10.1111/pbi.12279
53. Fernandez, A.L., Sheaffer, C.C., Wyse, D.L., Staley, C., Gould, T.J., Sadowsky, M.J. 2016a. Associations between soil bacterial community structure and nutrient cycling functions in long-term organic farm soils following cover crop and organic fertilizer amendment. Science of the Total Environment 566–567:949–959. http://dx.doi.org/10.1016/j.scitotenv.2016.05.073
54. Fernandez, A.L., Sheaffer, C.C., Wyse, D.L., Staley, C., Gould, T.J., Sadowsky, M.J. 2016b. Structure of bacterial communities in soil following cover crop and organic fertilizer incorporation. Applied Microbiology and Biotechnology 100:9331–9341. https://doi.org/10.1007/s00253-016-7736-9
55. Fierer, N., Bradford, M.A., Jackson, R.B. 2007. Toward an ecological classification of soil bacteria. Ecology 88:1354–1364. https://doi.org/10.1890/05-1839
56. Filion, M., St-Arnaud, M., Jajabi-Hare, S.H. 2003. Direct quantification of fungal DNA from soil substrate using real-time PCR. Journal of Microbiological Methods 53:67–76. https://doi.org/10.1016/S0167-7012(02)00225-7
57. Finlay, R.D., Thorn, R.G. 2019. The fungi in soil. In: van Elsas, J.D., Trevors, J.T., Rosado, A.S., Nannipieri, P. (eds) Modern Soil Microbiology. CRC Press, Boca Raton, FL. pp. 65–90.
58. Fraiser, I., Noellemeyer, E., Amiotti, N., Quiroga, A. 2017. Vetch-rye biculture is a sustainable alternative for enhanced nitrogen availability and low leaching losses in a no-till cover crop system. Field Crops Research 102:104–112. https://doi.org/10.1016/j.fcr.2017.08.016
59. Frøseth, R.B., Bleken, M.A. 2015. Effect of low temperature and soil type on the decomposition rate of soil organic carbon and clover leaves, and related priming effect. Soil Biology and Biochemistry 80:156–166. http://dx.doi.org/10.1016/j.soilbio.2014.10.004
60. Fu, S.-F., Wei, J.-Y., Chen, H.-W., Liu, Y.-Y., Lu, H.-Y., Chou, J.-Y. 2015. Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms. Journal of Plant Signaling and Behavior 10:e1048052. https://doi.org/10.1080/15592324.2015.1048052
61. Gallet, C., Keller, C. 1999. Phenolic composition of soil solutions: comparative study of lysimeter and centrifuge waters. Soil Biology and Biochemistry 31:1151–

1160. https://doi.org/10.1016/S0038-0717(99)00033-4

62. Geddes, C.M., Cavalieri, A., Daayf, F., Gulden, R.H. 2015. The allelopathic potential of hairy vetch (*Vicia villosa* Roth.) mulch. American Journal of Plant Sciences 6:2651–2663. http://dx.doi.org/10.4236/ajps.2015.616267
63. Geisseler, D., Joergense, R.G., Ludwig, B. 2012. Potential soil enzyme activities are decoupled from microbial activity in dry residue-amended soil. Pedobiologia 55:253–261. https://doi.org/10.1016/j.pedobi.2012.04.003
64. Gobran, G.R., Clegg, S. 1996. A conceptual model for nutrient availability in the mineral soil-root system. Canadian Journal of Soil Science 76:125–131. https://doi.org/10.4141/cjss96-019
65. Groβkopf, T., Soyer, O.S. 2014. Synthetic microbial communities. Current Opinion in Microbiology 18:72–77. http://dx.doi.org/10.1016/j.mib.2014.02.002
66. Haldar, S., Sengupta, S. 2015. Plant-microbe cross-talk in the rhizosphere: insight and biotechnological potential. The Open Microbiology Journal 9:1–7. https://doi.org/10.2174/1874285801509010001
67. Hamid, R., Khan, M.A., Ahmad, M., Ahmad, M.M., Abdin, M.Z., Musarrat, J., Javed, S. 2013. Chitinases: An update. Journal of Pharmacy and Bioallied Sciences 5:21–29. http://doi.org/10.4103/0975-7406.106559
68. Hardie, M., Doyle, R. 2012. Measuring soil salinity. In: Shabala, S., Cuin, T.A. (eds) Plant Salt Tolerance. Methods in Molecular Biology (Methods and Protocols) vol 913. Humana Press, Totowa, NJ. pp. 415–425. https://doi.org/10.1007/978-1-61779-986-0_28.
69. Hayano, K. 1973. A method for the determination of β-glucosidase activity in soil. Journal of Soil Science and Plant Nutrition. 19:103–108. https://doi.org/10.1080/00380768.1973.10432524
70. Hayatsu, M., K Tago, M Saito. 2008. Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Science and Plant Nutrition 54:33–45. https://doi.org/10.1111/j.1747-0765.2007.00195.x
71. He, Z.L., Yang, X.E., Baligar, V.C., Calvert, D.V. 2003. Microbiological and biochemical indexing systems for assessing quality of acid soils. Advances in Agronomy 78:89–138. https://doi.org/10.1016/S0065-2113(02)78003-6
72. Heller, P., Tripp, H.J., Turk-Kubo, K., Zehr, J.P. 2014. ARBitrator: A software pipeline for on-demand retrieval of auto-curated nifH sequences from GenBank. Bioinformatics 30:2883-90. http://doi.org/10.1093/bioinformatics/btu417
73. Hill, E.C., Ngouajio, M., Nair, M.G. 2007. Allelopathic potential of hairy vetch

(*Vicia villosa*) and cowpea (*Vigna unguiculata*) methanol and ethyl acetate extracts on weeds and vegetables. Weed Technology 21:437–444. https://www.jstor.org/stable/4495873

74. Hoque, M.M., Ajwa, H., Othman, M. 2010. Yield and postharvest quality of lettuce in response to nitrogen, phosphorus, and potassium fertilizers. HortScience 45:1539–1544. https://doi.org/10.21273/HORTSCI.45.10.1539.
75. Hu, S.J., van Bruggen, A.H.C., Grünwald, N.J. 1999. Dynamics of bacterial populations in relation to carbon availability in a residue-amended soil. Applied Soil Ecology 13:21–30. https://doi.org/10.1016/S0929-1393(99)00015-3
76. Ito, T., Araki, M., Komatsuzaki, M., Kaneko, N., Ohta, H. 2014. Soil nematode community structure affected by tillage systems and cover crop managements in organic soybean production. Applied Soil Ecology 86:137–147. http://dx.doi.org/10.1016/j.apsoil.2014.10.003
77. Jiang, S.-Q., Yu, Y.-N., Gao, R.-W., Wang, H., Zhang, J., Li, R., Long, X.-H., Shen, Q.-R., Chen, W., Cai, F. 2019. High-throughput absolute quantification sequencing reveals the effect of different fertilizer applications on bacterial community in a tomato cultivated coastal saline soil. Science of the Total Environment 687:601–609. https://doi.org/10.1016/j.scitotenv.2019.06.105
78. Jin, T., Zhang, T., Yan, Q. 2010. Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR. Applied Microbiology and Biotechnology 87:1167–1176. https://doi.org/10.1007/s00253-010-2595-2
79. Jones, H.A. 1927. Pollination and life history studies of lettuce (*Lactuca sativa* L.). Hilgardia 2:425–479. https://doi.org/10.3733/hilg.v02n13p425
80. Khan, M.I., Gwon, H.S., Alam, M.A., Song, H.J., Das, S., Kim, P.J. 2020. Short term effects of different green manure amendments on the composition of main microbial groups and microbial activity of a submerged rice cropping system. Applied Soil Ecology 147:103400. https://doi.org/10.1016/j.apsoil.2019.103400
81. Kim, M.J., Moon, Y., Tou, J.C., Mou, B., Waterland, N.L. 2016. Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). Journal of Food Composition and Analysis 49:19–34. http://dx.doi.org/10.1016/j.jfca.2016.03.004
82. Kim, N., Zabaloy, M.C., Guan, K., Villamil, M.B. 2020. Do cover crops benefit soil microbiome? A meta-analysis of current research. Soil Biology and Biochemistry 142:107701. https://doi.org/10.1016/j.soilbio.2019.107701
83. Komatsuzaki, M. 2011. Agro-ecological approach for developing a sustainable

farming and food system. Journal of Developments in Sustainable Agriculture 6:54–63. https://doi.org/10.11178/jsda.6.54.

84. Komatsuzaki, M., Ohta, H. 2013. Sustainable agriculture practices. In: Osaki, M., Braimoh, A.K., Nakagami, K. (eds) Designing Our Future: Local Perspective on Bioproduction, Ecosystems and Humanity. pp. 38–49. htpps://doi.org/10.18356/4f898e93-en.
85. Komatsuzaki, M., Ito, T., Zhao, T., Araki, H. 2020. Cover crop farming system. In: Tojo, S. (eds) Recycle Based Organic Agriculture in a City. pp. 159–172. https://doi.org/10.1007/978-981-32-9872-9_8.
86. Koranda, M., Kaiser, C., Fchslueger, L., Kitzler, B., Sessitsch, A., Zechmeister-Boltenstern, S., Richter, A. 2014. Fungal and bacterial utilization of organic substrates depends on substrate complexity and N availability. FEMS Microbiology Ecology 87:142–152. https://doi.org/10.1111/1574-6941.12214
87. Kuo, S., Sainju, U.M. 1998. Nitrogen mineralization and availability of mixed leguminous and non-leguminous cover crop residues in soil. Biology and Fertility of Soils 26:346–353. https://doi.org/10.1007/s003740050387
88. Kuo, S., Huang, B., Bembenek, R. 2001. Effect of winter cover crops on soil nitrogen availability, corn yield, and nitrate leaching. The Scientific World Journal 1:22–29. https://doi.org/10.1100/tsw.2001.267
89. Kuzyakov, Y., Blagodatskaya, E. 2015. Microbial hotspots and hot moments in soil: Concept and review. Soil Biol and Biochemistry 83:184–199. http://dx.doi.org/10.1016/j.soilbio.2015.01.025
90. Landriscini, M.R., Galantini, J.A., Duval, M.E., Capurro, J.E. 2019. Nitrogen balance in a plant-soil system under different cover crop-soybean cropping in Argentina. Applied Soil Ecology 133:124–131. https://doi.org/10.1016/j.apsoil.2018.10.005
91. Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V., Knight, R, Beiko, R.G., Huttenhower, C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotechnology 31:814–823. https://doi.org/10.1038/nbt.2676
92. Lawson, A., Fortuna, A.M., Cogger, C., Bary, A., Stubbs, T. 2012. Nitrogen contribution of rye–hairy vetch cover crop mixtures to organically grown sweet corn. Renewable Agriculture and Food Systems 28:59–69. https://doi.org/10.1017/S1742170512000014.

93. Layton, A.C., Dionisi, H., Kuo, H.-W., Robinson, K.G., Garrett, V.M., Meyers, A., Sayler, G.S. 2005. Emergence of competitive dominant ammonia-oxidizing bacterial populations in a full-scale industrial wastewater treatment plant. Applied and Environmental Microbiology 71:1105–1108. https://doi.org/10.1128/AEM.71.2.1105–1108.2005.
94. Lee, S., Yun, H., Gu, M., Sung, J., An, N., Lee, B., Cho, J., Jung, S., Choi, H. 2014. Effects of hairy vetch, rye, and alternating cultivation of rye-vetch cover crops on soil nutrient concentrations and the production of red pepper (*Capsicum annuum* L.). The Journal of Horticultural Science and Biotechnology 89:245–252. https://doi.org/10.1080/14620316.2014.11513075
95. Levy-Booth, D.J., Prescott, C.E., Grayston, S.J. 2014. Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. Soil Biology and Biochemistry 75:11–25. http://dx.doi.org/10.1016/j.soilbio.2014.03.021
96. Lévy-Frébault, V., Grandry, J., David, H.L. 1982. Evaluation of rapid tests for the identification of Mycobacteria. Journal of Medical Microbiology 15:575–577. https://doi.org/10.1099/00222615-15-4-575
97. Li, Y.-T., Rouland, C., Benedetti, M., Li, F., Pando, A., Lavelle, P., Dai, J. 2009. Microbial biomass, enzyme, and mineralization activity in relation to soil organic C, N, and P turnover influenced by acid metal stress. Soil Biology and Biochemistry 41:969–977. https://doi.org/10.1016/j.soilbio.2009.01.021
98. Li, W., Pan, K.W., Wu, N., Wang, J.C., Wang, Y.J., Zhang, L. 2014. Effect of litter type on soil microbial parameters and dissolved organic carbon in a laboratory microcosm experiment. Plant, Soil and Environment 60:170–176. https://doi.org/10.17221/39/2014-PSE
99. Li, J., Nedwell, D.B., Beddow, J., Dumbrell, A.J., McKew, B.A., Thorpe, E.L., Whitby, C. 2015. *amoA* gene abundances and nitrification potential rates suggest that benthic ammonia-oxidizing bacteria and not archaea dominate N cycling in the Colne Estuary, United Kingdom. Applied and Environmental Microbiology 81:159–165. https://doi.org/10.1128/AEM.02654-14.
100. Li, Z., Zu, C., Wang, C., Yang, J., Yu, H., Wu, H. 2016. Different responses of rhizosphere and non-rhizosphere soil microbial communities to consecutive *Piper ningrum* L. monoculture. Scientific Reports 6:35825. https://doi.org/10.1038/srep35825
101. Li, F., Chen, L., Redmile-Gordon, M., Zhang, J., Zhang, C., Ning, Q., Li, W. 2018. *Mortierella elongata*'s roles in organic agriculture and crop growth promotion in a

mineral soil. Land Degradation and Development 29:1642–1651. https://doi.org/10.1002/ldr.2965

102. Li, L.-J., Zhu-Barker, X., Ye, R., Doane, T.A., Horwath, W.R. 2018. Soil microbial biomass size and soil carbon influence the priming effect from carbon inputs depending on nitrogen availability. Soil Biology and Biochemistry 119:41–49. https://doi.org/10.1016/j.soilbio.2018.01.003.
103. Liang, S., Grossman, J., Shi, W. 2014. Soil microbial responses to winter legume cover crop management during organic transition. European Journal of Soil Biology 65:15–22. https://doi.org/10.1016/j.ejsobi.2014.08.007
104. Lindsay, E.A., Colloff, M.J., Gibb, N.L., Wakelin, S.A. 2010. The abundance of microbial functional genes in grassy woodlands is influences more by soil nutrient enrichment than by recent weed invasion or livestock exclusion. Applied and Environmental Microbiology 76:5547–5555. https://doi.org/10.1128/AEM.03054-09
105. López-Pérez, M., Rodruquez-Valera, F. 2014. The family *Alteromonadaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (eds) The Prokaryotes. Springer, Verlag, Berlin, Heidelberg. pp. 69–92. https://doi.org/10.1007/978-3-642-38922-1_233
106. Maloney, P.E., van Bruggen, A.H.C., Hu, S. 1997. Bacterial community structure in relation to the carbon environments in lettuce and tomato rhizospheres and in bulk soil. Microbial Ecology 34:103–117. https://doi.org/10.1007/s002489900040
107. Mampholo, B.M., Maboko, M.M., Soundy, P., Sivakumar, D. 2016. Phytochemicals and overall quality of leafy lettuce (*Lactuca sativa* L.) varieties grown in closed hydroponic system. Journal of Food Quality 39:805–815. http://doi.org/10.1111/jfq.12234
108. Manici, L.M., Caputo, F., Nicoletti, F., Leteo, F., Campanelli, G. 2018. The impact of legume and cereal cover crops on rhizosphere microbial communities of subsequent vegetable crops for contrasting crop decline. Biological Control 120:17–25. http://dx.doi.org/10.1016/j.biocontrol.2016.11.003
109. Marrou, H., Wéry, J., Dufour, L., Dupraz, C. 2013. Productivity and radiation use efficiency of lettuces grown in the partial shade of photovoltaic panels. European Journal of Agronomy 44:54–66. http://dx.doi.org/10.1016/j.eja.2012.08.003.
110. Matuo, T., Motohashi, S. 1967. On *Fusarium oxysporum* f.sp. *lactucae* n.f. causing root rot lettuce. Transactions of the Mycological Society of Japan 8:13–15.
111. Maul, J.E., Buyer, J.S., Lehman, R.M., Culman, S., Blackwood, C.B., Roberts, D.P., Zasada, I.A., Teasdale, J.R. 2014. Microbial community structure and abundance in

the rhizosphere and bulk soil of a tomato cropping system that includes cover crops. Applied Soil Ecology 77:42–50. http://dx.doi.org/10.1016/j.apsoil.2014.01.002

112. Mauro, R.P., Pesce, G.R., Mauromicale, G. 2013. The role of cover crops in agro-ecosystems management. In: Taab, A. (eds) Weeds and Their Ecological Functions, Chapter 5. Nova Science Publishers, Inc. pp. 115–152.
113. Mbuthia, L.W., Acosta-Martínez, V., DeBryun, J., Schaeffer, S., Tyler, D., Odoi, E., Mpheshea, M., Walker, F., Eash, N. 2015. Long term tillage, cover crop, and fertilization effects on microbial community structure, activity: implications for soil quality. Soil Biology and Biochemistry 89:24–34. http://dx.doi.org/10.1016/j.soilbio.2015.06.016.
114. McBride, M.J., Liu, W., Lu, X., Zhu, Y., Zhang, W. 2014. The family *Cytophagaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (eds) The Prokaryotes. Springer, Verlag, Berlin, Heidelberg. pp. 577–593. https://doi.org/10.1007/978-3-642-38954-2_382
115. McDaniel, M.D., Grandy, A.S. 2016. Soil microbial biomass and function are altered by 12 years of crop rotation. Soil 2:583–299. https://doi.org/10.5194/soil-2-583-2016
116. McIlvaine, T.C. 1921. A buffer solution for colorimetric comparison. Journal of Biological Chemistry 49:183–186. http://www.jbc.org/content/49/1/183.short.
117. Mendes, R., Garbeva, P., Raaijmakers, J.M. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiology Reviews 37:634–663. http://doi.org/10.1111/1574-6976.12028
118. Mendes, L.W., Raaijmakers, J.M., de Hollander, M., Mendes, R., Tsai, S.M. 2018. Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. The ISME Journal 12:212–224. http://doi.org/10.1038/ismej.2017
119. Mijangos, I., Becerril, J.M., Albizu, I., Epelde, L., Garbisu, C. 2009. Effect of glyphosate on rhizosphere soil microbial communities under two different plant compositions by cultivation-dependent and -independent methodologies. Soil Biology and Biochemistry 41:505–513. https://doi.org/10.1016/j.soilbio.2008.12.009
120. Miller, D.N., Bryant, J.E., Madsen, E.L., Ghiorse, W.C. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and Environmental Microbiology 65:4715–4724. https://doi.org/10.1128/AEM.65.11.4715-4724.1999

121. Miransari, M. 2013. Soil microbes and the availability of soil nutrients. Acta Physiologiae Plantarum 35:3075–3084. https://doi.org/10.1007/s11738-013-1338-2
122. Miura, S., Watanabe, Y. 2002. Growth and yield of sweet corn with legume living mulches. Japanese Journal of Crop Science 71:36–42. https://doi.org/10.1626/jcs.71.36
123. Miyashita, K., Fujii, T. 1993. Nucleotide sequence and analysis of a gene (*chiA*) for a chitinase from *Streptomyces lividans* 66. Bioscience, Biotechnology, and Biochemistry 57:1691–1698. https://doi.org/10.1271/bbb.57.1691
124. Moinet, G.Y.K., Moinet, M., Hunt, J.E., Rumpel, C., Chabbi, A., Millard, P. 2020. Temperature sensitivity of decomposition decreases with increasing soil organic matter stability. Science of The Total Environment 704:135460. https://doi.org/10.1016/j.scitotenv.2019.135460
125. Muchanga, R.A., Hirata, T., Araki, H. 2017. Hairy vetch becomes an alternative basal N fertilizer in low-input fresh-market tomato production in a plastic high tunnel. Horticulture Journal 86:493–500. https://doi.org/10.2503/hortj.OKD-020
126. Muchanga, R.A., Hirata, T., Uchida, Y., Hatano, R., Araki, H. 2020. Soil carbon and nitrogen and tomato yield response to cover crop management. Agronomy Journal 112:1636–1648. https://doi.org/10.1002/agj2.20098.
127. Nakamoto, T., Komatsuzaki, M., Hirata, T., Araki, H. 2012. Effects of tillage and winter cover cropping on microbial substrate-induced respiration and soil aggregation in two Japanese fields. Journal of Soil Science and Plant Nutrition 58:70–82. https://doi.org/10.1080/00380768.2011.650134.
128. Nannipieri, P., Ciardi, C., Palazzi, T., Badalucco, L. 1990. Short-term nitrogen reactions following the addition of urea to a grass-legume association. Soil Biology and Biochemistry 22:549–553. https://doi.org/10.1016/0038-0717(90)90192-3
129. Nannipieri, P., Ascheer, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G. 2003. Microbial diversity and soil functions. European Journal of Soil Science 54:655–670. https://doi.org//10.1046/j.1365-2389.2003.00556.x
130. Nevins, C.J., Nakatsu, C., Armstrong, S. 2018. Characterization of microbial community response to cover crop residue decomposition. Soil Biology and Biochemistry 127:39–49. https://doi.org/10.1016/j.soilbio.2018.09.015
131. Nevins, C.J., Lacey, C., Armstrong, S. 2020. The synchrony of cover crop decomposition, enzyme activity, and nitrogen availability in a corn agroecosystem in the Midwest United States. Soil and Tillage Research 197:104518. https://doi.org/10.1016/j.still.2019.104518.

132. Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., Schilling, J.S., Kennedy, P.G. 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20:241–248. https://doi.org/10.1016/j.funeco.2015.06.006
133. Nihorimbere, V., Ongena, M., Smargiassi, M., Thonart, P. 2011. Beneficial effect of the rhizosphere microbial community for plant growth and health. Biotechnology, Agronomy, Society and Environment 15:327–337.
134. Nivelle, E., Verzeaux, J., Habbib, H., Kuzyakov, Y., Decocq, G., Roger, D., Lacoux, J., Duclercq, J., Spicher, F., Nava-Saucedo, J.-E., Catterou, M., Dubois, F., Tetu, T. 2016. Functional response of soil microbial communities to tillage, cover crops and nitrogen fertilization. Applied Soil Ecology 108:147–155. http://dx.doi.org/10.1016/j.apsoil.2016.08.004
135. Oberholster, T., Vikram, S., Cowan, D., Valverde, A. 2018. Key microbial taxa in the rhizosphere of sorghum and sunflower grown in crop rotation. Science of the Total Environment 624:530–539. https://doi.org/10.1016/j.scitotenv.2017.12.170
136. Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H. 2017. Package 'vegan'. Community Ecology Package, Version 2.4-2. http://cran.r-project.org/web/packages/vegan/index.html
137. Osganian, S.K., Stamfer, M.J., Rimm, E., Spiegelman, D., Hu, F.B., Manson, J.E., Willet, W.C. 2003. Vitamin C and risk of coronary heart disease in women. Journal of the American College of Cardiology 42:246–252. https://doi.org/10.1016/S0735-1097(03)00575-8
138. Oyedara, O.O., Luna-Santillana, E.D.J.D., Olguin-Rodriquez, O., Guo, X., Mendoza-Villa, M.A., Menchaca-Arrendodo, J.L., Elufisan, T.O., Garza-Hernandez, J.A., Leon, I.G., Rodriquez-Perez, M.A. 2016. Isolation of *Bdellovibrio* sp. from soil samples in Mexico and their potential applications in control of pathogens. MicrobiologyOpen 5:992–1002. https://doi.org/10.1002/mbo3.382
139. Pandin, C., Védie, R., Rousseau, T., Coq, D.L., Aymerich, S., Briandet, R. 2018 Dynamics of compost microbiota during the cultivation of *Agaricus bisporus* in the presence of *Bacillus velezensis* QST713 as biocontrol agent against *Trichoderma aggressivum*. Biological Control 127:39–54. https://doi.org/10.1016/j.biocontrol.2018.08.022
140. Platas, G., Morón, R., González, I., Collado, J., Genilloud, O., Peáez, F., Diez, M.T. 1998. Production of antibacterial activities by members of the family Pseudonocardiaceae: influence of nutrients. World Journal of Microbiology and

Biotechnology 14:521–527. https://doi.org/10.1023/A:1008874203344

141. Poly, F., Monrozier, L.J., Bally, R. 2001. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Research in Microbiology 152:95–103. https://doi.org/10.1016/S0923-2508(00)01172-4
142. Poudel, R., Jumpponen, A., Schlatter, D.C., Paulitz, T.C., Gardener, B.B.McS., Kinkel, L.L., Garrett, K.A. 2016. Microbiome networks: a systems framework for identifying candidate microbial assemblages for disease management. Phytopathology 106:1083–1096. http://dx.doi.org/10.1094/PHYTO-02-16-0058-FI
143. Raid, RN. 2004. Lettuce diseases and their management. In: Naqvi, S.A.M.H. (eds.) Diseases of Fruits and Vegetables: Volume II. Springer, Dordrecht. pp. 121–147. https://doi.org/10.1007/1-4020-2607-2_5
144. Ramaiah, N., Hill, R.T., Chun, J., Ravel, J., Matte, M.H., Straube, W.L., Colwell, R.R. 2000. Use of a *chiA* probe for detection of chitinase genes in bacteria from the Chesapeake Bay. FEMS Microbiology Ecology 34:63–71. https://doi.org/10.1111/j.1574-6941.2000.tb00755.x
145. Ren, M., Zhang, Z., Wang, X., Zhou, Z., Chen, D., Zeng, H., Zhao, S., Chen, L., Hu, Y., Zhang, C., Liang, Y., She, Q., Zhang, Y., Peng, N. 2018. Diversity and contributions to nitrogen cycling and carbon fixation of soil salinity shaped microbial communities in Tarim Basin. Frontiers in Microbiology 9:431. https://doi.org/10.3389/fmicb.2018.00431
146. Robačer, M., Canali, S., Kristensen, H.L., Bavec, F., Mlakar, S.G., Jakop, M., Bavec, M. 2016. Cover crops in organic field vegetable production. Scientia Horticulturae 208:104–110. https://doi.org/10.1016/j.scienta.2015.12.029
147. Rösch, C., Mergel, A., Bothe, H. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Applied and Environmental Microbiology 68:3818–3829. https://doi.org/10.1128/AEM.68.8.3818–3829.2002
148. Rosecrance, R.C., McCarty, G.W., Shelton, D.R., Teasdale, J.R. 2000. Denitrification and N mineralization from hairy vetch (*Vicia villosa* Roth) and rye (*Secale cereale* L.) cover crop monocultures and bicultures. Plant Soil 227:283–290. https://doi.org/10.1023/A:1026582012290
149. Rotthauwe, J.-H., Witzel, K.-P., Liesack, W. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology 63:4704–4712. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC168793/
150. Sagova-Mareckova, M., Cermak, L., Novotna, J., Plhackova, K., Forstova, J.,

Kopecky, J. 2008. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. Applied and Environmental Microbiology 74:2902–2907. https://doi.org/10.1128/AEM.02161-07

151. Sainju, U.M., Singh, B.P., Whitehead, W.F. 2001. Comparison of the effects of cover crops and nitrogen fertilization on tomato yield, root growth, and soil properties. Scientia Horticulturae 91:201–214. https://doi.org/10.1016/S0304-4238(01)00264-3
152. Samad, A., Brader, G., Pfaffenbichler, N., Sessitsch, N. 2019. Plant-associated bacteria and the rhizosphere. In: van Elsas, J.D., Trevors, J.T., Rosado, A.S., Nannipieri, P. (eds) Modern Soil Microbiology. CRC Press, Boca Raton, FL. pp. 163–178.
153. Sapkota, S., Sapkota, S., Liu, Z. 2019. Effects of nutrient composition and lettuce cultivar on crop production in hydroponic culture. Horticulturae 5:1–8. https://doi.org/10.3390/horticulturae5040072.
154. Sapirstein, H.D., Bushuk, W. 2016. Rye grain: its genetic, production, and utilization. In: Wrigley, C., Corke, H., Seetharaman,K., Faubion, J. (eds). Encyclopedia of Food Grains. Academic Press. pp. 159–167. https://doi.org/10.1016/b978-0-12-394437-5.00017-6
155. Savvas, D., Passam, H.C., Olympios, C., Nasi, E., Moustaka, E., Mantzos, N., Barouchas, P. 2006. Effects of ammonium nitrogen on lettuce grown on pumice in a closed hydroponic system. Horticulture Science 41:1667–1673. http://doi.org/10.21273/HORTSCI.41.7.1667
156. Schirawski, J., Perlin, M.H. 2018. Plant–microbe interaction 2017 – the good, the bad and the diverse. International Journal of Molecular Sciences 19:1374. http://doi.org/10.3390/ijms19051374
157. Schmidt, R., Mitchell, J., Scow, K. 2019. Cover cropping and no-till increase diversity and symbiotroph:saprotroph ratios of soil fungal communities. Soil Biology and Biochemistry 129:99–109. https://doi.org/10.1016/j.soilbio.2018.11.010
158. Schmidt, J.E., Vannette, R.L., Igwe, A., Blundell, R., Casteel, C.L., Gaudin, A.C.M. 2019. Effects of agricultural management on rhizosphere microbial structure and function in processing tomato plants. Applied and Environmental Microbiology 85:e01064-19. https://doi.org/10.1128/AEM.01064-19.
159. Sharma, P., Singh, A., Kahlon, C.S., Brar, A.S., Grover, K.K., Dia, M., Steiner, R.L. 2018. The role of cover crops towards sustainable soil health and agriculture–A

review paper. American Journal of Plant Sciences 9:1935–1951. https://doi.org/10.4236/ajps.2018.99140

160. Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G. 2001. Bulk and rhizosphere soil bacterial communities studies by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology 67:4742–4751. https://doi.org/10.1128/AEM.67.10.4742–4751.2001
161. Smets, W., Leff, J.W., Bradford, M.A., McCulley, R.L., Lebeer, S., Fierer, N. 2016. A method for simultaneous measurement of soil bacterial abundances and community composition via 16S rRNA gene sequencing. Soil Biology and Biochemistry 96:145–151. https://doi.org/10.1016/j.soilbio.2016.02.003
162. Soltys, D., Bogatek, R., Gniazdowska, A. 2012. Phytotoxic effects of cyanamide on seed germination and seedling growth of weed and crop species. Acta Biologica Cracoviensia s. Botanica 54:87–92. https://doi.org/10.2478/v10182-012-0025-8
163. Sorensen, J.N., Thorup-Kristense, K. 2003. Undersowing legume crops for green manuring of lettuce. Biological Agriculture and Horticulture 21:399–414. https://doi.org/10.1080/01448765.2003.9755281.
164. Sugihara, S., Funakawa, S., Kilasara, M., Kosaki, T. 2010. Dynamics of microbial biomass nitrogen in relation to plant nitrogen uptake during the crop growth period in a dry tropical cropland in Tanzania. Journal of Soil Science and Plant Nutrition 56:105–114. https://doi.org/10.1111/j.1747-0765.2009.00428.x.
165. Sugihara, Y., Ueno, H., Hirata, T., Komatsuzaki, M., Araki, H. 2016. Contribution of N derived from a hairy vetch incorporated in the previous year to tomato N uptake under hairy vetch-tomato rational cropping system. The Horticulture Journal 85:1–7. https://doi.org/10.2503/hortj.MI-073.
166. Tei, F., Benincasa, P., Guiducci, M. 2000. Effect of nitrogen availability on growth and nitrogen uptake in lettuce. Acta Horticulturae 533:385–392. https://doi.org/10.17660/ActaHortic.2000.533.47
167. Throbäck, I.N., Enwall, K., Jarvis, Å., Hallin, S. 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology 49:401–417. https://doi.org/10.1016/j.femsec.2004.04.011
168. Tian, Y., Zhang, X., Wang, J., Gao, L. 2013. Soil microbial communities associated with the rhizosphere of cucumber under different summer cover crops and residue management: a 4-year field. Scientia Horticulturae 150:100–109. http://dx.doi.org/10.1016/j.scienta.2012.10.025

169. Tkacz, A., Hortala, M., Poole. P.S. 2018. Absolute quantitation of microbiota abundance in environmental samples. Microbiome 6:110. https://doi.org/10.1186/s40168-018-0491-7
170. Toda, M., Uchida, Y. 2017. Long-term use of green manure legume and chemical fertilizer affect soil bacterial community structures but not the rate of soil nitrate decrease when excess carbon and nitrogen are applied. Soil Research 55:524–533. http://dx.doi.org/10.1071/SR17109
171. Treseder, K.K., Lennon, J.T. 2015. Fungal traits that drive ecosystem dynamics on land. Microbiology and Molecular Biology Reviews 79:243 – 262. https://doi.org/10.1128/MMBR.00001-15
172. van der Heijden, M.G.A., Hartmann, M. 2016. Networking in the plant microbiome. PloS Biology 14:e1002378. http://doi.org/10.1371/journal.pbio.1002378
173. van Groenigen, J.W., Huygens, D., Boeckx, P., Kuyper, Th.W., Lubbers, I.M., Rütting, T., Goffman, P.M. 2015. The soil N cycle: new insights and key challenges. SOIL 1:235–256. https://doi.org/10.5194/soil-1-235-2015
174. Vance, E.D., Brookes, P.C., Jenkinson, D.S. 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry 19:703–707. https://doi.org/10.1016/0038-0717(87)90052-6.
175. Verma, P., Yadav, A.N., Khannam, K.S., Kumar, S., Saxena, A.K., Suman, A. 2016. Molecular diversity and multifarious plant growth promoting attributes of Bacilli associated with wheat (Triticum aestivum L.) rhizosphere from six diverse agro-ecological zones of India. Journal of Basic Microbiology 56:44–58. https://doi.org/10.1002/jobm.201500459.
176. Wallenstein, M.D. 2017. Managing and manipulating the rhizosphere microbiome for plant health: a systems approach. Rhizosphere 3:230–232. http://dx.doi.org/10.1016/j.rhisph.2017.04.004
177. Wang, Y., Uchida, Y., Shimomura, Y., Akiyama, H., Hayatsu, M. 2017. Responses of denitrifying bacterial communities to short-term waterlogging of soils. Scientific Reports 7:803. https://doi.org/10.1038/s41598-017-00953-8
178. Warren, N.D., Smith, R.G., Sideman, R.G. 2015. Effects of living mulch and fertilizer on the performance of broccoli in plasticulture. HortScience 50:218–224. https://doi.org/10.21273/HORTSCI.50.2.218
179. Watson, C.A., Atkinson, D. 1999. Using nitrogen budgets to indicate nitrogen use efficiency and losses from whole farm systems: a comparison of three methodological approaches. Nutrient Cycling in Agroecosystems 53:259–267. https://doi.org/10.1023/A:1009793120577

180. Wattenburger, C.J., Halverson, L.J., Hofmockel, K.S. 2019. Agricultural management affects root-associated microbe recruitment over maize development. Phytobiomes Journal 3:260–272. https://doi.org/10.1094/PBIOMES-03-19-0016-R
181. White, T.J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., USA, pp. 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1
182. Widdison, P.E., Burt, T.P. 2010. Nitrogen cycle. *In* Global Ecology (ed. SE Jørgensen, BD Fath). Academic Press, Amsterdam, The Netherland. pp.190–197.
183. Yang, X., Zhang, J., Guo, D., Xiong, X., Chang, L., Niu, Q., Huang, D. 2016. Measuring and evaluating anthocyanins in lettuce leaf based on color information. IFAC-PapersOnLine 49-16:096–099. http://doi.org/10.1016/j.ifacol.2016.10.018
184. Yang, C., Liu, N., Zhang, Y. 2019. Soil aggregates regulate the impact of soil bacterial and fungal communities on soil respiration. Geoderma 337:444–452. https://doi.org/10.1016/j.geoderma.2018.10.002
185. Zhang, X., Zhang, R., Gao, J., Wang, X., Fan, F., Ma, X., Yin, H., Zhang, C., Feng, K., Deng, Y. 2017. Thirty-one years of rice-rice-green manure rotations shape the rhizosphere microbial community and enrich beneficial bacteria. Soil Biology and Biochemistry 104:208–217. http://dx.doi.org/10.1016/j.soilbio.2016.10.023
186. Zang, X., Liu, M., Fan, Y., Xu, J., Xu, X., Li, H. 2018. The structural and functional contributions of β-glucosidase-producing microbial communities to cellulose degradation in composting. Biotechnology for Biofuels 11:51. https://doi.org/10.1186/s13068-018-1045-8
187. Zheng, W., Zhao, Z., Gong, Q., Zhai, B., Li, Z. 2018. Responses of fungal-bacterial community and network to organic inputs vary among different spatial habitats in soil. Soil Biology and Biochemistry 125:54–63. https://doi.org/10.1016/j.soilbio.2018.06.029
188. Zhou, Y., Lai, R., Li, W.-J. 2014. The family *Solimonadaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (eds) The Prokaryotes Springer, Verlag, Berlin, Heidelberg. pp. 627–638. https://doi.org/10.1007/978-3-642-38922-1_373