IMPACTS OF COVER CROPS ON SOIL HEALTH, SOIL NITROGEN DYNAMICS, AND CYTOKININ PROFILES

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ABSTRACT

Impact of Cover Crops on Soil Health, Soil Nitrogen Dynamics, and Cytokinin Profiles

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In Ontario, the dominant cash crop rotations consist of soybean (SB), which is a leguminous crop grown in rotation with maize (MZ) and winter wheat (WW). In addition to these crops, some farmers integrate cover crops (CC) into crop rotation, especially during the fallow period and winter seasons, to reduce nitrogen (N) losses via nitrate (NO_3^-) leaching and emission of N_2 and the greenhouse gas nitrous oxide (N_2O). This thesis focused on understanding the impact of crop phases in a MZ-(SB-WW)-CC rotation on the abundance of N-cycling bacterial communities that mediate nitrification and denitrification pathways. In addition, the influence of CCs on soil cytokinin (CK) profiles, which are plant growth-promoting hormones, were studied in a greenhouse trial to assess their potential impacts when integrating CCs into crop rotations. In particular, the relationship between traditional soil health parameters and the soil CK profiles was studied to understand how CKs might reflect biotic interactions and soil vitality. Results indicate N fertilizer application mono ammonium phosphate (MAP) and starter N:P: K (24:6:24) during WW planting in fall largely supported nitrifying bacterial communities (*amo*A) and potentially contributed to NO_3^- leaching. Management of MZ, which included spring-applied MAP resulted in larger denitrifying (*nir*K) bacterial communities, increasing the potential risk of N-loss via emission of dinitrogen gas (N_2) and greenhouse gas N_2O. However, CC soils had significantly lower *nir*K than MZ, reflecting the importance of strong and deep root systems of

CCs, which have a higher ability to scavenge the substrates for denitrifying communities (NO_3^-). This highlights the importance of growing CCs in reducing the potential risk for N-loss via leaching and denitrification. Additionally, in the greenhouse trial, the ability of CCs to affect CK was detected, highlighting the importance of integrating CC in crop rotations. This is particularly noteworthy, given that total CK profiles showed strong associations with traditional soil health parameters such as labile or active carbon and soil microbial community diversity. It was concluded that total soil CK can be used as a novel and dynamic soil health measure. Future research on quantifying N_2O fluxes and levels of NO_3^- in leachates would provide a more precise understanding of the impact of different crop rotation phases on N-dynamics in these fields. Further studies on single or combined measures of soil CKs are warranted to develop its potential as a practical and effective soil health parameter.

Keywords: Cytokinin, agriculture, maize-soybean-winter wheat- cover crops, nitrate leaching, greenhouse gas

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Abbreviations

AC-Active Carbon

BG- β-glucosidase

CB- Cereal Rye-Bulk

CC- Cover crops

CK-Cytokinin

CR- Cereal Rye-Rhizosphere

EEA- Extra cellular Enzyme Activities

GHG- Greenhouse gas

HRMS- High resolution mass spectrometry

MAP- Monoammonium phosphate

MZ-Maize

N-Nitrogen

N_2- Di-nitrogen gas

NH_4^+- ammonium ions

NO- nitric oxide

NO_2^-- nitrite

NO_3^- -Nitrate

N_2O - Nitrous Oxide

PO- phosphatase

qPCR- Quantitative Polymerase Chain Reaction

SB- Soybean

SMC- Soil microbial communities

SOC- Soil organic carbon

TB- Tillage Radish-Bulk

TR- Tillage Radish-Rhizosphere

WSA- Wet Stable Aggregates

WW- Winter wheat

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Chapter 1: General Introduction

1.1 Context

In terms of feeding the ever-growing world population, improving crop yields has become one of the major goals in agriculture (FAO 2020). Yet, the limitation of agricultural land is a pressing global constraint on expanding agriculture; for instance, in Canada, agricultural lands account for only 7% of the total land area (Statistics Canada, 2017). As such, intensive agricultural management and application of nitrogen (N)-fertilizers are commonly used methods that optimize crop yields (Rop et al., 2019; Olaniyi et al., 2008; Singh, 2018).

A common management practice for improved yields and yield stability is the use of diversified crop rotations, whereby different crops are grown in succession over time (Zhang et al., 2021; Chamberlain et al., 2020; Martinez et al., 2020; Lu et al., 2018; Castellazzi et al., 2008). Crop rotations are established mainly to overcome the adverse impacts of monoculture on the depletion of soil health (Pervaiz et al., 2020). For instance, soybean (SB), a leguminous crop grown widely in Canada and Midwest USA, produces low biomass and residues with a low carbon to nitrogen (C: N) ratio that may deplete soil organic carbon (SOC) stocks, and harm soil health (Agomoh et al., 2021; Martinez et al., 2020; Córdova et al., 2019). Therefore, growing high biomass producing crops with high C: N ratios, (e.g. maize (MZ), winter-wheat (WW) or cover crops (CC)), would enhance soil health (Agomoh et al., 2021; Studdert and Echeverría, 2000), which is defined as “the continued capacity of a soil to function as a living system within the ecosystem and land-use boundaries to sustain biological productivity, maintain environmental quality (water and air), and promote plant, animal and human health” (Prabha et al. 2020; Doran and Zeiss, 2000).

Despite the soil health benefits of integrating MZ into crop rotations, studies have repeatedly reported adverse impacts on the environment due to leaching of NO_3^- to freshwater bodies. This is a result of higher amounts of N-fertilizers applied during planting for the establishment of MZ, and the low density of living roots to trap NO_3^- during the early growing seasons (Gholamhoseini et al., 2013; Andraski et al., 2000; Jemison and Fox, 1994). However, studies have shown that integration of leguminous crops inputs biologically fixed N and improves N-mineralization (Vachon and Oelbermann, 2011) into rotation with MZ can reduce the amount of N-fertilizer application and, as a result, it decreases the risk of NO_3^- leaching (Pasley et al., 2021; Koropeckyj-Cox et al., 2021; Zhu and Fox, 2003). However, applying N-fertilizers above the recommended levels caused N-loss in MZ-SB rotations For example Randall and Vetsch (2005) observed 54% of NO_3^- loss from MZ phase and 46% in SB phase during their growing seasons.

The adoption of CC in crop rotations has become an important management strategy that improves both soil health and environmental quality by enhancing the levels of SOC, active carbon (AC), and the abundance and diversity of the soil microbial community (SMC), while reducing soil erosion, weeds, soil-borne pathogens and NO_3^- leaching (Samaddar et al., 2021; Ghimire et al., 2019; Van Eerd, 2018; Belfry et al., 2017; Coombs et al., 2017; Finney et al., 2017). In contrast, a minority of studies have documented neither advantages nor disadvantages of integrating CC on soil health, while still other studies reported that CC inclusion might increase N_2O emissions - depending on the CC species integrated (Linton et al., 2020). Because of conflicting evidence, the effect of CCs in different crop rotations on soil health and N-dynamics needs more detailed investigation.

Currently, different parameters are used to measure soil health. These are known as soil health parameters, and they are comprised of a measurable set of physicochemical and biological

attributes that can respond to changes in agricultural management practices (Prabha et al., 2020). Some commonly used soil health parameters are: pH, water-stable aggregates (WSA), SOC, AC, extracellular enzyme activities (EEA), and nutrient levels such as nitrate (NO_3^-) and ammonium (NH_4^+) (Bonfante et al., 2020; Lehmann et al. 2020). Although NO_3^- and NH_4^+ levels themselves can serve as N-status indicators in soil, the analyses of the abundance of N-cycling SMC provide more insight into soil N-dynamics that leads to N-losses through nitrification and denitrification processes that produce NO_3^- ions, and N_2O gas, respectively (Kuypers et al. 2018; Snider et al., 2015; Robertson and Groffman, 2006; Wallenstein et al. 2006).

Phytohormones can be produced and released by living organisms, including plants and microorganisms, and they play crucial roles in crop growth promotion and yield improvement. One prime example is the group of phytohormones known as cytokinins (CKs) (Stirk and Staden 2010, Hluska et al. 2021). CKs are adenine derivatives with a side chain at the N^6 position and are a widely studied group of plant growth promoters (Jameson and Song 2015). They are also produced and released by many soil organisms such as fungi, bacteria, protists, and nematodes (Wong et al., 2015; Stirk and van Staden, 2010). Although a common role of CKs is to induce cell division (High et al. 2019; Stirk and Staden 2010), their many more probable roles are largely undefined, despite being present in many animals, protists, fungi, and bacteria (Seegobin et al., 2018; Aoki et al., 2019; Chanclud et al., 2016).

The biological roles of CK have been intensively studied in plants over the years, and it has found that CKs are major compounds involved in multiple developmental processes. Their effects include orchestrating the growth of roots, promoting initiation and growth of shoots, and the maintenance of shoot apical dominance. They also enhance the development of chloroplasts and chlorophyll production and reduce leaf senescence (Stirk and Staden 2010; Kieber and

Schaller 2014). Furthermore, CKs play a key role during mutual symbiotic associations of microorganisms with plants. For example, *Rhizobia* spp. induce the formation of N-fixing root nodules in symbiotic association with legumes via the secretion of CKs (Fonouni-Farde et al., 2017). In addition, Goh et al. (2019) illustrated the importance of CKs released by the arbuscular mycorrhizal fungi *Rhizophagus irregularis* during the colonization of pea. Besides benefits, CKs released by pathogenic microorganisms can negatively impact plants. For example, CKs released by the fungus *Magnaporthe oryzae* enable rice blast infection of rice (Chanclud et al., 2016). Similarly, CKs released by *Ustilago maydis* regulate tumour formation during the progression of MZ-smut disease (Morrison et al., 2017). In addition, Moffatt et al. (2017) demonstrated the involvement of CKs during the pathogenesis of the double-host cedar-apple rust caused by the fungus *Gymnosporangium juniper-virginianae* Schwein.

However, little is known about the persistence of CKs in agricultural soil matrices or the impact of different agricultural management strategies such as CC species on soil CK profiles. The potential ability of these hormones to improve crop growth and benefit non-plant organisms that synthesize and respond to the levels of phytohormones highlights the importance of understanding the occurrence and biological role of CK profiles in soil. Interestingly, bio-fertilizers rich in CK-producing beneficial microbes are currently available on the market and have shown promising results in crop improvement, further reinforcing the importance of studying CK profiles in agricultural soil (Jorge et al., 2019; Palberg et al., 2022).

1.2 Thesis format and research objectives

This thesis is arranged as five main chapters in manuscript style. Due to each study's complimentary but independent nature, specific objectives are stated within each chapter. The overall aim of this thesis was to investigate the effects of CCs on soil health, N-cycling SMC sizes, and soil CK profiles. **Chapter 2** is a literature review highlighting agricultural management, including cover cropping related to soil health, N-dynamics, and CK occurrence and flow through the environment. In **Chapter 3**, I investigated the impact of different crop phases, including CC mixtures in a long-term cropping system under the rotation of MZ-(SB-WW)-CC on soil health parameters and communities of total bacteria (*16S* rRNA), nitrifying bacteria (*amo*A), and denitrifying bacteria (*nir*S, *nir*K) using quantitative polymerase chain reaction (qPCR). In **Chapter 4,** the impacts of CCs on soil CK profiles were investigated. QExactive Orbitrap-high resolution mass spectrometry (HRMS) was used to identify and quantify soil CKs in rhizosphere and bulk soil grown under two types of CCs with different root structures in a greenhouse trial. In addition, I also looked at how CK profiles differed between rhizosphere and bulk soils and examined the relationships between soil CKs and traditional soil health parameters to determine whether soil CKs are useful as a novel soil health parameter. In summary, the scope of this thesis addressed the following objectives:

1. To assess the impact of CCs in a long-term MZ-(SB-WW)-CC rotation on soil health and N-cycling SMCs (**Chapter 3**).
2. To study the impact of CC sp. tillage radish (T) and cereal rye (C) which possess different taproot and fibrous root systems, on soil health parameters and soil CK profiles between rhizosphere and bulk soil (**Chapter 4**).

1.3 Bibliography

Agomoh, I. V., Drury, C. F., Yang, X., Phillips, L. A., and Reynolds, W. D. (2021). Crop rotation enhances soybean yields and soil health indicators. Soil Science Society of America Journal, 85(4), 1185–1195. https://doi.org/10.1002/saj2.20241

Andraski, T. W., Bundy, L. G., and Brye, K. R. (2000). Crop Management and Corn Nitrogen Rate Effects on Nitrate Leaching. Journal of Environmental Quality, 29(4), 1095–1103. https://doi.org/10.2134/jeq2000.00472425002900040009x

Aoki, M. M., Seegobin, M., Kisiala, A., Noble, A., Brunetti, C., and Emery, R. J. N. (2019). Phytohormone metabolism in human cells: Cytokinins are taken up and interconverted in HeLa cell culture. FASEB BioAdvances, 1(5), 320–331. https://doi.org/10.1096/fba.2018-00032

Belfry, K. D., Trueman, C., Vyn, R. J., Loewen, S. A., and Van Eerd, L. L. (2017). Winter cover crops on processing tomato yield, quality, pest pressure, nitrogen availability, and profit margins. PLoS ONE, 12(7), 1–17. https://doi.org/10.1371/journal.pone.0180500

Bonfante, A., Basile, A., and Bouma, J. (2020). Targeting the soil quality and soil health concepts when aiming for the United Nations Sustainable Development Goals and the EU Green Deal. Soil, 6(2), 453–466. https://doi.org/10.5194/soil-6-453-2020

Castellazzi, M. S., Wood, G. A., Burgess, P. J., Morris, J., Conrad, K. F., and Perry, J. N. (2008). A systematic representation of crop rotations. Agricultural Systems, 97(1–2), 26–33. https://doi.org/10.1016/j.agsy.2007.10.006

Chamberlain, L. A., Bolton, M. L., Cox, M. S., Suen, G., Conley, S. P., and Ané, J. M. (2020).

Crop rotation, but not cover crops, influenced soil bacterial community composition in a corn-soybean system in southern Wisconsin. Applied Soil Ecology, 154(December 2019), 103603. https://doi.org/10.1016/j.apsoil.2020.103603

Chanclud, E., Kisiala, A., Emery, N. R. J., Chalvon, V., Ducasse, A., Romiti-Michel, C., Gravot, A., Kroj, T., and Morel, J. B. (2016). Cytokinin Production by the Rice Blast Fungus Is a Pivotal Requirement for Full Virulence. PLoS Pathogens, 12(2), 1–19. https://doi.org/10.1371/journal.ppat.1005457

Coombs, C., Lauzon, J. D., Deen, B., and Van Eerd, L. L. (2017). Legume cover crop management on nitrogen dynamics and yield in grain corn systems. Field Crops Research, 201, 75–85. https://doi.org/10.1016/j.fcr.2016.11.001

Córdova, S. C., Castellano, M. J., Dietzel, R., Licht, M. A., Togliatti, K., Martinez-Feria, R., and Archontoulis, S. V. (2019). Soybean nitrogen fixation dynamics in Iowa, USA. Field Crops Research, 236(November 2018), 165–176. https://doi.org/10.1016/j.fcr.2019.03.018

FAO. (2020). Tracking progress on food and agriculture-related SDG indicators. Food and Agriculture Organization of the United Nations.

Finney, D. M., Murrell, E. G., White, C. M., Baraibar, B., Barbercheck, M. E., Bradley, B. A., Cornelisse, S., Hunter, M. C., Kaye, J. P., Mortensen, D. A., Mullen, C. A., and Schipanski, M. E. (2017). Ecosystem Services and Disservices Are Bundled in Simple and Diverse Cover Cropping Systems. Agricultural & Environmental Letters, 2(1), 170033. https://doi.org/10.2134/ael2017.09.0033

Fonouni-Farde, C., Kisiala, A., Brault, M., Emery, R. J. N., Diet, A., and Frugier, F. (2017). DELLA1-mediated gibberellin signaling regulates cytokinin-dependent symbiotic

nodulation. Plant Physiology, 175(4), 1795–1806. https://doi.org/10.1104/pp.17.00919

Goh, D. M., Cosme, M., Kisiala, A. B., Mulholland, S., Said, Z. M. F., Spíchal, L., Emery, R. J. N., Declerck, S., and Guinel, F. C. (2019). A stimulatory role for cytokinin in the arbuscular mycorrhizal symbiosis of pea. Frontiers in Plant Science, 10(March), 1–18. https://doi.org/10.3389/fpls.2019.00262

Ghimire, R., Ghimire, B., Mesbah, A. O., Sainju, U. M., and Idowu, O. J. (2019). Soil health response of cover crops in winter wheat–fallow system. Agronomy Journal, 111(4), 2108–2115. https://doi.org/10.2134/agronj2018.08.0492

Gholamhoseini, M., AghaAlikhani, M., Modarres Sanavy, S. A. M., and Mirlatifi, S. M. (2013). Interactions of irrigation, weed and nitrogen on corn yield, nitrogen use efficiency and nitrate leaching. Agricultural Water Management, 126, 9–18. https://doi.org/10.1016/j.agwat.2013.05.002

Hartwig, N. L., and Ammon, H. U. (2002). Cover crops and living mulches. Weed Science, 50(6), 688–699. https://doi.org/10.1614/0043-1745(2002)050[0688:aiacca]2.0.co;2

High, K. E., Ashton, P. D., Nelson, M., Rylott, E. L., Thomas-Oates, J. E., and Hodson, M. E. (2019). New approaches using mass spectrometry to investigate changes to cytokinin and abscisic acid (ABA) concentrations in soil. Soil Biology and Biochemistry, 135(April), 108–116. https://doi.org/10.1016/j.soilbio.2019.04.017

Hluska, T., Hlusková, L., and Emery, R. J. N. (2021). The hulks and the deadpools of the cytokinin universe: A dual strategy for cytokinin production, translocation, and signal transduction. Biomolecules, 11(2), 1–40. https://doi.org/10.3390/biom11020209

Jemison, J. M., and Fox, R. H. (1994). Nitrate Leaching from Nitrogen-Fertilized and Manured Corn Measured with Zero-Tension Pan Lysimeters. Journal of Environmental Quality, 23(2), 337–343. https://doi.org/10.2134/jeq1994.00472425002300020018x

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., and Emery, R. J. N. (2019). Endosymbiotic Methylobacterium oryzae mitigates the impact of limited water availability in lentil (Lens culinaris Medik.) by increasing plant cytokinin levels. Environmental and Experimental Botany, 162(June), 525–540. https://doi.org/10.1016/j.envexpbot.2019.03.028

Kieber, J. J., and Schaller, G. E. (2014). Cytokinins. The Arabidopsis Book, 12(February 2015), e0168. https://doi.org/10.1199/tab.0168

Koropeckyj-Cox, L., Christianson, R., D., and Yuan, Y. (2021). Effectiveness of Conservation Crop Rotation For Water Pollution Reduction From Agricultural Areas. American Society of Agricultural and Biological Engineers. 64(2): 691-704. https://doi.org/10.13031/trans.14017 691

Kuypers, M. M. M., Marchant, H. K., and Kartal, B. (2018). The microbial nitrogen-cycling network. Nature Reviews Microbiology, 16(5), 263–276. https://doi.org/10.1038/nrmicro.2018.9

Lehman, R. M., Osborne, S. L., and Duke, S. E. (2017). Diversified No-Till Crop Rotation Reduces Nitrous Oxide Emissions, Increases Soybean Yields, and Promotes Soil Carbon Accrual. Soil Science Society of America Journal, 81(1), 76–83. https://doi.org/10.2136/sssaj2016.01.0021

Linton, N. F., Ferrari Machado, P. V., Deen, B., Wagner-Riddle, C., and Dunfield, K. E. (2020). Long-term diverse rotation alters nitrogen cycling bacterial groups and nitrous oxide

emissions after nitrogen fertilization. Soil Biology and Biochemistry, 149(April), 107917. https://doi.org/10.1016/j.soilbio.2020.107917

Lu, S., Lepo, J. E., Song, H. X., Guan, C. Y., and Zhang, Z. H. (2018). Increased rice yield in long-term crop rotation regimes through improved soil structure, rhizosphere microbial communities, and nutrient bioavailability in paddy soil. Biology and Fertility of Soils, 54(8), 909–923. https://doi.org/10.1007/s00374-018-1315-4

Martinez, J. P., Crespo, C., Sainz Rozas, H., Echeverría, H., Studdert, G., Martinez, F., Cordone, G., and Barbieri, P. (2020). Soil organic carbon in cropping sequences with predominance of soya bean in the argentinean humid Pampas. Soil Use and Management, 36(1), 173–183. https://doi.org/10.1111/sum.12547

Moffatt, K., Flores, C.M., Andreas, P., Kisiala, A., and Emery, R.J.N. (2017). Phytohormone profiling reveals fungal signatures and strong manipulation of infection cycle in the Gymnosporangium juniper-virginianae dual-host plant system. 1011, 1–27.

Morrison, E. N., Emery, R. J. N., and Saville, B. J. (2017). Fungal derived cytokinins are necessary for normal Ustilago maydis infection of maize. Plant Pathology, 66(5), 726–742. https://doi.org/10.1111/ppa.12629

Muhammad, I., Sainju, U. M., Zhao, F., Khan, A., Ghimire, R., Fu, X., and Wang, J. (2019). Regulation of soil CO2 and N2O emissions by cover crops: A meta-analysis. Soil and Tillage Research, 192(November 2018), 103–112. https://doi.org/10.1016/j.still.2019.04.020

Olaniyi, J. O., Adelasoye, K., and Jegede, C. O. (2008). Influence of Nitrogen Fertilizer on the Growth , Yield and Quality of Grain Amaranth Varieties. World Journal of Agricultural

Sciences, 4(4), 506–513.

Palberg, D., Kisiała, A., Jorge, G. L., and Emery, R. J. N. (2022). A survey of Methylobacterium species and strains reveals widespread production and varying profiles of cytokinin phytohormones. BMC Microbiology, 22(1), 1–17. https://doi.org/10.1186/s12866-022-02454-9

Pasley, H., Nichols, V., Castellano, M., Baum, M., Kladivko, E., Helmers, M., and Archontoulis, S. (2021). Rotating maize reduces the risk and rate of nitrate leaching. Environmental Research Letters, 16(6). https://doi.org/10.1088/1748-9326/abef8f

Pervaiz, Z. H., Iqbal, J., Zhang, Q., Chen, D., Wei, H., and Saleem, M. (2020). Continuous Cropping Alters Multiple Biotic and Abiotic Indicators of Soil Health. Soil Systems, 4(4), 59. https://doi.org/10.3390/soilsystems4040059

Prabha, A. C. S., Senthivelu, M., and Paramasivam, A. (2020). Soil quality indicators for different land uses : A review. International Journal of Plant and Soil.17, 379-387. https://www.researchgate.net/publication/342551296

Randall, G. W., and Vetsch, J. A. (2005). Corn production on a subsurface-drained mollisol as affected by fall versus spring application of nitrogen and nitrapyrin. Agronomy Journal, 97(2), 472–478. https://doi.org/10.2134/agronj2005.0472

Robertson, G. P., and Groffman, P. M. (2006). Nitrogen transformations. In Soil Microbiology, Ecology and Biochemistry: Third Edition (4th ed.). Elsevier Inc. https://doi.org/10.1016/b978-0-12-415955-6.00014-1

Rocha, K. F., Kuramae, E. E., Borges, B. M. F., Leite, M. F. A., and Rosolem, C. A. (2020).

Microbial N-cycling gene abundance is affected by cover crop specie and development stage in an integrated cropping system. Archives of Microbiology, 202(7), 2005–2012. https://doi.org/10.1007/s00203-020-01910-2

Rop, K., Karuku, G. N., Mbui, D., Njomo, N., and Michira, I. (2019). Evaluating the effects of formulated nano-NPK slow release fertilizer composite on the performance and yield of maize, kale and capsicum. Annals of Agricultural Sciences, 64(1), 9–19. https://doi.org/10.1016/j.aoas.2019.05.010

Seegobin, M., Kisiala, A., Noble, A., Kaplan, D., Brunetti, C., and Emery, R. J. N. (2018). Canis familiaris tissues are characterized by different profiles of cytokinins typical of the tRNA degradation pathway. FASEB Journal, 32(12), 6575–6581. https://doi.org/10.1096/fj.201800347

Singh, B. (2018). Are Nitrogen Fertilizers Deleterious to Soil Health? Agronomy, 8(4). https://doi.org/10.3390/agronomy8040048

Snider, D., Thompson, K., Wagner-Riddle, C., Spoelstra, J., and Dunfield, K. (2015). Molecular techniques and stable isotope ratios at natural abundance give complementary inferences about N2O production pathways in an agricultural soil following a rainfall event. Soil Biology and Biochemistry, 88, 197–213. https://doi.org/10.1016/j.soilbio.2015.05.021

Statistics Canada. (2017) Table 32-10-0411-01: Land practices and land features, Land use and environmental practices, Statistics Canada.

Stirk, W. A., and van Staden, J. (2010). Flow of cytokinins through the environment. Plant Growth Regulation, 62(2), 101–116. https://doi.org/10.1007/s10725-010-9481-x

Studdert, G. A., and Echeverría, H. E. (2000). Crop Rotations and Nitrogen Fertilization to Manage Soil Organic Carbon Dynamics. Soil Science Society of America Journal, 64(4), 1496–1503. https://doi.org/10.2136/sssaj2000.6441496x

Stubner, S. (2002). Enumeration of 16S rDNA of Desulfotomaculum lineage 1 in rice field soil by real-time PCR with SybrGreenTM detection. Journal of Microbiological Methods, 50(2), 155–164. https://doi.org/10.1016/S0167-7012(02)00024

Vachon, K., and Oelbermann, M. (2011). Crop residue input and decomposition in a temperate maize-soybean intercrop system. Soil Science, 176(4), 157–163. https://doi.org/10.1097/SS.0b013e3182104213

Van Eerd, L. L. (2018). Nitrogen dynamics and yields of fresh bean and sweet corn with different cover crops and planting dates. Nutrient Cycling in Agroecosystems, 111(1), 33–46. https://doi.org/10.1007/s10705-018-9914-x

Wallenstein, M. D., McNulty, S., Fernandez, I. J., Boggs, J., and Schlesinger, W. H. (2006). Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. Forest Ecology and Management, 222(1–3), 459–468. https://doi.org/10.1016/j.foreco.2005.11.002

Zhang, K., Maltais-Landry, G., and Liao, H. L. (2021). How soil biota regulate C cycling and soil C pools in diversified crop rotations. Soil Biology and Biochemistry, 156. https://doi.org/10.1016/j.soilbio.2021.108219

Zhu, Y., and Fox, R. H. (2003). Corn-soybean rotation effects on nitrate leaching. Agronomy Journal, 95(4), 1028–1033. https://doi.org/10.2134/agronj2003.1028

Chapter 2: Literature review

2.1 Soil health

Soil is composed of organic substrate (plant residues and microbial decomposed products), inorganic substrate (rock and mineral particles), biota, water and air (Prabha et al., 2014; Pulleman et al., 2012). Soil is recognized as a natural, non-renewable, and dynamic ecosystem, in which complex interactions among plants, microorganisms, and abiotic factors take place to provide vital ecosystem services such as cycling of nutrients, food production, and degradation of organic matter (Grzadziel et al., 2018; Stockdale and Watson, 2009; Doran and Zeiss, 2000). Soil provides numerous habitats for a wide range of life on earth, such as SMCs, insects, earthworms, and nematodes (Prabha et al., 2014; Pulleman et al., 2012; Nannipieri et al., 2003; Brussaard, 1997). It also acts as a plant growth substrate by providing physical support, and the nutrients and water required for plants' physiology and survival (Stockdale and Watson, 2009). Therefore, soil health is crucial for the well-being of plants and animals (Grzadziel et al., 2018). The depletion of soil health due to intensive agriculture has become a significant problem worldwide (Andrén and Kätterer, 2018) and, in turn, has caused a reduction in crop yields and an increase in environmental pollution via the emission of greenhouse gases (GHG) and NO_3^- leaching. Therefore, soil health assessment in agricultural fields is crucially important to understand the impact of various management strategies on soil functioning.

2.1.1 Parameters in determining soil health in agroecosystems

A set of measurable physiochemical and biological attributes, known as soil health parameters, are used to assess soil health (Prabha et al., 2020). Around 42 different soil health parameters that respond to agricultural management strategies have been identified so far. However, most of

them lack consistent results and show low responsiveness to agricultural management practices (Stewart et al., 2018). Frequently used physical soil health parameters include: soil texture, soil aggregate stability, porosity, bulk density, and water holding capacity. Chemical parameters include: soil pH, SOM, SOC, and N content. Finally, biological parameters include attributes that reflect living organisms in the soil, e.g., microbial biomass, SMC diversity, abundance and composition, and microbial EEA (Turbé et al., 2010; Gregorich, 1996). For two significant reasons, changes in SMC matrics (e.g. abundance, structure and diversity) have been recognized as key indicators in assessing soil health with respect to agricultural management strategies; first, the SMC mediates 80-90% of the functioning of the soil, including the cycling of nutrients by their participation in SOM decomposition and nutrient transformation reactions (Wang et al., 2022; Grzadziel et al., 2018; Nannipieri et al., 2003), and, second, SMC respond in a timely way to spatial and temporal environmental changes (Barreiro et al., 2015; Garland 1997). Therefore, measures of SMC abundance, microbial biomass, and EEA are helpful indicators in monitoring ecosystem functioning, especially for monitoring the impact of agricultural management practices on soil health (Kardol et al., 2010; Brookes, 2001).

SOM is one of the major components in soil and is composed of microbial, plant and animal detritus matter such as senescing plant matter and animal feces, root exudates, living organisms, and microbial necromass, and microbial products (Dixon and Tilston, 2010; Turbé et al., 2010; Lützow et al., 2006). SOM is, therefore, rich in elements such as C, N, O, P, K and S (Dixon and Tilston, 2010; Brookes, 2001), and is a primary source of energy for SMC (Pulleman et al., 2012). Turnover of SOM occurs via the processes of decomposition and mineralization, which is mainly driven by the SMC, while a small portion (< 5%) is decomposed via abiotic chemical oxidation (Dixon and Tilston, 2010; Lützow et al., 2006). During decomposition, with the action

of various extracellular enzymes secreted by SMC, complex organic compounds - like polysaccharides (starch, cellulose), polyphenols, lipids, and proteins - degrade into small inorganic molecules that plants and soil biota can utilize, including water (H_2O), organic C, NH_4^+, phosphates (PO_4^{3-}), sulphates (SO_4^{3-}) (Brookes, 2001; Lützow et al., 2006).

2.2 Impact of sustainable agricultural management practices on soil health

2.2.1 Diversified crop rotations

Crop rotation and cover cropping are two sustainable agricultural management practices used in agriculture with primary aim of improving soil health and crop yields (Behnke et al., 2018). Several studies have reported the capacity of diverse crop rotations to improve: soil health parameters, SOM (Martinez et al., 2020; Zuber et al., 2018), SOC (Liu et al., 2020; Martinez et al., 2020; Ghimire et al., 2019; Maiga et al., 2019), total C (Garcia et al., 2013), soil nutrient content (Zhang et al., 2019), soil structure (Oliveira et al., 2019), and reduced weed pressure (Weisberger et al., 2019). This in turn, has improved crop yields (Agomoh et al., 2021; Behnke et al., 2018). Studies have even shown the capacity of diverse crop rotations to improve soil health and crop yields under abiotic stresses such as drought (Bowles et al., 2020; Degani et al., 2019).

Although the influence of crop rotations on soil health and crop yields are intensively studied, little is known about the response of belowground SMC to CC management. Studies to date have reported an ability of diverse crop rotations to increase the abundance and activity of SMC (Behnke et al., 2021; D'Acunto et al., 2018) and the diversity of belowground SMC (Linton et al., 2020; D'Acunto et al., 2018). Moreover, diversified cropping systems have imposed changes in SMC compositions (Li et al., 2021; Mayer et al., 2019). Using a meta-analysis of 122 studies,

McDaniel et al. (2014) found that diverse crop rotations significantly increased soil microbial biomass C and N compared to monocultures. Russell et al. (2006) observed similar effects on microbial biomass in a diverse crop rotation (MZ/MZ/oat/alfa-alfa) established in the Midwestern USA. Crop rotations of rice-rice-rape have significantly improved soil health parameters, including SOM, AC, WSA, and soil microbial biomass and composition and EEA (Town et al., 2022; Lu et al., 2018). Crop species, the composition of crop residues, and root exudates are major determinants of soil physiochemical characteristics such as SOM, SOC, and WSA etc., which may directly impact SMCs (Town et al., 2022; Lu et al., 2018). In support of this idea, Samaddar et al. (2021) reported the presence of significantly different rhizosphere microbial communities associated with varying crop species.

2.2.2 Impact of cover cropping on soil health

Cover crops are a group of plants commonly used as living ground covers that are integrated into crop rotations, especially during the fallow period (Justes, 2017; Fageria et al., 2005; Hartwig and Ammon, 2002). Although in the past, CCs were used primarily as forage crops to feed cattle (Hartwig and Ammon, 2002), an increasing number of recent studies have reported dual benefits on soil health and crop yields (Ali et al., 2019; Belfry et al., 2017; Raphael et al., 2016). Multiple studies have reported the ability of CCs to improve soil health parameters such as SOM, SOC (Finney et al., 2017; Zhang et al., 2021), N content (Zhang et al., 2021; Finney et al., 2017; Belfry et al., 2017), and soil physical properties such as: infiltration rates, saturated hydraulic conductivity, and soil aggregates (Nouri et al., 2019). As a result of enhancing soil health parameters, CCs can increase crop yields. Integration of oilseed CC into a 3-year tomato cropping system had significantly higher tomato yields than yields from no CC controls (Belfry et al., 2017). Van Eerd (2018) observed different cash crop yields when different CC were

grown. For instance, integration of oat CC has increased bean yields by 10.5 %, and MZ yields increased by 6.9% with all CC species tested (oat, cereal rye, oilseed radish, forage pea, and hairy vetch), emphasizing the effectiveness of combinations of CC and cash crop spp. on crop yields (Van Eerd, 2018). The growing of CC increases aboveground biomass accumulation (Belfry and Van Eerd, 2016), which reflects the improvement of C and N stocks, as well as the reduction of rain-induced soil erosion due to retardation of water flow (Dabney et al., 2001). A CC with a deep and intense root system further reduces soil erosion by keeping the soil in place during heavy precipitation events and over winter (De Baets et al., 2011). Moreover, a brassica species CC, commonly known as oil seed radish, can suppress weeds and soilborne pathogens (Zukalová & Vašák, 2002; Boydston & Hang, 1995). The isothiocyanates, known as mustard oils produced by brassica; including cabbage, canola, and oilseed rape (*Brassica napus*) have fungicidal and bactericidal activity (Vig et al., 2009). It is also known that oilseed rape (*Brassica napus*) and sundan grass can suppress weeds (Boydston and Hang, 1995).

In contrast to considerable literature concerning the influence of CC on soil physicochemical parameters and crop yields, the impact of CC on the abundance, diversity, and functioning of belowground SMCs is much less studied. Recent studies have revealed the ability of CC to enhance SMC abundance (Thapa et al., 2021; Zhang et al., 2021), diversity (Ali et al., 2019), and activity (Wegner et al., 2018). Furthermore, Finney et al. (2017) have shown a significant impact of CCs in improving soil microbial activity and structure relative to a no CC controls. Integration of CCs had a substantial effect on microbial EEA of β-glucosidase (BG), β-glucosaminidase, and phosphatase (PO) (Thapa et al., 2021), and β-xylosidase, xylosidase, and cellobiohydrolase (Zhang et al., 2021). The activities of microbially produced extracellular enzymes reflect the functioning of SMCs, including nutrient cycling in the ecosystem.

2.3 Nutrient transformation reactions: Soil N-dynamics and N-cycling SMC in agroecosystems

Nitrogen is a major macronutrient essential for the survival of living organisms (Guo et al. 2018; Kraiser et al. 2011; Kuypers et al. 2018; Orr et al. 2011) and is found in biological compounds such as nucleic acids, proteins, hormones, and chlorophyll present in living organisms (Takai, 2019; Kuypers et al. 2018; Leghari et al. 2016; Espenberg et al. 2017). A large number of studies to date have reported the roles of N in several critical physiological and metabolic processes in plants, such as: nutrient uptake, signaling pathways, photosynthesis, synthesis of phytohormones, and respiration in plants (Guo et al., 2018; Leghari et al. 2016; Jones 2014; Silva and Uchida 2000). When lacking adequate N, plants show N-deficiency symptoms such as stunted growth, early maturity, chlorophyll reduction (chlorosis), reduction of leaf area, and reduction in protein content of seeds and vegetative parts that lead to a decrease in crop yield and quality (Zhao et al. 2005; Silva and Uchida 2000). The long-term application of N in a WW-MZ cropping system in the North China plain significantly improved SOC stocks and crop yields compared to no fertilizer treatment (Gai et al., 2018). Studies have also reported the importance of N-application in improving drought tolerance of WW and MZ cultivars (Sedri et al., 2019; Song et al., 2019) and improving lodging resistance of rice cultivars and WW (Chen et al., 2018; Pan et al., 2019; Zhang et al., 2019). These results emphasize the importance of adequate N supply, especially in agricultural fields, for meeting crop demands and producing good yields. Although N occurs in abundance (78%) in the atmosphere in the form of N_2 (Jones, 2014; Robertson and Groffman, 2006; Kraiser et al., 2011), the plant-available forms of N, such as ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) are limited in nature (Gruber and Galloway, 2008). The only natural process that converts atmospheric N_2 to plant-available forms is through biological N-fixation,

mainly occuring in leguminous plants with rhizobial associations (Abalos et al., 2020; Kraiser et al., 2011; Robertson and Groffman, 2006). For this reason, N has become a major limiting factor of plant growth, especially in agricultural systems for which biomass is harvested, so less plant tissue N (residues) is returned to the soil (Gruber and Galloway, 2008; Takai, 2019).

2.3.1 Microbially-mediated N-transformations in soil

Loss of N via NO_3^- leaching (reviewed in Di Capua et al., 2019) and via emission of the GHG N_2O are two major pathways by which N is lost from agroecosystems, which has become a concerning global, environmental issue. Agricultural production systems are recognized as the major sites of N_2O emissions worldwide, while in Canada, agriculture accounts for 75% of Canada's total N_2O production (Environment and Climate Change Canada, 2022). Intensive agricultural management strategies and massive applications of inorganic N-fertilizers are often highlighted as the primary causes (Wang et al., 2018). Therefore, understanding the dynamics of N in agroecosystems is crucial to mitigating N losses. The primary microbial processes involved in the loss of N from these systems are nitrification and denitrification, which are mediated by nitrifying and denitrifying SMCs, respectively.

2.3.1.1 Nitrification

Nitrification is a process whereby NH_4^+ ions are sequentially oxidized to hydroxyl-amines (NH_2OH), NO_2^- and finally to plants' highly utilizable form, NO_3^-, by the action of nitrifying SMC (Snider et al., 2015; Robertson and Groffman, 2006) (**Fig. 2.1**). Chemoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are the two major groups of microbial communities involved in this process. AOB, for e.g., *Nitrosomonas* spp. and *Nitrosococcus* spp., are involved in the first rate-limiting oxidation step whereby NH_4^+ ions are oxidized to NH_2OH. This reaction is catalyzed by the enzyme ammonia

monooxygenase (denoted by gene targets *amoA* in bacteria and *crenamoA* in archaea), followed by further oxidation of NH_2OH to NO_2^- with the action of hydroxylamine oxidoreductase enzymes (reviewed in Kuypers et al., 2018; Snider et al. 2015).

2.3.1.2 Denitrification

Denitrification is a series of microbial-mediated reduction reactions whereby NO_3^- is sequentially reduced to NO_2^-, nitric oxide (NO), N_2O, and N_2 gas (Kuypers et al., 2018; Snider et al., 2015; Wallenstein et al., 2006). These reactions are mediated by autotrophic and heterotrophic microorganisms ranging from archaea, bacteria, and protozoa to fungi present in anoxic and NO_3^- rich environments, such as soil (Maia & Moura, 2014; Moreno-Vivián et al., 1999).

Nitrate is a major N source for many life forms, including bacteria and archaea (Moreno-Vivián et al., 1999). Nitrate reduction to NO_2^- is the major pathway that generates NO_2^- and this reaction is crucial in the processes of NO_3^- assimilation, whereby organisms use NO_3^- for their growth and dissimilatory reactions such as respiration (Moreno-Vivián et al., 1999). This reaction is mediated by microorganisms capable of producing nitrate reductase (NAR) enzymes. There are two types of NAR enzymes, namely, 1. Membrane-bound NAR-, which catalyzes the reduction of NO_3^- present in the cytoplasm of microorganisms, and 2. Periplasmic NAR, which catalyzes the reduction of NO_3^- in the periplasm. Nitrate-reducing microorganisms possess one or both enzymes (Moreno-Vivián et al., 1999).

Nitrites act as the substrate for denitrifying microorganisms that produce one of the haem-containing cadmium nitrite reductases encoded by *nir*S genes or copper-containing nitrite reductase encoded by *nir*K genes. These enzymes catalyze the reduction of NO_2^- to NO, which acts as an intermediate in denitrification and nitrification reactions (**Fig. 2.1**). The *nir*K and *nir*S

genes coding for the nitrite reducing enzymes are used as gene markers to identify denitrifiers. These microorganisms can be found in many environments, including wastewater treatments, agricultural soils etc. (reviewed in Kuypers et al., 2018).

Nitric oxide is a toxic molecule that undergoes either a detoxification reaction or respiration inside microorganisms by the action of nitric oxide reductases (NOR), and produces N_2O gas, an ozone-depleting and GHG molecule. In addition to this reaction, two other pathways that produce N_2O gas are, as a by-product of hydroxylamine oxidation by AOB during nitrification, and via the reduction of NO in the nitrifier-denitrification pathway (**Fig. 2.1**). The reduction of N_2O is a major reaction that produces N_2 gas, and this reaction is catalyzed by nitrous oxide reductase (NOS) encoded by the *nos*Z gene. Compared to other N-reducing enzymes, NOS is more sensitive to abiotic factors such as pH, oxygen levels etc. (reviewed in Kuypers et al., 2018; Maia & Moura, 2014; Moreno-Vivián et al., 1999).

2.3.2 Environmental drivers of N-cycling

N-cycling dynamics also depend on environmental factors, since N-cycling SMC are sensitive to environmental changes (Chamberlain et al., 2020; Li et al., 2019). Higher SMC biomass, total N, mean annual precipitation (Li et al., 2019), atmospheric carbon dioxide (CO_2) levels (Zak et al., 1993), and SOC (Miao et al., 2019) have improved available N levels in soil, while higher soil pH levels have decreased available N levels in the soil. In addition, Wang et al. (2022) have reported the direct impact of changes in physicochemical properties such as pH and SOM due to effects of different crop management systems (e.g. crop rotations and tillage) on the functioning of N-cycling SMC. Furthermore, different plant root exudates (Moreau et al., 2019) and SOC (Pereg et al., 2018) impact the abundance of denitrifying SMC. Higher salinity negatively impacts the abundance and activity of denitrifiers and nitrifiers (AOB) (Wang et al., 2018).

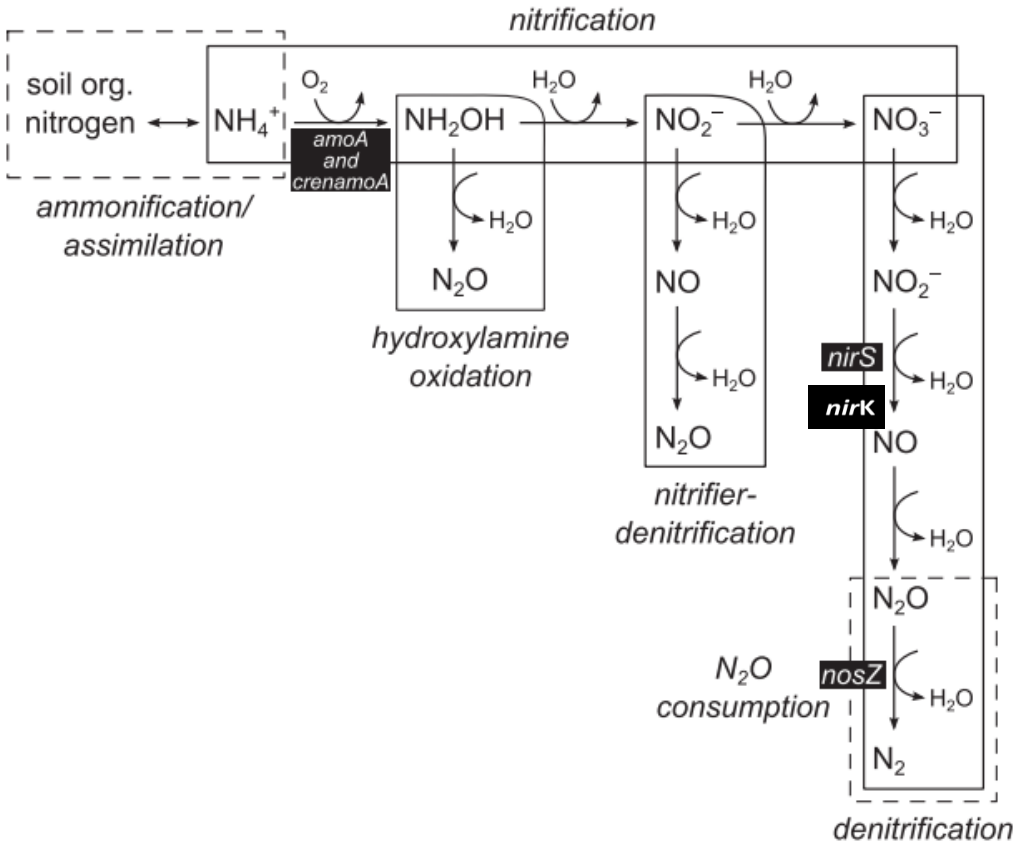

Fig. 2.1. Nitrification and denitrification reactions mediated by nitrifying (*amo*A and *crenamo*A) and denitrifying (*nir*S, *nir*K, and *nos*Z) soil microbial communities (Snider et al. 2015).

2.3.3 Impact of crop rotation and cover cropping on mitigation of N-loss in agroecosystems

Recent evidence indicates crop rotations and adoption of CC are a solution to reduce N-loss, from NO_3^- via leaching and as N_2O emissions, because crop sequences impact the abundance and functioning of N-cycling SMCs (Romillac et al., 2022). In a greenhouse experiment, Bowles et al. (2022) found that a complex rotation allowed 80% more N uptake by MZ than monoculture MZ, reducing the risk of N-loss. Furthermore, Bay et al. (2021) reported a reduction in AOB

diversity, potentially leading a decrease in nitrification in a diverse crop rotation. In addition to the impact of crop rotation on N-cycling SMCs, abiotic stresses such as drought also impact the N-cycle. Bowles et al. (2022) reported an increase in N-mineralization and nitrification during water deficit conditions, which worsened the risk of N-loss as NO_3- ions via leaching after soil moisture was restored. However, studies have shown the ability of CCs to minimize N-loss even under changing environments. For example, Tonitto et al. (2006) demonstrated the ability of a winter CC in a diverse cropping system to recover soil N before precipitation in spring, thereby reflecting the importance of CCs in a rotation to reduce the risk of N-loss during dry seasons.

CCs play an important role in determining N-levels in agricultural fields. Poaceae spp. such as oats (*Avena sativa* L.) and rye grass (*Secale cereale* L.), have extensive root systems that can scavenge excess nutrients such as N in soil (Snapp et al., 2005). Cover crops retain scavenged NO_3^- as forage proteins for eventual release and uptake by the following cash cropping after CC termination (Dabney et al., 2001), reducing the potential of NO_3^- leaching to underground and surface freshwater bodies (Tonitto et al., 2006), and N-losses via denitrification (Thapa et al., 2018). Several studies have reported cereal rye, forage radish (*Raphanus sativus* L.), and canola (*Brassica rapa* L.) as CCs with a relatively higher ability to scavenge NO_3^- (Dabney et al., 2001; Teixeira et al., 2016; Thapa et al., 2018; Tonitto et al., 2006). A meta-analysis of data from long-term studies demonstrated a 70% decrease in leaching in fallow agricultural lands after the adoption of non-leguminous CCs such as cereal rye, annual rye (*Lolium multiflorum* Lamarck), oat, or oilseed radish (Tonitto et al., 2006). Comparably, Thapa et al. (2018) reported a 56% reduction in NO_3^- in leachate when non-leguminous CCs, such as grasses and broad-leaf species, were used in cropping systems. Teixeira et al. (2016) observed a decrease of NO_3^- in the leachate in fallow fields with well-grown CC compared to bare fallow fields and fallow fields with poorly

grown CC. Although, recent studies have focused more on understanding the importance of CC in relation to soil health and soil N-management. No attention has been given to exploring the impact of CCs on the soil phytohormone profiles, which are another crucial factor for plant growth and survival.

2.4 phytohormones in plants

Phytohormones, or plant hormones, are naturally occurring signalling molecules with a low molecular weight that play crucial roles in crops by regulating their growth and mediating biological and physiological processes (Smith et al., 2017; Mubarik et al., 2021; Jan et al., 2019). Some commonly known phytohormones are abscisic acid, cytokinins, ethylene, gibberellin, auxin, jasmonic acid, and salicylic acid (Jan et al., 2019). Among them, the cytokinins (CKs) are considered a prominent growth regulator involved in improving crop growth and crop yields (High et al., 2019; Jameson and Song, 2016).

2.4.1 Cytokinins

Cytokinins are adenine derivatives with a side chain at the N^6 position, with a defining role in inducing cell division/cytokinesis (Kakimoto 2003; Hwang and Sakakibara 2006; Stirk and Staden 2010). The first unambiguous CK discovered was ‘Kinetin’ isolated from autoclaved herring sperm, and this compound induced cell division in wounded tobacco callus tissues (Miller and Skoog, 1955). Later, Kinetin was identified as the compound responsible for coconut milk's growth-promoting ability (Ge et al., 2005). Since then, several natural and synthetic CKs have been discovered in various forms of life, including microorganisms. Some examples include: *Methylobacteria* (Palberg et al., 2022; Jorge et al., 2019), *Sinorhizobium meliloti* (Kisiala et al., 2013), *Paenibacillus polymyxa* (Timmusk et al., 1999), *Bacillus subtilis* (Hussain and Hasnain, 2015; Kudoyarova et al., 2014; Liu et al., 2013; Arkhipova et al., 2007),

Pseudomonas aeruginosa (Hussain and Hasnain, 2015), *Agrobacterium tumefaciens* and *Pseudomonas solanacearum* (Akiyoshi et al., 1987).

2.4.1.1 Different types of CKs and their biosynthesis pathways

To date, more than 32 different CK forms have been identified (Kisiala et al., 2019), which are sorted into two major classes based on the configuration of their side chain, namely: isoprenoid CKs and aromatic CKs (**Fig. 2.2**). Modification in these side chains has resulted in different CK types and biological roles (reviewed in Hluska et al., 2021). Isoprenoid type CKs bear an isoprenoid side chain at the N^6 position of the adenine ring, while aromatic type CKs contain an aromatic group at the N^6 position of the adenine ring (Kieber and Schaller 2014; Stirk and Staden 2010). Isoprenoid type CKs are the most common CKs found in nature, and they include isopentenyl adenine (iP), zeatin (Z), dihydrozeatin (DHZ), and their riboside and nucleotide forms. Zeatin was the first natural type of CK detected, and both cis (cZ) or trans (tZ) isomers of Z have been detected in living tissues (reviewed in Hluska et al., 2021). Trans-Zeatin is the most active form of CK, while cZ has shown lower CK activity in bioassays. However, cZ has been recognized as the predominant form of CK in plants and fungi, and it also mediates stress responses in plants (reviewed in Hluska et al., 2021). Aromatic CKs include kinetin, N^6-benzyl adenine (BA), topolins (To), and purines (MPP) (reviewed in Hluska et al., 2021; Kieber and Schaller 2014; Stirk and Staden 2010).

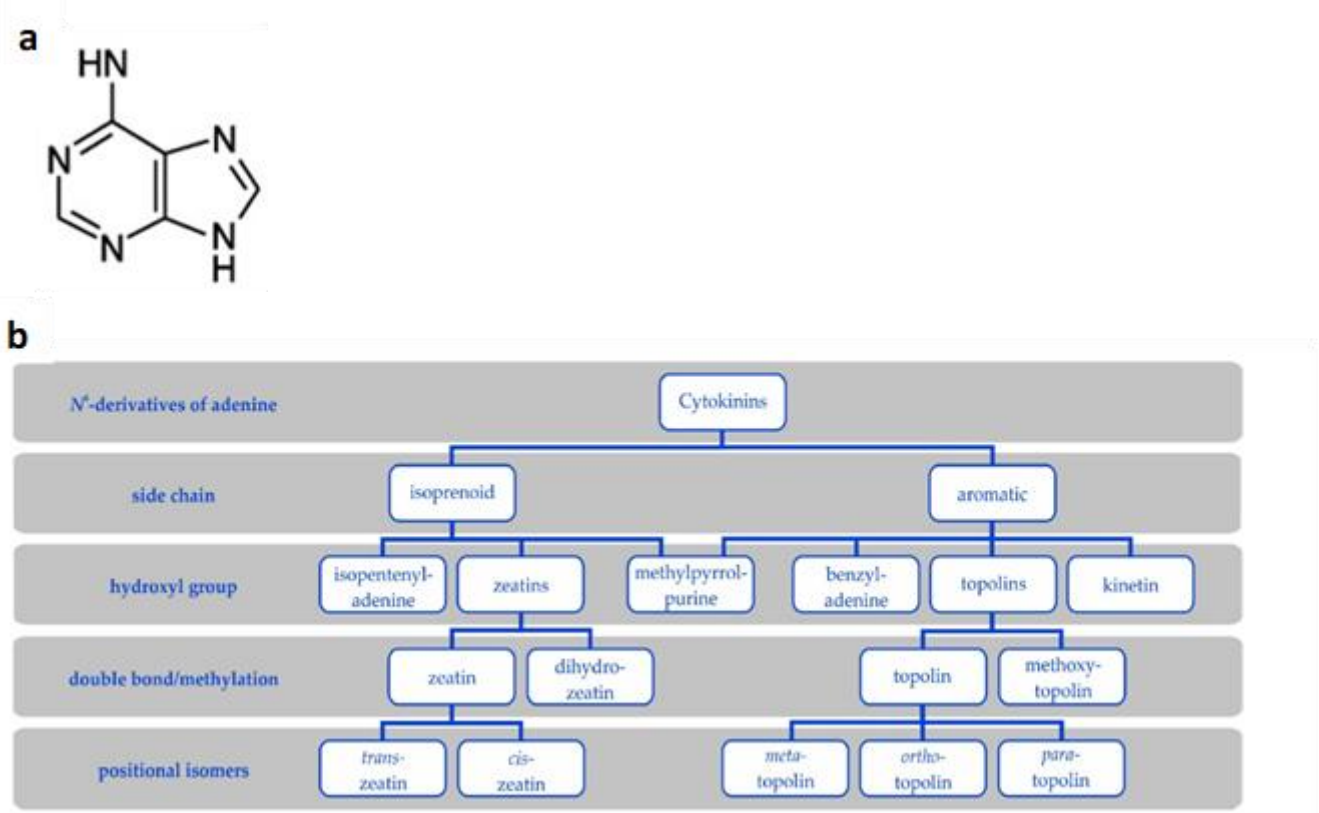

Fig. 2.2. a. Structure of cytokinin, **b**. Different types of cytokinins, and their classification based the configuration of their side chain as isoprenoid cytokinins and aromatic cytokinins. Based on the hydroxyl group, isoprenoid cytokinins are further classified into isopentenyl adenine and zeatins, while aromatic cytokinins are grouped into benzyl adenine, topolins and kinetin. Although methylpyrrol purine originate from trans-zeatin, it has an aromatic side chain; therefore, it shares both isoprenoid and aromatic cytokine groups. Zeatins are further grouped into zeatin and dihydrozeatin because of the position of a double bond., and zeatins occur as either trans or cis isomers (Hluska et al., 2021).

2.4.1.2 Evolution of CK biosynthesis genes

Pathways of CK biosynthesis have been discovered in almost all kingdoms of life, ranging from low-level organisms- to cyanobacterial species, e.g., *Synechocystis*, *Chroococcidiopsis*, *Anabaena*, *Phormidium* and *Oscillatoria* spp. (Hussain et al., 2010; Stirk and Koma, 1999) to higher levels organisms such as plants, for e.g., *Arabidopsis thaliana* (Takei et al. 2001). Biosynthesis of isoprenoid-type CKs occurs via two pathways, 1. Isopentylation of free adenosine nucleotides (ATP, ADP or AMP) (direct pathway), and 2. tRNA degradation pathway

(indirect pathway) (Gray et al. 1996; Sakakibara 2010; Hluska et al. 2021) (**Fig. 2.3**). The first step of the direct pathway involves N-prenylation of adenosine 5'-phosphate (ATP, ADP or AMP) at the N^6 position with either dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP) (Hwang and Sakakibara 2006; Kamada-Nobusada and Sakakibara 2009). Depending on the prenyl acceptor (ATP/ ADP/AMP) used, this reaction produces iP riboside 5'-triphosphate (iPRTP), iP riboside 5'-diphosphate (iPRDP) or iP riboside 5'-monophosphate (iPRMP) and this reaction is the rate-limiting step of CK biosynthesis and is catalyzed by the enzyme adenosine phosphate-isopentenyl transferase (IPT) (Kamada-Nobusada and Sakakibara 2009; Sugawara et al. 2007; Hwang and Sakakibara 2006; Kakimoto, 2003).

An IPT gene was first discovered at the *tmr* locus in the Ti plasmid of *Agrobacterium tumefaciens* (Barry et al., 1984) and later in other organisms, including *Arabidopsis thaliana* (Miyawaki et al., 2006). Homology between IPT genes in plants and cyanobacteria suggests that CK genes evolved from cyanobacteria via horizontal gene transfer (Frébortová et al., 2015; Spíchal, 2012; Frébort et al., 2011). With the reveal of the full genome sequence of *Arabidopsis thaliana,* nine IPT encoding genes (*AtIPT*1-*AtIPT*9) were identified (Sakakibara, 2010). Miyawaki et al. (2006) reported that these genes produce two types of IPTs that utilize different substrates, adenosine phosphate-IPT (AP-IPT) (*AtIPT1*, *AtIPT3*, *AtIPT5* and *AtIPT7*), and tRNA-IPT (*AtIPT*2, *AtIPT*9). Nishii et al., (2018) have classified plant IPTs into two classes based on their protein sequences, namely, class I tRNA-IPTs originated from bacterial *mia*A genes, and class II tRNA-IPTs and adenosine-IPT (AD-IPT) evolved from eukaryotic genes in prasinophyte algae and in euphyllophytes. AD-IPTs catalyze the first step of the direct pathway (Kasahara et al. 2004), while tRNA-IPT- modify the adenosine residue at the 37^{th} position located next to the 3' end of the anticodon starting from uracil in the tRNA molecule (Nishii et al., 2018).

The tumour-inducing *Agrobacterium tumefaciens Tmr* gene is responsible for the synthesis of IPT enzyme, which involves CK production during plant infection Akiyoshi et al. (1984, 1985). Sakakibara et al. (2005) reported that the Tmr enzyme, encoded by the T-DNA region of *A. tumefaciens* functions in the plastid of the infected plant in a similar way to the IPT enzyme. Furthermore, this study has revealed that Tmr creates a new shortcut to producing tZ cytokinin directly by primarily utilizing HMBDP (an intermediate of MEP pathway in plastids) as the prenyl donor and AMP as the prenyl acceptor (**Fig. 2.3**). This pathway eliminates the P450 monooxygenase-mediated hydroxylation step in the normal tZ biosynthesis pathway (Sakakibara 2006). Another IPT transferase gene, known as TZS, has been reported in the virulence region of *A. tumefaciens* (Akiyoshi et al. 1985). The affinity of two bacterial IPT genes (Km values) for both the substrates are similar; therefore, unlike plant IPTs, bacterial IPTs can utilize either DMAPP or HMBDP as prenyl donors (Hwang and Sakakibara 2006; Kamada-Nobusada and Sakakibara 2009). When the bacterial IPTs use DMAPP in the presence of AMP to synthesize iP type CKs, while HMBDP with AMP to synthesize tZ type CKs (Kamada-Nobusada and Sakakibara 2009), the major pathway of tZ synthesis in higher plants is through trans-hydroxylation of the side chain of iP-type cytokinins (iPRTP, iPRDP or iPRMP) in the presence of the enzyme P450 monooxygenase (CYP735A) (**Fig. 2.3**; Hwang and Sakakibara 2006; Miyawaki et al. 2006).

The tRNA degradation pathway regulates the biosynthesis of cZ-type CKs. In this pathway, a tRNA molecule acts as a prenyl acceptor and undergoes isoprenylation in the presence of the prenyl donor DMAPP, and this reaction is catalyzed by the enzyme tRNA- isopentenyl transferase (Kamada-Nobusada and Sakakibara 2009). The tRNA isopentenyltransferase enzyme can modify the adenosine residue at the 37^{th} position, which is situated next to the 3' end of the

anticodon starting from U in the tRNA molecule (Nishii et al., 2018). These modifications of adenosine include N6-isopentenyl adenosine (i6A, or iPR), and modification of guanosine, to N1-methylguanosine (m1G) or wybutosine (yW) (Schweizer et al., 2017; Miyawaki et al. 2006; Golovco et al. 2002; Motorin et al. 1997; Gray et al. 1996). Therefore, degradation of these tRNA can produce iP and cZ type CKs (**Fig. 2.3**; Miyawaki et al. 2006; Hluska et al., 2021). A lesser studied group of CKs, are the methylthiolated CKs (2MeS-CKs) conjugates. They are CKs derived through the addition of a thiol group –SH to position 2 of the adenine ring in tRNA-bound iP or Z-type CKs (**Fig. 2.4**) and produce 2-methyl-thio i6A (ms2i6A, or 2MeSiPR), 6-hydroxy ms2i6A (ms2io6A, or 2MeScZR), N6-threonylcarbamoyladenosine (t6A, or ms2t6A) (reviewed in Gibb et al., 2020). The 2MeS-CK conjugates have been detected across kingdoms ranging from microorganisms to macro-organisms, including plants and animals.

In this thesis, I focused on determining and quantifying different CK forms including, active CKs, riboside CKs forms, 2MeS-CKs (MeSZ, MeSiP, MeSZR, and MeSiPR), and aromatic CKs in cover crop grown soil.

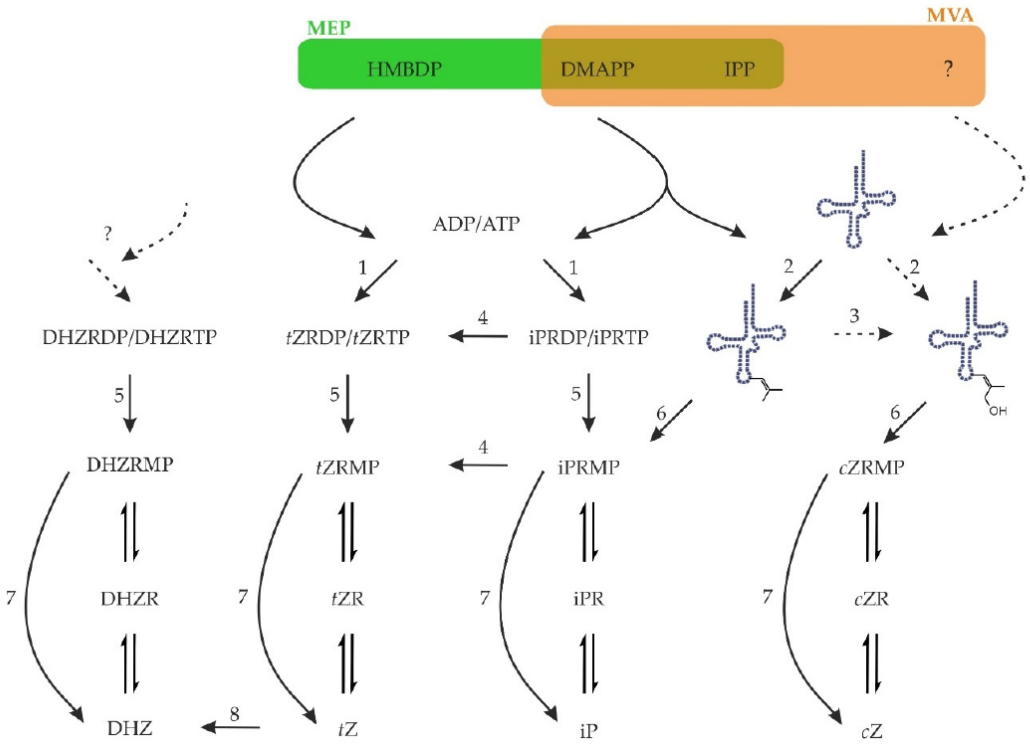

Fig. 2.3. Biosynthesis and activation of cytokinins in plants. Precursors for CK biosynthesis, HMBDP and DMAPP, come either from the methylerythritol (MEP) or mevalonate (MVA) pathways. Plant adenylate IPTs (1) utilize mostly ADP or ATP whereas tRNA IPTs (2) use the adenine in position 37 of certain tRNAs as acceptor substrate. cis-Zeatin is known to originate from tRNA, but its synthesis is unclear, as neither a cis-hydroxylated precursor nor cis-hydroxylase (3) have yet been identified in plants. Nucleotides of iP may be hydroxylated by cytochrome P450 (4) to form tZ. Upon hydrolysis of γ- and β-phosphates (5) or tRNA hydrolysis (6), the resulting monophosphates may be activated in one step by CK-specific phosphoribohydrolase named 'Lonely guy' (7). Alternatively, the nucleotides, nucleosides, and nucleobases are probably interconverted by enzymes of purine metabolism. The free base tZ may be reduced to DHZ by zeatin reductase (8). Whether there is any de novo biosynthesis of DHZ is currently unknown. Zeatin cis-trans isomerase does not exist. (Hluska et al., 2021)

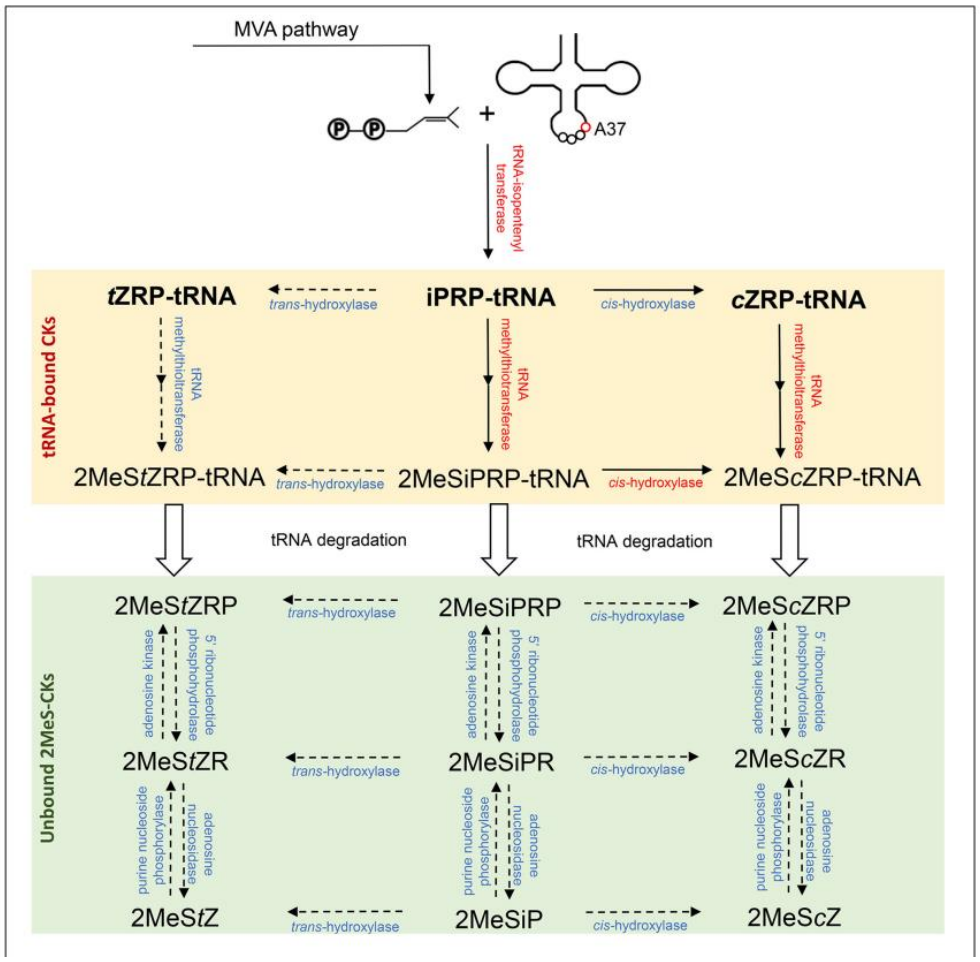

Fig. 2.4. Biosynthesis pathway of the methylthiolated cytokinins (2MeS-CKs) proposed based on the microbial, plant and animal 2MeS-CK metabolite profiles obtained at the Water Quality Centre, Trent University and from other laboratories. Three groups of enzymes (red font) involved in the production of 2MeS-CKs have been previously identified across kingdoms of life including: tRNA-isopentenyltransferases (e.g., miaA, MOD5, IPT2 and 9, TRIT1), tRNA-methylthiotransferases (e.g., miaB + C, CDK5RAP1), and cis-hydroxylases (e.g., miaE) (Dabravolski, 2020). The inferred enzymes involved in further modification of tRNA-bound and free 2MeS-CKs (blue font, dashed arrows) were predicted based on the information available from other CK producing pathways (Sakakibara, 2006; Spíchal, 2012; Morrison et al., 2017; Aoki et al., 2019a). Mevalonate (MVA) pathway is the main source of the isoprenoid substrate for tRNA-isopentenyltranserases; however, a small pool of the prenyl chain molecules can originate from the methylerythritol phosphate (MEP) pathway. If tRNA degradation occurs prior to methylthiolation of the prenylated adenine nucleobase (A37), tRNA-bound iPRP, tZRP, and cZRP (bold font) are released and act as the precursors of classical CKs (iP, tZ and cZ) (reviewed in Gibb et al., 2020).

2.4.2 Impact of soil N status on CK biosynthesis in plants

CK biosynthesis in plants is affected by various abiotic factors, like soil nutrient status, e.g., soil NO_3^-/NH_4^+ levels (Sakakibara, 2021). The N-signaling pathway in plants is the way levels and status of N-in soil are communicated to plants for the up or down-regulation of N-metabolism genes (Sakakibara, 2021). It involves a two-component signalling system, which is activated by CK (Sakakibara et al. 2000). The His-Asp phosphorelay (HAP), is a common signalling pathway found in prokaryotes, which mediates multiple signal transductions in response to environmental signals (Sakakibara et al. 2000; Hwang et al. 2002; Asakura et al. 2003). A HAP signalling system consists of three components: His- kinase protein (HK), His-containing phosphotransfer protein (HP), and a response regulator (RR) (Sakakibara et al. 2000; Hwang et al. 2002; Asakura et al. 2003; Sakakibara 2003) (**Fig. 2.5**).

The N-dependent CK biosynthesis in plants has been shown in several early studies. Takei et al. (2004) reported the correlation of the *Arabidopsis* IPT gene transcripts, *AtIPT3*, and *AtIPT5* with NO_3^-/NH_4^+ levels. Some early works showed that N-depleted MZ leaves that were treated with CK resulted in enhanced RR gene expression involved in the N-signal transduction pathway (Takei et al. 2001; Sakakibara et al., 1998). Similarly, there is increased expression of N-signal transduction genes (*ARR*) (Taniguchi et al., 1998) and *ZmCip*1 (Sakakibara et al., 1998) in leaves of *Arabidopsis* and maize, respectively. In addition to RR genes, CKs have also accumulated in roots as a response to NO_3^- resupply, suggesting the involvement of CKs in N-signalling (Sakakibara et al., 1998). Moreover, it was reported that the accumulation of CKs in roots and shoots depends on the type of N supplied (NO_3^- or NH_4^+) (Kamada-Nobusada et al. 2013). Furthermore, Takei et al. (2001) have shown a transient accumulation of iPMP in roots, and Z-

type CKs in both shoots and leaves in response to NO_3^- resupply. This emphasizes the role of CK in N-signal transduction from roots-leaves via xylem.

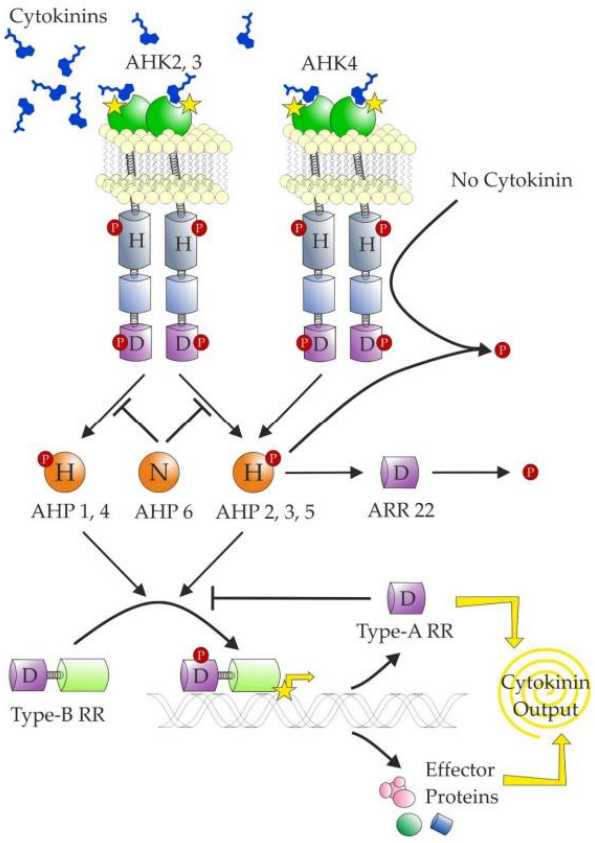

Fig. 2. 5. Schematic representation of current knowledge of cytokinin signalling. The transmembrane cytokinin receptors (histidine kinases) auto-phosphorylate in the presence of cytokinins and subsequently transfer the phosphate to the histidine phosphotransfer proteins (AHPs). However, in the absence of cytokinins, AHK4 has phosphatase activity and dephosphorylates all AHPs. The transfer is inhibited by AHP6, which has a substitution of the conserved Asp for Asn. AHP2,3 and 5 are rapidly dephosphorylated by ARR22. The AHPs transfer the phosphate group to type-B response regulators, which work as transcription factors. One of their targets are type-A response regulators, which inhibit the phosphotransfer from AHPs to type-B RRs. The conserved residues are marked (H—histidine; D—aspartate; N—asparagine). The phosphate group is marked by a P contained in a red circle. The extracytosolic CHASE domains are shown in green, kinase domains are shown in grey, and receiver and receiver-like domains are respectively shown in violet (marked with a D) and light blue (no D) (Hluska et al., 2021).

2.4.3 Cycling of CK in the soil matrix

CKs are biosynthesized inside living organisms; however, CKs inside living organisms may be released to their outside environments, such as soil, water etc. Knowledge of the occurrence of CKs and their roles in soil is crucially important since this may provide insight towards understanding plant health and crop yields, especially agroecosystems, with respect to different agricultural management strategies such as cover cropping, tillage, inter-cropping, and integrated pest and nutrient approaches. Furthermore, this will also reveal the relationship between soil CKs and soil health.

Organisms including plants, SMCs, and other soil-dwelling organisms such as nematodes, earthworms, and insects can all be sources of soil CKs (**Fig. 2.6**). In addition, CKs can be added to the soil with the input of fertilizers containing CKs or CK precursors, microbial biofertilizers or organic materials such as manures or plant residues.

Plants are a core component in agroecosystems, and therefore, they should be major contributors to soil CK profiles. Previous studies report the presence of free base-CKs (iP, cZ, tZ), riboside-CKs (cZR, tZR), and conjugated-tZ in root exudates of rice plants collected on cotton swabs (Murofushi et al., 1983; Soejima et al., 1992). Like the ability of roots to release CKs to the soil medium, they also can uptake CK from the soil for their own metabolism. In addition to roots, leaves are another common reservoir of CK (Wu et al., 2021; Janečková et al., 2018; Hewett & Wareing, 1973), highlighting the likelihood that senescing and degrading plant matter are also contributing to the pool of soil CKs.

Another possible source of soil CKs are SMCs, which produce CKs. A vast number of studies have documented the ability of beneficial microorganisms, e.g. *Methylobacterium* (Palberg et al., 2022; Jorge et al., 2019), *Trichoderma* (Bean et al., 2021), and *Rhizobia* (Fonouni-Farde et al.,

2017) spp., and arbuscular mycorrhizal fungi, e.g. *Rhizophagus irregularis* (Goh et al., 2019), and as well as pathogenic microorganisms, e.g. fungus *Magnaporthe oryzae* (Chanclud et al., 2016), *Ustilago maydis* (Morrison et al., 2017), and fungus *Gymnosporangium juniper-virginianae* Schwein (Moffatt et al. 2017) to synthesize CKs. Furthermore, early studies have reported the ability of microorganisms, for e.g., N_2 fixing, symbiotic *Rhizobium japonicum* and *Rhizobium leguminosarum* (Phillips & Torrey, 1972), free-living *Rhizobacterium*, *Paenibacillus polymyxa* (Timmusk et al., 1999), and mycorrhizal fungi (Crafts & Miller, 1974) to release CKs to their growth medium. In addition to plants and SMC, other CK-producing soil-dwelling organisms such nematodes (De Meutter et al., 2003), earthworms (High et al., 2019), and insects (Andreas et al., 2020) could be potential sources of soil CKs. CKs have ample opportunity to feedback and impact plant or non-plant organisms alike once they are in the soil. However, studies on soil CKs are limited, and there is a poor understanding of the potential role of soil CK functioning in the soil ecosystem.

Very few studies have reported on the occurrence of CKs in the soil to date. Those include High et al. (2019), who identified adenine, iP, and tZ type CKs in soils and have reported the positive impact of earthworms on soil CK profiles. van Staden and Dimalla (1976), have extracted and identified zeatin and zeatin riboside-like CKs in four types of acidic soils, where different plants (*Acacia mearnsii, Pinus patula, Carya illinoensis*) were cultivated in association with rhizosphere microorganisms (Rhizobium sp., and mycorrhizal fungi; *Lycoperdon* sp. and *Scleroderma* sp.). Furthermore, exogenous application of the extracted CKs on soybean callus cultures confirms the CKs present in soil have the same biological activity (van Staden & Dimalla 1976). Moreover, Nieto & Frankenberger (1989) reported that the exogenous application of CK precursors adenine (ADE) and isoprenoid alcohol (IA) together with the

inoculum of rhizosphere microorganisms (*Azotobacter chroococcum*) improve the levels of zeatin ribosides (ZR) and t-zeatin CKs in soil. The findings of Nieto & Frankenberger (1989) showed that the input of CK precursors enhances CK biosynthesis in soil microorganisms, which in turn improves CK levels in the soil. CK levels in agricultural soil are affected by climatic conditions, plant growth stage, and agricultural management practices (Rossi et al., 1984). Rossi et al. (1984) showed that the addition of fertilizers and herbicide atrazine had lowered the levels of CK in both rhizosphere and bulk soil. Given the paucity of knowledge on soil CK occurrence in agricultural soils, it is not surprising that the impact of cover cropping management on soil CK levels and the impact of different cover crop species on soil CK levels are completely unknown. Moreover, the effect of soil CKs on soil dwelling organisms and their functioning, especially SMCs that participate in major nutrient cycling such as the N- cycle, is unknown.

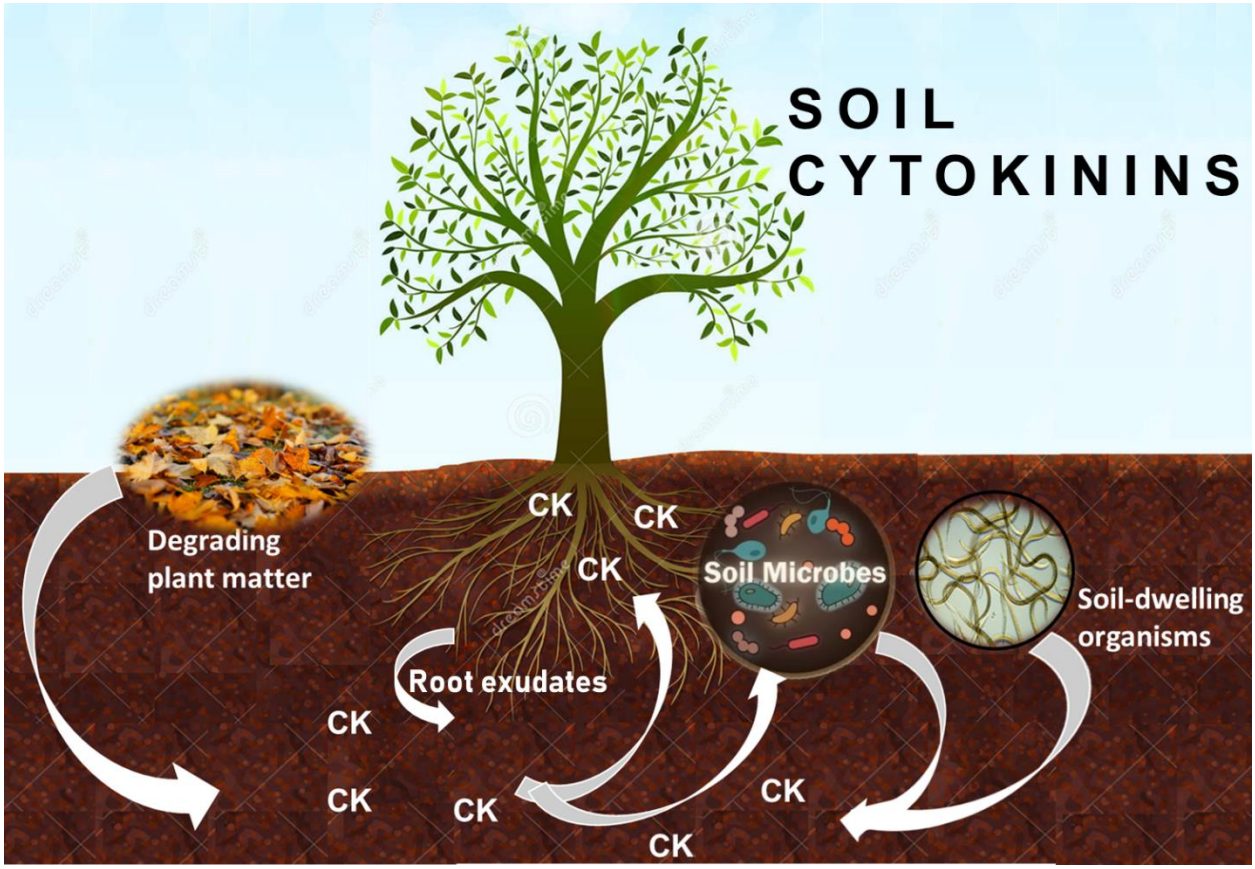

Fig. 2.6. Cytokinin flow in soil. Organisms including plants, soil microbial communities, and other soil dwelling organisms such as nematodes, earthworms, and insects could be the sources of soil cytokinins. Moreover, plant degrading matter can contribute to the pool of cytokinins in soil. Cytokinins present in soil are uptake by plant roots and other soil-dwelling organisms for their metabolism.

2.5 Bibliography

Abalos, D., Liang, Z., Dörsch, P., & Elsgaard, L. (2020). Trade-offs in greenhouse gas emissions across a liming-induced gradient of soil pH: Role of microbial structure and functioning. *Soil Biology and Biochemistry*, *150*(March). https://doi.org/10.1016/j.soilbio.2020.108006

Agomoh, I. V., Drury, C. F., Yang, X., Phillips, L. A., & Reynolds, W. D. (2021). Crop rotation enhances soybean yields and soil health indicators. *Soil Science Society of America Journal*, *85*(4), 1185–1195. https://doi.org/10.1002/saj2.20241

Akiyoshi, D. E., Kleet, H., Amasinot, R. M., Nestert, E. W., & Gordon, M. P. (1984). T-DNA of Agrobacterium tumefaciens encodes an enzyme of cytokinin biosynthesis (crown gall/tumor-inducing plasmid/tmr gene/plant tumorigenesis). In *Proc. Nati. Acad. Sci. USA* (Vol. 81).

Akiyoshi, D. E., Regier, D. A., & Gordon, M. P. (1987). Cytokinin production by Agrobacterium and Pseudomonas spp. *Journal of Bacteriology*, *169*(9), 4242–4248. https://doi.org/10.1128/jb.169.9.4242-4248.1987

Ali, A., Ghani, M. I., Li, Y., Ding, H., Meng, H., & Cheng, Z. (2019). Hiseq base molecular characterization of soil microbial community, diversity structure, and predictive functional profiling in continuous cucumber planted soil ffected by diverse cropping systems in an intensive greenhouse region of northern China. *International Journal of Molecular Sciences*, *20*(11). https://doi.org/10.3390/ijms20112619

Andrén, O., & Kätterer, T. (2018). Agriculture systems. *Encyclopedia of Ecology*, *2*, 401–406. https://doi.org/10.1016/B978-0-444-63768-0.00313-9

Andreas, P., Kisiala, A., Neil Emery, R. J., De Clerck-Floate, R., Tooker, J. F., Price, P. W., Miller, D. G., Chen, M. S., & Connor, E. F. (2020). Cytokinins are abundant and widespread among insect species. *Plants*, *9*(2). https://doi.org/10.3390/plants9020208

Asakura, Y., Hagino, T., Ohta, Y., Aoki, K., Yonekura-Sakakibara, K., Deji, A., Yamaya, T., Sugiyama, T., & Sakakibara, H. (2003). Molecular characterization of His-Asp phosphorelay signaling factors in maize leaves: Implications of the signal divergence by cytokinin-inducible response regulators in the cytosol and the nuclei. In *Plant Molecular Biology* (Vol. 52).

Barc, P., Arshad, M., & Frankenberger, W. T. (1991). *Microbial production of plant hormones*.

Barreiro, A., Fontúrbel, M. T., Lombao, A., Martín, A., Vega, J. A., Fernández, C., Carballas, T., & Díaz-Raviña, M. (2015). Using phospholipid fatty acid and community level physiological profiling techniques to characterize soil microbial communities following an experimental fire and different stabilization treatments. *Catena*, *135*, 419–429. https://doi.org/10.1016/j.catena.2014.07.011

Bay, G., Lee, C., Chen, C., & Mahal, N. K. (n.d.). *Agricultural Management Affects the Active Rhizosphere Bacterial Community Composition and Nitri fi cation*.

Barry, G. F., Rogers, S. G., Fraley, R. T., & Brand, L. (1984). Identification of a cloned cytokinin biosynthetic gene. *Proceedings of the National Academy of Sciences*, *81*(15), 4776–4780. https://doi.org/10.1073/pnas.81.15.4776

Bean, K. M., Kisiala, A. B., Morrison, E. N., & Emery, R. J. N. (2021). Trichoderma Synthesizes Cytokinins and Alters Cytokinin Dynamics of Inoculated Arabidopsis Seedlings. *Journal of Plant Growth Regulation*, *0123456789*. https://doi.org/10.1007/s00344-021-10466-4

Behnke, G. D., Kim, N., Zabaloy, M. C., Riggins, C. W., Rodriguez-Zas, S., & Villamil, M. B. (2021). Soil microbial indicators within rotations and tillage systems. *Microorganisms*, *9*(6). https://doi.org/10.3390/microorganisms9061244

Behnke, G. D., Zuber, S. M., Pittelkow, C. M., Nafziger, E. D., & Villamil, M. B. (2018a). Long-term crop rotation and tillage effects on soil greenhouse gas emissions and crop production in Illinois, USA. *Agriculture, Ecosystems and Environment*, *261*(January), 62–70. https://doi.org/10.1016/j.agee.2018.03.007

Behnke, G. D., Zuber, S. M., Pittelkow, C. M., Nafziger, E. D., & Villamil, M. B. (2018b). Long-term crop rotation and tillage effects on soil greenhouse gas emissions and crop production in Illinois, USA. *Agriculture, Ecosystems and Environment*, *261*(March), 62–70. https://doi.org/10.1016/j.agee.2018.03.007

Behnke, G. D., Zuber, S. M., Pittelkow, C. M., Nafziger, E. D., & Villamil, M. B. (2018c). Long-term crop rotation and tillage effects on soil greenhouse gas emissions and crop production in Illinois, USA. *Agriculture, Ecosystems and Environment*, *261*(January), 62–70. https://doi.org/10.1016/j.agee.2018.03.007

Belfry, K. D., Trueman, C., Vyn, R. J., Loewen, S. A., & Van Eerd, L. L. (2017). Winter cover crops on processing tomato yield, quality, pest pressure, nitrogen availability, and profit margins. *PLoS ONE*, *12*(7), 1–17. https://doi.org/10.1371/journal.pone.0180500

Belfry, K. D., & Van Eerd, L. L. (2016). Establishment and impact of cover crops intersown into corn. *Crop Science*, *56*(3), 1245–1256. https://doi.org/10.2135/cropsci2015.06.0351

Bodner, G., Himmelbauer, M., Loiskandl, W., & Kaul, H. P. (2010). Improved evaluation of cover crop species by growth and root factors. *Agronomy for Sustainable Development*,

30(2), 455–464. https://doi.org/10.1051/agro/2009029

Bonfante, A., Basile, A., & Bouma, J. (2020). Targeting the soil quality and soil health concepts when aiming for the United Nations Sustainable Development Goals and the EU Green Deal. *Soil*, *6*(2), 453–466. https://doi.org/10.5194/soil-6-453-2020

Bowles, T. M., Jilling, A., Mor, K., & Grandy, A. S. (2022). *Crop rotational complexity affects plant-soil nitrogen cycling during water deficit*. *166*. https://doi.org/10.1016/j.soilbio.2022.108552

Bowles, T. M., Mooshammer, M., Socolar, Y., Calderón, F., Cavigelli, M. A., Culman, S. W., Deen, W., Drury, C. F., Garcia y Garcia, A., Gaudin, A. C. M., Harkcom, W. S., Lehman, R. M., Osborne, S. L., Robertson, G. P., Salerno, J., Schmer, M. R., Strock, J., & Grandy, A. S. (2020). Long-Term Evidence Shows that Crop-Rotation Diversification Increases Agricultural Resilience to Adverse Growing Conditions in North America. *One Earth*, *2*(3), 284–293. https://doi.org/10.1016/j.oneear.2020.02.007

Boydston, R. A., & Hang, A. (1995). Rapeseed (Brassica napus) Green Manure Crop Suppresses Weeds in Potato (Solanum tuberosum) . *Weed Technology*, *9*(4), 669–675. https://doi.org/10.1017/s0890037x00024039

Brookes, P. (2001). The Soil Microbial Biomass: Concept, Measurement and Applications in Soil Ecosystem Research. *Microbes and Environments*, *16*(3), 131–140. https://doi.org/10.1264/jsme2.2001.131

Brussaard, L., (1997). Biodiversity and Ecosystem Functioning in Soil. Springer. 26, 563-570.

Cai, A., Xu, M., Wang, B., Zhang, W., Liang, G., Hou, E., & Luo, Y. (2019). Manure acts as a

better fertilizer for increasing crop yields than synthetic fertilizer does by improving soil fertility. *Soil and Tillage Research*, *189*(February 2018), 168–175. https://doi.org/10.1016/j.still.2018.12.022

Castellazzi, M. S., Wood, G. A., Burgess, P. J., Morris, J., Conrad, K. F., & Perry, J. N. (2008). A systematic representation of crop rotations. *Agricultural Systems*, *97*(1–2), 26–33. https://doi.org/10.1016/j.agsy.2007.10.006

Chamberlain, L. A., Bolton, M. L., Cox, M. S., Suen, G., Conley, S. P., and Ané, J. M. (2020). Crop rotation, but not cover crops, influenced soil bacterial community composition in a corn-soybean system in southern Wisconsin. Applied Soil Ecology, 154(December 2019), 103603. https://doi.org/10.1016/j.apsoil.2020.103603

Chanclud, E., Kisiala, A., Emery, N. R. J., Chalvon, V., Ducasse, A., Romiti-Michel, C., Gravot, A., Kroj, T., & Morel, J. B. (2016). Cytokinin Production by the Rice Blast Fungus Is a Pivotal Requirement for Full Virulence. *PLoS Pathogens*, *12*(2), 1–25. https://doi.org/10.1371/journal.ppat.1005457

Chen, X., Wang, J., Wang, Z., Li, W., Wang, C., Yan, S., Li, H., Zhang, A., Tang, Z., & Wei, M. (2018). Optimized nitrogen fertilizer application mode increased culms lignin accumulation and lodging resistance in culms of winter wheat. Field Crops Research, 228(August), 31–38. https://doi.org/10.1016/j.fcr.2018.08.019

Crafts, C. B., & Miller, C. O. (1974). Detection and Identification of Cytokinins Produced by Mycorrhizal Fungi. *Plant Physiology*, *54*(4), 586–588. https://doi.org/10.1104/pp.54.4.586

Dabney, S. M., Delgado, J. A., & Reeves, D. W. (2001). Using winter cover crops to improve soil and water quality. *Communications in Soil Science and Plant Analysis*, *32*(7–8), 1221–

1250. https://doi.org/10.1081/CSS-100104110

De Baets, S., Poesen, J., Meersmans, J., & Serlet, L. (2011). Cover crops and their erosion-reducing effects during concentrated flow erosion. *Catena*, *85*(3), 237–244. https://doi.org/10.1016/j.catena.2011.01.009

De Meutter, J., Tytgat, T., Witters, E., Gheysen, G., Van Onckelen, H., & Gheysen, G. (2003). Identification of cytokinins produced by the plant parasitic nematodes Heterodera schachtii and Meloidogyne incognita. *Molecular Plant Pathology*, *4*(4), 271–277. https://doi.org/10.1046/j.1364-3703.2003.00176.x

Degani, E., Leigh, S. G., Barber, H. M., Jones, H. E., Lukac, M., Sutton, P., & Potts, S. G. (2019). Crop rotations in a climate change scenario: short-term effects of crop diversity on resilience and ecosystem service provision under drought. *Agriculture, Ecosystems and Environment*, *285*(August), 106625. https://doi.org/10.1016/j.agee.2019.106625

Di Capua, F., Pirozzi, F., Lens, P. N. L., & Esposito, G. (2019). Electron donors for autotrophic denitrification. *Chemical Engineering Journal*, *362*(3), 922–937. https://doi.org/10.1016/j.cej.2019.01.069

Dixon, G. R., & Tilston, E. L. (2010). Soil microbiology and sustainable crop production. In *Soil Microbiology and Sustainable Crop Production*. https://doi.org/10.1007/978-90-481-9479-7

Doran, J. W., & Zeiss, M. R. (2000). DigitalCommons @ University of Nebraska - Lincoln Soil health and sustainability : managing the biotic component of soil quality Soil health and sustainability : managing the biotic component of soil quality. *Applied Soil Ecology*, *June*.

Eerd, L. L. Van. (2018). Nitrogen dynamics and yields of fresh bean and sweet corn with

different cover crops and planting dates. *Nutrient Cycling in Agroecosystems*, *111*(1), 33–46. https://doi.org/10.1007/s10705-018-9914-x

Environment and Climate Change Canada. (2022). National inventory report : greenhouse gas sources and sinks in Canada : executive summary.

Fageria, N. K., Baligar, V. C., & Bailey, B. A. (2005). Role of cover crops in improving soil and row crop productivity. *Communications in Soil Science and Plant Analysis*, *36*(19–20), 2733–2757. https://doi.org/10.1080/00103620500303939

Ferreira, N. S., Matos, G. F., Meneses, C. H. S. G., Reis, V. M., Rouws, J. R. C., Schwab, S., Baldani, J. I., & Rouws, L. F. M. (2020). Interaction of phytohormone-producing rhizobia with sugarcane mini-setts and their effect on plant development. *Plant and Soil*, *451*(1–2), 221–238. https://doi.org/10.1007/s11104-019-04388-0

Finney, D. M., Buyer, J. S., & Kaye, J. P. (2017). Living cover crops have immediate impacts on soil microbial community structure and function. *Journal of Soil and Water Conservation*, *72*(4), 361–373. https://doi.org/10.2489/jswc.72.4.361

Fonouni-Farde, C., Kisiala, A., Brault, M., Emery, R. J. N., Diet, A., & Frugier, F. (2017). DELLA1-mediated gibberellin signaling regulates cytokinin-dependent symbiotic nodulation. *Plant Physiology*, *175*(4), 1795–1806. https://doi.org/10.1104/pp.17.00919

Frébortová, J., Greplová, M., Seidl, M. F., Heyl, A., & Frébort, I. (2015). Biochemical characterization of putative adenylate dimethylallyltransferase and cytokinin dehydrogenase from Nostoc sp. PCC 7120. *PLoS ONE*, *10*(9), 1–21. https://doi.org/10.1371/journal.pone.0138468

Frébort, I., Kowalska, M., Hluska, T., Frébortová, J., & Galuszka, P. (2011). Evolution of cytokinin biosynthesis and degradation. *Journal of Experimental Botany*, *62*(8), 2431–2452. https://doi.org/10.1093/jxb/err004

Frugier, F., Kosuta, S., Murray, J. D., Crespi, M., & Szczyglowski, K. (2008). Cytokinin: secret agent of symbiosis. *Trends in Plant Science*, *13*(3), 115–120. https://doi.org/10.1016/j.tplants.2008.01.003

Gai, X., Liu, H., Liu, J., Zhai, L., Yang, B., Wu, S., Ren, T., Lei, Q., & Wang, H. (2018). Long-term benefits of combining chemical fertilizer and manure applications on crop yields and soil carbon and nitrogen stocks in North China Plain. *Agricultural Water Management*, *208*(12), 384–392. https://doi.org/10.1016/j.agwat.2018.07.002

Garcia, R. A., Li, Y., & Rosolem, C. A. (2013). Soil Organic Matter and Physical Attributes Affected by Crop Rotation Under No-till. *Soil Science Society of America Journal*, *77*(5), 1724–1731. https://doi.org/10.2136/sssaj2012.0310

Garland, J. L., and Mills, A. L., (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level soil-carbon source utilization. Applied and Environmental Microbiology.

Gawronska, H., Deji, A., Sakakibara, H., & Sugiyama, T. (2003). Hormone-mediated nitrogen signaling in plants: Implication of participation of abscissic acidin negative regulation of cytokinin-inducible expression of maize response regulator. *Plant Physiology and Biochemistry*, *41*(6–7), 605–610. https://doi.org/10.1016/S0981-9428(03)00059-7

Ge, L., Yong, J. W. H., Goh, N. K., Chia, L. S., Tan, S. N., & Ong, E. S. (2005). Identification of kinetin and kinetin riboside in coconut (Cocos nucifera L.) water using a combined approach of liquid chromatography-tandem mass spectrometry, high performance liquid

chromatography and capillary electrophoresis. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *829*(1–2), 26–34. https://doi.org/10.1016/j.jchromb.2005.09.026

Ghimire, R., Ghimire, B., Mesbah, A. O., Sainju, U. M., & Idowu, O. J. (2019). Soil health response of cover crops in winter wheat–fallow system. *Agronomy Journal*, *111*(4), 2108–2115. https://doi.org/10.2134/agronj2018.08.0492

Gibb, M., Kisiala, A. B., Morrison, E. N., & Emery, R. J. N. (2020). The Origins and Roles of Methylthiolated Cytokinins: Evidence From Among Life Kingdoms. Frontiers in Cell and Developmental Biology, 8(November). https://doi.org/10.3389/fcell.2020.605672

Goh, D. M., Cosme, M., Kisiala, A. B., Mulholland, S., Said, Z. M. F., Spíchal, L., Emery, R. J. N., Declerck, S., & Guinel, F. C. (2019). A stimulatory role for cytokinin in the arbuscular mycorrhizal symbiosis of pea. *Frontiers in Plant Science*, *10*(March), 1–18. https://doi.org/10.3389/fpls.2019.00262

Goicoechea, N., Dolezal, K., Antolin, M. C., Strnad, M., & Sanchez-Diaz, M. (1995). Influence of mycorrhizae and Rhizobium on cytokinin content in drought-stressed alfalfa. In *Journal of Experimental Botany* (Vol. 46, Issue 291).

Golovko, A., Sitbon, F. and Nicander, B. (2002). Identification of a tRNA isopentenyltransferase gene from Arabidopsis thaliana. 49, 161-169. https://www.researchgate.net/publication/11372174

Gregorich, E. G. (1996). An Ecological View of Soil Quality. *Soil Quality Is in the Hands of the Land Manager*, 42–46. http://onlinelibrary.wiley.com/doi/10.1002/cbdv.200490137/abstract

Gruber, N., & Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature*, *451*(7176), 293–296. https://doi.org/10.1038/nature06592

Grzadziel, J., Furtak, K., & Galazka, A. (2018). Community-level physiological profiles of microorganisms from different types of soil that are characteristic to poland-a long-term microplot experiment. *Sustainability (Switzerland)*, *11*(1). https://doi.org/10.3390/su11010056

Hachiya, T., & Sakakibara, H. (2017). Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants. In *Journal of Experimental Botany* (Vol. 68, Issue 10, pp. 2501–2512). Oxford University Press. https://doi.org/10.1093/jxb/erw449

Hartwig, N. L., & Ammon, H. U. (2002). Cover crops and living mulches. *Weed Science*, *50*(6), 688–699. https://doi.org/10.1614/0043-1745(2002)050[0688:aiacca]2.0.co;2

Hee Lee, J., Takei, K., Sakakibara, H., Sun Cho, H., Myung Kim, D., Sung Kim, Y., Ran Min, S., Taek Kim, W., Young Sohn, D., Pyo Lim, Y., & Pai, H.-S. (2003). CHRK1, a chitinase-related receptor-like kinase, plays a role in plant development and cytokinin homeostasis in tobacco. In *Plant Molecular Biology* (Vol. 53).

Hewett, A. E. W., Wareing, P. F. (1973). Cytokinins in Populus×robusta: Qualitative Changes during Development. *Physiologia Plantarum*, *29*(3), 386–389. https://doi.org/10.1111/j.1399-3054.1973.tb04835.x

Hewett, A. E. W., Wareing, P. F., & Planta, S. (2021). Cytokinins in Populus × robusta Schneid : A Complex in Leaves. *Published by : Springer Stable URL : https://www.jstor.org/stable/23370732 REFERENCES Linked references are available on*

JSTOR for this article : You may need to log in to JSTOR to access the l. *112*(3), 225–233.

High, K. E., Ashton, P. D., Nelson, M., Rylott, E. L., Thomas-Oates, J. E., & Hodson, M. E. (2019). New approaches using mass spectrometry to investigate changes to cytokinin and abscisic acid (ABA) concentrations in soil. *Soil Biology and Biochemistry*, *135*, 108–116. https://doi.org/10.1016/j.soilbio.2019.04.017

Hirose, N., Makita, N., Kojima, M., Kamada-Nobusada, T., & Sakakibara, H. (2007). Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. *Plant and Cell Physiology*, *48*(3), 523–539. https://doi.org/10.1093/pcp/pcm022

Hirose, N., Makita, N., Yamaya, T., & Sakakibara, H. (2005). Functional characterization and expression analysis of a gene, OsENT2, encoding an equilibrative nucleoside transporter in rice suggest a function in cytokinin transport. *Plant Physiology*, *138*(1), 196–206. https://doi.org/10.1104/pp.105.060137

Hluska, T., Hlusková, L., & Emery, R. J. N. (2021). The hulks and the deadpools of the cytokinin universe: A dual strategy for cytokinin production, translocation, and signal transduction. Biomolecules, 11(2), 1–40. https://doi.org/10.3390/biom11020209

Hussain, A., & Hasnain, S. (2015). *Cytokinin production by some bacteria : its impact on cell division in cucumber cotyledons . Afr J Microbiol Res 3 : 704-712 Cytokinin production by some bacteria : Its impact on cell division in cucumber cotyledons*. *3*(OCTOBER 2009), 704–712.

Hussain, A., Khan, A. W., & Hasnain, S. (2009). Cytokinin production by some bacteria: Its impact on cell division in cucumber cotyledons Anthropometric and metabolic indices in assessment of type and severity of dyslipidemia View project Clinical Microbiology View

project Cytokinin production by some bacteria: Its impact on cell division in cucumber cotyledons. *Article in African Journal of Microbiology Research*, *3*(11), 704–712. http://blast.ncbi.nlm.nih.gov/Blast.cgi

Hwang, I., Chen, H. C., & Sheen, J. (2002). Two-component signal transduction pathways in Arabidopsis. *Plant Physiology*, *129*(2), 500–515. https://doi.org/10.1104/pp.005504

Hwang, I., & Sakakibara, H. (2006). Cytokinin biosynthesis and perception. In *Physiologia Plantarum* (Vol. 126, Issue 4, pp. 528–538). https://doi.org/10.1111/j.1399-3054.2006.00665.x

Hwang, I., & Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. In *NATURE* (Vol. 413).

Immanen, J., Nieminen, K., Smolander, O. P., Kojima, M., Alonso Serra, J., Koskinen, P., Zhang, J., Elo, A., Mähönen, A. P., Street, N., Bhalerao, R. P., Paulin, L., Auvinen, P., Sakakibara, H., & Helariutta, Y. (2016). Cytokinin and Auxin Display Distinct but Interconnected Distribution and Signaling Profiles to Stimulate Cambial Activity. *Current Biology*, *26*(15), 1990–1997. https://doi.org/10.1016/j.cub.2016.05.053

Ichihashi, Y., Ichihashi, Y., Date, Y., Date, Y., Shino, A., Shimizu, T., Shibata, A., Kumaishi, K., Funahashi, F., Wakayama, K., Yamazaki, K., Umezawa, A., Sato, T., Kobayashi, M., Kamimura, M., Kusano, M., Kusano, M., Che, F. S., O'Brien, M., … Nihei, N. (2020). Multi-omics analysis on an agroecosystem reveals the significant role of organic nitrogen to increase agricultural crop yield. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(25), 14552–14560. https://doi.org/10.1073/pnas.1917259117

Itoh, J. I., Hibara, K. I., Kojima, M., Sakakibara, H., & Nagato, Y. (2012). Rice DECUSSATE controls phyllotaxy by affecting the cytokinin signaling pathway. *Plant Journal*, *72*(6), 869–881. https://doi.org/10.1111/j.1365-313x.2012.05123.x

Jameson, P. E., & Song, J. (2016). Cytokinin: A key driver of seed yield. *Journal of Experimental Botany*, *67*(3), 593–606. https://doi.org/10.1093/jxb/erv461

Jan, S., Abbas, N., Ashraf, M., & Ahmad, P. (2019). Roles of potential plant hormones and transcription factors in controlling leaf senescence and drought tolerance. *Protoplasma*, *256*(2), 313–329. https://doi.org/10.1007/s00709-018-1310-5

Janečková, H., Husičková, A., Ferretti, U., Prčina, M., Pilařová, E., Plačková, L., Pospíšil, P., Doležal, K., & Špundová, M. (2018). The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. *Plant Cell and Environment*, *41*(8), 1870–1885. https://doi.org/10.1111/pce.13329

Jiang, C. J., Shimono, M., Sugano, S., Kojima, M., Liu, X., Inoue, H., Sakakibara, H., & Takatsuji, H. (2013). Cytokinins act synergistically with salicylic acid to activate defense gene expression in rice. *Molecular Plant-Microbe Interactions*, *26*(3), 287–296. https://doi.org/10.1094/MPMI-06-12-0152-R

Jin, S. H., Ma, X. M., Kojima, M., Sakakibara, H., Wang, Y. W., & Hou, B. K. (2013). Overexpression of glucosyltransferase UGT85A1 influences trans-zeatin homeostasis and trans-zeatin responses likely through O-glucosylation. *Planta*, *237*(4), 991–999. https://doi.org/10.1007/s00425-012-1818-4

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., & Emery, R. J. N. (2019). Endosymbiotic Methylobacterium oryzae mitigates the impact of limited water availability

in lentil (Lens culinaris Medik.) by increasing plant cytokinin levels. *Environmental and Experimental Botany*, *162*, 525–540. https://doi.org/10.1016/j.envexpbot.2019.03.028

Justes, E. (2017). Cover crops for sustainable farming. In *Cover Crops for Sustainable Farming*. https://doi.org/10.1007/978-94-024-0986-4

Kakimoto, T. (2003). Biosynthesis of cytokinins. *Journal of Plant Research*.

Kamada-Nobusada, T., Makita, N., Kojima, M., & Sakakibara, H. (2013). Nitrogen-dependent regulation of de novo cytokinin biosynthesis in rice: The role of glutamine metabolism as an additional signal. *Plant and Cell Physiology*, *54*(11), 1881–1893. https://doi.org/10.1093/pcp/pct127

Kamada-Nobusada, T., & Sakakibara, H. (2009). Molecular basis for cytokinin biosynthesis. In *Phytochemistry* (Vol. 70, Issue 4, pp. 444–449). https://doi.org/10.1016/j.phytochem.2009.02.007

Kardol, P., Cregger, M. A., Campanv, C. E., & Classen, A. T. (2010). Soil ecosystem functioning under climate change: Plant species and community effects. *Ecology*, *91*(3), 767–781. https://doi.org/10.1890/09-0135.1

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., Yamaguchi, S., & Sakakibara, H. (2004). Distinct Isoprenoid Origins of cis- and trans-Zeatin Biosyntheses in Arabidopsis. *Journal of Biological Chemistry*, *279*(14), 14049–14054. https://doi.org/10.1074/jbc.M314195200

Kiba, T., Aoki, K., Sakakibara, H., & Mizuno, T. (2004). Arabidopsis Response Regulator, ARR22, Ectopic Expression of Which Results in Phenotypes Similar to the wol Cytokinin-

Receptor Mutant. In *Plant Cell Physiol* (Vol. 45, Issue 8). https://academic.oup.com/pcp/article-abstract/45/8/1063/1837056

Kiba, T., Feria-Bourrellier, A. B., Lafouge, F., Lezhneva, L., Boutet-Mercey, S., Orsel, M., Bréhaut, V., Miller, A., Daniel-Vedele, F., Sakakibara, H., & Krapp, A. (2012). The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. *Plant Cell*, *24*(1), 245–258. https://doi.org/10.1105/tpc.111.092221

Kiba, T., Kudo, T., Kojima, M., & Sakakibara, H. (2011). Hormonal control of nitrogen acquisition: Roles of auxin, abscisic acid, and cytokinin. In *Journal of Experimental Botany* (Vol. 62, Issue 4, pp. 1399–1409). https://doi.org/10.1093/jxb/erq410

Kiba, T., Naitou, T., Koizumi, N., Yamashino, T., Sakakibara, H., & Mizuno, T. (2005). Combinatorial microarray analysis revealing Arabidopsis genes implicated in cytokinin responses through the His→Asp phosphorelay circuitry. *Plant and Cell Physiology*, *46*(2), 339–355. https://doi.org/10.1093/pcp/pci033

Kieber, J. J., & Schaller, G. E. (2014). Cytokinins. *The Arabidopsis Book*, *12*, e0168. https://doi.org/10.1199/tab.0168

Kisiala, A., Laffont, C., Emery, R. J. N., & Frugier, F. (2013). Bioactive cytokinins are selectively secreted by sinorhizobium meliloti nodulating and nonnodulating strains. *Molecular Plant-Microbe Interactions*, *26*(10), 1225–1231. https://doi.org/10.1094/MPMI-02-13-0054-R

Kisiala, A., Kambhampati, S., Stock, N. L., Aoki, M., & Emery, R. J. N. (2019). Quantification of Cytokinins Using High-Resolution Accurate-Mass Orbitrap Mass Spectrometry and Parallel Reaction Monitoring (PRM). Analytical Chemistry, 91(23), 15049–15056. https://doi.org/10.1021/acs.analchem.9b03728

Ko, D., Kang, J., Kiba, T., Park, J., Kojima, M., Do, J., Kim, K. Y., Kwon, M., Endler, A., Song, W. Y., Martinoia, E., Sakakibara, H., & Lee, Y. (2014). Arabidopsis ABCG14 is essential for the root-to-shoot translocation of cytokinin. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(19), 7150–7155. https://doi.org/10.1073/pnas.1321519111

Kudo, T., Kiba, T., & Sakakibara, H. (2010). Metabolism and long-distance translocation of cytokinins. In *Journal of Integrative Plant Biology* (Vol. 52, Issue 1, pp. 53–60). https://doi.org/10.1111/j.1744-7909.2010.00898.x

Kudo, T., Makita, N., Kojima, M., Tokunaga, H., & Sakakibara, H. (2012). Cytokinin activity of cis-Zeatin and phenotypic alterations induced by Overexpression of putative cis-Zeatin-O-glucosyltransferase in rice. *Plant Physiology*, *160*(1), 319–331. https://doi.org/10.1104/pp.112.196733

Kudoyarova, G. R., Korobova, A. V., Akhiyarova, G. R., Arkhipova, T. N., Zaytsev, D. Y., Prinsen, E., Egutkin, N. L., Medvedev, S. S., and Veselov, S. Y. (2014). Accumulation of cytokinins in roots and their export to the shoots of durum wheat plants treated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP). *Journal of Experimental Botany*, *65*(9), 2287–2294. https://doi.org/10.1093/jxb/eru113

Kuypers, M. M. M., Marchant, H. K., and Kartal, B. (2018). The microbial nitrogen-cycling network. Nature Reviews Microbiology, 16(5), 263–276. https://doi.org/10.1038/nrmicro.2018.9

Lee, B. H., Johnston, R., Yang, Y., Gallavotti, A., Kojima, M., Travençolo, B. A. N., Costa, L. D. F., Sakakibara, H., & Jackson, D. (2009). Studies of aberrant phyllotaxy1 mutants of maize indicate complex interactions between auxin and cytokinin signaling in the shoot

apical meristem 1[W][OA]. *Plant Physiology*, *150*(1), 205–216. https://doi.org/10.1104/pp.109.137034

Lehmann, J., Bossio, D. A., Kögel-Knabner, I., & Rillig, M. C. (2020). The concept and future prospects of soil health. *Nature Reviews Earth and Environment*, *1*(10), 544–553. https://doi.org/10.1038/s43017-020-0080-8

Lezhneva, L., Kiba, T., Feria-Bourrellier, A. B., Lafouge, F., Boutet-Mercey, S., Zoufan, P., Sakakibara, H., Daniel-Vedele, F., & Krapp, A. (2014). The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. *Plant Journal*, *80*(2), 230–241. https://doi.org/10.1111/tpj.12626

Li, M., Guo, J., Ren, T., Luo, G., Shen, Q., Lu, J., Guo, S., & Ling, N. (2021). Crop rotation history constrains soil biodiversity and multifunctionality relationships. *Agriculture, Ecosystems and Environment*, *319*(June), 107550. https://doi.org/10.1016/j.agee.2021.107550

Li, Z., Tian, D., Wang, B., Wang, J., Wang, S., Chen, H. Y. H., Xu, X., Wang, C., He, N., & Niu, S. (2019). Microbes drive global soil nitrogen mineralization and availability. In *Global Change Biology* (Vol. 25, Issue 3). https://doi.org/10.1111/gcb.14557

Linton, N. F., Ferrari Machado, P. V., Deen, B., Wagner-Riddle, C., & Dunfield, K. E. (2020). Long-term diverse rotation alters nitrogen cycling bacterial groups and nitrous oxide emissions after nitrogen fertilization. *Soil Biology and Biochemistry*, *149*(April), 107917. https://doi.org/10.1016/j.soilbio.2020.107917

Liu, F., Xing, S., Ma, H., Du, Z., & Ma, B. (2013). Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in Platycladus orientalis container seedlings. *Applied Microbiology and Biotechnology*, *97*(20), 9155–9164.

https://doi.org/10.1007/s00253-013-5193-2

Liu, K., Bandara, M., Hamel, C., Knight, J. D., & Gan, Y. (2020). Intensifying crop rotations with pulse crops enhances system productivity and soil organic carbon in semi-arid environments. *Field Crops Research*, *248*(October 2019), 107657. https://doi.org/10.1016/j.fcr.2019.107657

Lomin, S. N., Yonekura-Sakakibara, K., Romanov, G. A., & Sakakibara, H. (2011). Ligand-binding properties and subcellular localization of maize cytokinin receptors. *Journal of Experimental Botany*, *62*(14), 5149–5159. https://doi.org/10.1093/jxb/err220

Lu, S., Lepo, J. E., Song, H. X., Guan, C. Y., & Zhang, Z. H. (2018). Increased rice yield in long-term crop rotation regimes through improved soil structure, rhizosphere microbial communities, and nutrient bioavailability in paddy soil. *Biology and Fertility of Soils*, *54*(8), 909–923. https://doi.org/10.1007/s00374-018-1315-4

Lützow, M. V., Kögel-Knabner, I., Ekschmitt, K., Matzner, E., Guggenberger, G., Marschner, B., & Flessa, H. (2006). Stabilization of organic matter in temperate soils: Mechanisms and their relevance under different soil conditions - A review. *European Journal of Soil Science*, *57*(4), 426–445. https://doi.org/10.1111/j.1365-2389.2006.00809.x

Maeda, Y., Konishi, M., Kiba, T., Sakuraba, Y., Sawaki, N., Kurai, T., Ueda, Y., Sakakibara, H., & Yanagisawa, S. (2018). A NIGT1-centred transcriptional cascade regulates nitrate signalling and incorporates phosphorus starvation signals in Arabidopsis. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-03832-6

Maia, L. B., & Moura, J. J. G. (2014). How biology handles nitrite. *Chemical Reviews*, *114*(10), 5273–5357. https://doi.org/10.1021/cr400518y

Maiga, A., Alhameid, A., Singh, S., Polat, A., Singh, J., Kumar, S., & Osborne, S. (2019). Responses of soil organic carbon, aggregate stability, carbon and nitrogen fractions to 15 and 24 years of no-till diversified crop rotations. *Soil Research*, *57*(2), 149–157. https://doi.org/10.1071/SR18068

Martinez, J. P., Crespo, C., Sainz Rozas, H., Echeverría, H., Studdert, G., Martinez, F., Cordone, G., & Barbieri, P. (2020). Soil organic carbon in cropping sequences with predominance of soya bean in the argentinean humid Pampas. *Soil Use and Management*, *36*(1), 173–183. https://doi.org/10.1111/sum.12547

Mayer, Z., Sasvári, Z., Szentpéteri, V., Rétháti, B. P., Vajna, B., & Posta, K. (2019). Effect of long-term cropping systems on the diversity of the soil bacterial communities. *Agronomy*, *9*(12), 1–10. https://doi.org/10.3390/agronomy9120878

McDaniel, M. D., Tiemann, L. K., & Grandy, A. S. (2014). Does agricultural crop diversity enhance soil microbial biomass and organic matter dynamics? A meta-analysis. *Ecological Applications*, *24*(3), 560–570. https://doi.org/10.1890/13-0616.1

Messenger, S., Keller, L. P., & Lauretta, D. S. (2005). Geochemistry: Supernova olivine from cometary dust. *Science*, *309*(5735), 737–741. https://doi.org/10.1126/science.1109602

Miki, T., Yokokawa, T., Ke, P. J., Hsieh, I. F., Hsieh, C. hao, Kume, T., Yoneya, K., & Matsui, K. (2018). Statistical recipe for quantifying microbial functional diversity from EcoPlate metabolic profiling. *Ecological Research*, *33*(1), 249–260. https://doi.org/10.1007/s11284-017-1554-0

Miller, C.O., Skoog, F., von Saltza, M.H., and Strong, M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. **77,** 1329–1334.

Miri, M., Janakirama, P., Held, M., Ross, L., & Szczyglowski, K. (2016). Into the Root: How Cytokinin Controls Rhizobial Infection. In *Trends in Plant Science* (Vol. 21, Issue 3, pp. 178–186). Elsevier Ltd. https://doi.org/10.1016/j.tplants.2015.09.003

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., & Kakimoto, T. (2006). Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(44), 16598–16603. https://doi.org/10.1073/pnas.0603522103

Mok, M. C., Martin, R. C., Dobrev, P. I., Vanková, R., Ho, P. S., Keiko, Y. S., Sakakibara, H., & Mok, D. W. S. (2005). Topolins and hydroxylated thidiazuron derivatives are substrates of cytokinin O-glucosyltransferase with position specificity related to receptor recognition. *Plant Physiology*, *137*(3), 1057–1066. https://doi.org/10.1104/pp.104.057174

Moreau, D., Bardgett, R. D., Finlay, R. D., Jones, D. L., & Philippot, L. (2019). A plant perspective on nitrogen cycling in the rhizosphere. *Functional Ecology*, *33*(4), 540–552. https://doi.org/10.1111/1365-2435.13303

Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R., & Castillo, F. (1999). Prokaryotic nitrate reduction: Molecular properties and functional distinction among bacterial nitrate reductases. *Journal of Bacteriology*, *181*(21), 6573–6584. https://doi.org/10.1128/jb.181.21.6573-6584.1999

Morrison, E. N., Emery, R. J. N., & Saville, B. J. (2015). Phytohormone involvement in the Ustilago maydis- Zea mays pathosystem: Relationships between abscisic acid and cytokinin levels and strain virulence in infected cob tissue. *PLoS ONE*, *10*(6). https://doi.org/10.1371/journal.pone.0130945

Morrison, E. N., Knowles, S., Hayward, A., Greg Thorn, R., Saville, B. J., & Emery, R. J. N. (2015). Detection of phytohormones in temperate forest fungi predicts consistent abscisic acid production and a common pathway for cytokinin biosynthesis. *Mycologia*, *107*(2), 245–257. https://doi.org/10.3852/14-157

Motorin, Y., Bec, G., Tewari, R. and Grosjean, H. (1997). Transfer RNA recognition by the Escherichia coli isopenttnyl-pyrophosphate:tRNA isopentynyl transferase: Dependence on the anticodon arm structure. 3:721-733.

Mubarik, M. S., Khan, S. H., Sajjad, M., Raza, A., Hafeez, M. B., Yasmeen, T., Rizwan, M., Ali, S., & Arif, M. S. (2021). A manipulative interplay between positive and negative regulators of phytohormones: A way forward for improving drought tolerance in plants. *Physiologia Plantarum*, *172*(2), 1269–1290. https://doi.org/10.1111/ppl.13325

Nam, Y. J., Tran, L. S. P., Kojima, M., Sakakibara, H., Nishiyama, R., & Shin, R. (2012). Regulatory Roles of Cytokinins and Cytokinin Signaling in Response to Potassium Deficiency in Arabidopsis. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0047797

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G., & Renella, G. (2003). Microbial diversity and soil functions. *European Journal of Soil Science*, *54*(4), 655–670. https://doi.org/10.1046/j.1351-0754.2003.0556.x

Nieto, K. F., & Frankenberger, W. T. (1989). Biosynthesis of Cytokinins in Soil. *Soil Science Society of America Journal*, *53*(3), 735–740. https://doi.org/10.2136/sssaj1989.03615995005300030016x

Nishii, K., Wright, F., Chen, Y. Y., & Möller, M. (2018). Tangled history of a multigene family: The evolution of ISOPENTENYLTRANSFERASE genes. *PLoS ONE*, *13*(8), 1–23.

https://doi.org/10.1371/journal.pone.0201198

Noel, I. T. C., Sheng, C., Yost, C. K., Pharis, R. P., Hynes, M. F., & Noel, T. C. (n.d.). *I growth-promoting rhizobacterium: direct growth promotion of canola and lettuce*.

Nouri, A., Lee, J., Yin, X., Tyler, D. D., & Saxton, A. M. (2019). Thirty-four years of no-tillage and cover crops improve soil quality and increase cotton yield in Alfisols, Southeastern USA. *Geoderma*, *337*, 998–1008. https://doi.org/10.1016/j.geoderma.2018.10.016

Novara, A., Minacapilli, M., Santoro, A., Rodrigo-Comino, J., Carrubba, A., Sarno, M., Venezia, G., & Gristina, L. (2019). Real cover crops contribution to soil organic carbon sequestration in sloping vineyard. *Science of the Total Environment*, *652*, 300–306. https://doi.org/10.1016/j.scitotenv.2018.10.247

Olaniyi, J. O., Adelasoye, K. a, & Jegede, C. O. (2008). Influence of Nitrogen Fertilizer on the Growth , Yield and Quality of Grain Amaranth Varieties. *World Journal of Agricultural Sciences*, *4*(4), 506–513.

Oliveira, M., Barré, P., Trindade, H., & Virto, I. (2019). Different efficiencies of grain legumes in crop rotations to improve soil aggregation and organic carbon in the short-term in a sandy Cambisol. *Soil and Tillage Research*, *186*(September 2018), 23–35. https://doi.org/10.1016/j.still.2018.10.003

Osugi, A., Kojima, M., Takebayashi, Y., Ueda, N., Kiba, T., & Sakakibara, H. (2017). Systemic transport of trans-zeatin and its precursor have differing roles in Arabidopsis shoots. *Nature Plants*, *3*(8). https://doi.org/10.1038/nplants.2017.112

Osugi, A., & Sakakibara, H. (2015). Q and A: How do plants respond to cytokinins and what is their importance? *BMC Biology*, *13*(1). https://doi.org/10.1186/s12915-015-0214-5

Palberg, D., Kisiała, A., Jorge, G. L., & Emery, R. J. N. (2022). A survey of Methylobacterium species and strains reveals widespread production and varying profiles of cytokinin phytohormones. *BMC Microbiology*, *22*(1), 1–17. https://doi.org/10.1186/s12866-022-02454-9

Pan. J., Zhao, J., Liu, Y., Huang, N., Tian, K., Shah, F., Liang, K, Zhong, X. and Liu, B. (2019). *Scientific reports.*

Pereg, L., Morugán-Coronado, A., McMillan, M., & García-Orenes, F. (2018). Restoration of nitrogen cycling community in grapevine soil by a decade of organic fertilization. *Soil and Tillage Research*, *179*(July 2017), 11–19. https://doi.org/10.1016/j.still.2018.01.007

Phillips, D. A., & Torrey, J. G. (1972). Studies on Cytokinin Production by Rhizobium . *Plant Physiology*, *49*(1), 11–15. https://doi.org/10.1104/pp.49.1.11

Prabha, A. C. S., Senthivelu, M., and Paramasivam, A. (2020). Soil quality indicators for different land uses : A review. International Journal of Plant and Soil.17, 379-387. https://www.researchgate.net/publication/342551296

Pulleman, M., Creamer, R., Hamer, U., Helder, J., Pelosi, C., Pérès, G., & Rutgers, M. (2012). Soil biodiversity, biological indicators and soil ecosystem services-an overview of European approaches. *Current Opinion in Environmental Sustainability*, *4*(5), 529–538. https://doi.org/10.1016/j.cosust.2012.10.009

Radhika, V., Ueda, N., Tsuboi, Y., Kojima, M., Kikuchi, J., Kudo, T., & Radhika, V. (2015). Methylated cytokinins from the phytopathogen Rhodococcus fascians mimic plant hormone activity. *Plant Physiology*, *169*(2), 1118–1126. https://doi.org/10.1104/pp.15.00787

Raphael, J. P. A., Calonego, J. C., Milori, D. M. B. P., & Rosolem, C. A. (2016). Soil organic matter in crop rotations under no-till. *Soil and Tillage Research*, *155*, 45–53. https://doi.org/10.1016/j.still.2015.07.020

Robertson, G. P., & Groffman, P. M. (2006). Nitrogen transformations. In *Soil Microbiology, Ecology and Biochemistry: Third Edition* (4th ed.). Elsevier Inc. https://doi.org/10.1016/b978-0-12-415955-6.00014-1

Romillac, N., Slezack-Deschaumes, S., Amiaud, B., & Piutti, S. (2022). Soil Microbial Communities Involved in N and S Cycling are More Influenced by Interannual Variability than by Crop Sequence. *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.4000299

Rop, K., Karuku, G. N., Mbui, D., Njomo, N., & Michira, I. (2019). Evaluating the effects of formulated nano-NPK slow release fertilizer composite on the performance and yield of maize, kale and capsicum. *Annals of Agricultural Sciences*, *64*(1), 9–19. https://doi.org/10.1016/j.aoas.2019.05.010

Rose, R. J., Damoc, E., Denisov, E., Makarov, A., & Heck, A. J. R. (2012). High-sensitivity Orbitrap mass analysis of intact macromolecular assemblies. *Nature Methods*, *9*(11), 1084–1086. https://doi.org/10.1038/nmeth.2208

Rossi, W., Grafpelli, A., & Pietrosanti, W. (1984). Phytohormones in Soil after Atrazine Application. In *Folia Microbiol* (Vol. 29).

Russell, A. E., Laird, D. A., & Mallarino, A. P. (2006). Nitrogen Fertilization and Cropping System Impacts on Soil Quality in Midwestern Mollisols. *Soil Science Society of America Journal*, *70*(1), 249–255. https://doi.org/10.2136/sssaj2005.0058

Samaddar, S., Schmidt, R., Tautges, N. E., & Scow, K. (2021a). Adding alfalfa to an annual crop

rotation shifts the composition and functional responses of tomato rhizosphere microbial communities. *Applied Soil Ecology*, *167*(June), 104102. https://doi.org/10.1016/j.apsoil.2021.104102

Samaddar, S., Schmidt, R., Tautges, N. E., & Scow, K. (2021b). Adding alfalfa to an annual crop rotation shifts the composition and functional responses of tomato rhizosphere microbial communities. *Applied Soil Ecology*, *167*(October 2020), 104102. https://doi.org/10.1016/j.apsoil.2021.104102

Sanchez, J. E., Willson, T. C., Kizilkaya, K., Parker, E., & Harwood, R. R. (2001). Enhancing the Mineralizable Nitrogen Pool Through Substrate Diversity in Long Term Cropping Systems. *Soil Science Society of America Journal*, *65*(5), 1442–1447. https://doi.org/10.2136/sssaj2001.6551442x

Sakakibara, H. (2021). Cytokinin biosynthesis and transport for systemic nitrogen signaling. *Plant Journal*, *105*(2), 421–430. https://doi.org/10.1111/tpj.15011

Sakakibara, H. (2010). Cytokinin biosynthesis and metabolism. *Plant Hormones: Biosynthesis, Signal Transduction, Action!*, *35*, 95–114. https://doi.org/10.1007/978-1-4020-2686-7_5

Sakakibara, H. (2005). Cytokinin Biosynthesis and Regulation. In *Vitamins and Hormones* (Vol. 72, pp. 271–287). https://doi.org/10.1016/S0083-6729(05)72008-2

Sakakibara, H. (2006). Cytokinins: Activity, biosynthesis, and translocation. In *Annual Review of Plant Biology* (Vol. 57, pp. 431–449). https://doi.org/10.1146/annurev.arplant.57.032905.105231

Sakakibara, H. (2003). Nitrate-specific and cytokinin-mediated nitrogen signaling pathways in plants. *Journal of Plant Research*, *116*(3), 253–257. https://doi.org/10.1007/s10265-003-0097-3

Sakakibara, H., Kasahara, H., Ueda, N., Kojima, M., Takei, K., Hishiyama, S., Asami, T., Okada, K., Kamiya, Y., Yamaya, T., Yamaguchi, S., & MacMillan, J. (2005). Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. In *PNAS July* (Vol. 12, Issue 28). www.pnas.orgcgidoi10.1073pnas.0500793102

Sakakibara, H., Kobayashi, K., Deji, A., & Sugiyama, T. (1997). Partial Characterization of the Signaling Pathway for the Nitrate-Dependent Expression of Genes for Nitrogen-Assimilatory Enzymes Using Detached Maize Leaves. In *Plant Cell Physiol* (Vol. 38, Issue 7). https://academic.oup.com/pcp/article-abstract/38/7/837/1849189

Sakakibara, H., Suzuki, M., Takei, K., Deji, A., Taniguchi, M., & Sugiyama, T. (1998). A response-regulator homologue possibly involved in nitrogen signal transduction mediated by cytokinin in maize. In *The Plant Journal* (Vol. 14, Issue 3).

Sakakibara, H., Takei, K., & Hirose, N. (2006). Interactions between nitrogen and cytokinin in the regulation of metabolism and development. In *Trends in Plant Science* (Vol. 11, Issue 9, pp. 440–448). https://doi.org/10.1016/j.tplants.2006.07.004

Sakakibara, H., Taniguchi, M., & Sugiyama, T. (2000). His-Asp phosphorelay signaling: a communication avenue between plants and their environment. In *Plant Molecular Biology* (Vol. 42).

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y., & Matsuoka, M. (2006). Ectopic expression of KNOTTED1-like homeobox protein induces

expression of cytokinin biosynthesis genes in rice. *Plant Physiology*, *142*(1), 54–62. https://doi.org/10.1104/pp.106.085811

Sasaki, E., Ogura, T., Takei, K., Kojima, M., Kitahata, N., Sakakibara, H., Asami, T., & Shimada, Y. (2013). Uniconazole, a cytochrome P450 inhibitor, inhibits trans-zeatin biosynthesis in Arabidopsis. *Phytochemistry*, *87*, 30–38. https://doi.org/10.1016/j.phytochem.2012.11.023

Sasaki, T., Suzaki, T., Soyano, T., Kojima, M., Sakakibara, H., & Kawaguchi, M. (2014). Shoot-derived cytokinins systemically regulate root nodulation. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms5983

Sedri, M. H., Amini, A., & Golchin, A. (2019). Evaluation of Nitrogen Effects on Yield and Drought Tolerance of Rainfed Wheat using Drought Stress Indices. *Journal of Crop Science and Biotechnology*, *22*(3), 235–242. https://doi.org/10.1007/s12892-018-0037-0

Silva, J.A., & Uchida, R. (2000). Chapter 0: Plant Nutrient Management in Hawaii's Soils: Approaches for Tropical and Subtropical Agriculture.

Singh, B. (2018). Are Nitrogen Fertilizers Deleterious to Soil Health? *Agronomy*, *8*(4). https://doi.org/10.3390/agronomy8040048

Smith, S. M., Li, C., & Li, J. (2017). Hormone function in plants. In *Hormone Metabolism and Signaling in Plants*. Jiayang Li, Chuanyou Li and Steven Smith. https://doi.org/10.1016/B978-0-12-811562-6.00001-3

Snapp, S. S., Swinton, S. M., Labarta, R., Mutch, D., Black, J. R., Leep, R., Nyiraneza, J., & O'Neil, K. (2005). Evaluating cover crops for benefits, costs and performance within cropping system niches. *Agronomy Journal*, *97*(1), 322–332. https://doi.org/10.2134/agronj2005.0322a

Snider, D., Thompson, K., Wagner-Riddle, C., Spoelstra, J., & Dunfield, K. (2015). Molecular techniques and stable isotope ratios at natural abundance give complementary inferences about N2O production pathways in an agricultural soil following a rainfall event. *Soil Biology and Biochemistry*, *88,* 197–213. https://doi.org/10.1016/j.soilbio.2015.05.021

Soejima, H., Sugiyama, T., & Ishihara, K. (1992). Changes in cytokinin activities and mass spectrometric analysis of cytokinins in root exudates of rice plant (Oryza sativa L.): Comparison between cultivars Nipponbare and Akenohoshi. *Plant Physiology*, *100*(4), 1724–1729. https://doi.org/10.1104/pp.100.4.1724

Song, Y., Li, J., Liu, M., Meng, Z., Liu, K., & Sui, N. (2019). Nitrogen increases drought tolerance in maize seedlings. *Functional Plant Biology*, *46*(4), 350–359. https://doi.org/10.1071/FP18186

Spíchal, L. (2012). Cytokinins - Recent news and views of evolutionally old molecules. *Functional Plant Biology*, *39*(4), 267–284. https://doi.org/10.1071/FP11276

Stewart, D.R., Jian, J. J., Gyawali, A.J., Thomason, W. E., Badgley, B. D., Reiter, M. S., and Strickland, M. S. (2018). What We Talk about When We Talk about Soil Health. Agricultural and Environmental Letters. https://doi.org/10.2134/ael2018.06.0033

Stirk, W.A. and van Staden. (2010). Flow of cytokinins through the environment. *Plant Growth regulation*.

Stockdale, E. A., & Watson, C. A. (2009). Biological indicators of soil quality in organic farming systems. *Renewable Agriculture and Food Systems*, *24*(4), 308–318. https://doi.org/10.1017/S1742170509990172

Stubner, S. (2002). Enumeration of 16S rDNA of Desulfotomaculum lineage 1 in rice field soil by real-time PCR with SybrGreen^{TM} detection. *Journal of Microbiological Methods*, *50*(2),

155–164. https://doi.org/10.1016/S0167-7012(02)00024

Studdert, G. A., & Echeverría, H. E. (2000). Crop Rotations and Nitrogen Fertilization to Manage Soil Organic Carbon Dynamics. *Soil Science Society of America Journal*, *64*(4), 1496–1503. https://doi.org/10.2136/sssaj2000.6441496x

Sturtevant, D. B., & Taller, B. J. (1989). Cytokinin Production by Bradyrhizobium japonicum'. In *Plant Physiol* (Vol. 89).

Sugawara, H., Ueda, N., Kojima, M., Makita, N., Yamaya, T., Sakakibara, H., & Macmillan, J. (2008). *Structural insight into the reaction mechanism and evolution of cytokinin biosynthesis*. www.pnas.orgcgidoi10.1073pnas.0707374105

Sun, J., Hirose, N., Wang, X., Wen, P., Xue, L., Sakakibara, H., & Zuo, J. (n.d.). *Arabidopsis SOI33/AtENT8 Gene Encodes a Putative Equilibrative Nucleoside Transporter That Is Involved in Cytokinin Transport In Planta*.

Takai, K. (2019). The nitrogen cycle: A large, fast, and mystifying cycle. *Microbes and Environments*, *34*(3), 223–225. https://doi.org/10.1264/jsme2.ME3403rh

Takei, K., Sakakibara, H., Taniguchi, M., & Sugiyama, T. (2001). Nitrogen-Dependent Accumulation of Cytokinins in Root and the Transloca-tion to Leaf: Implication of Cytokinin Species that Induces Gene Expression of Maize Response Regulator. In *Plant Cell Physiol* (Vol. 42, Issue 1).

Takei, K., Takahashi, T., Sugiyama, T., Yamaya, T., & Sakakibara, H. (n.d.). *Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin*.

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T., &

Sakakibara, H. (2004). AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant and Cell Physiology*, *45*(8), 1053–1062. https://doi.org/10.1093/pcp/pch119

Takei, K., Yamaya, T., & Sakakibara, H. (2004). Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyse the biosynthesis of trans-Zeatin. *Journal of Biological Chemistry*, *279*(40), 41866–41872. https://doi.org/10.1074/jbc.M406337200

Tallerand, B. 1, & Stur1evant, D. B. (1991). *CYTOKININ PRODUCTION BY RHIZOBIA*.

Taniguchi, M., Kiba, T., Sakakibara, H., Ueguchi, C., Mizuno, T., & Sugiyama, T. (1998). Expression of Arabidopsis response regulator homologs is induced by cytokinins and nitrate. *FEBS Letters*, *429*(3), 259–262. https://doi.org/10.1016/S0014-5793(98)00611-5

Teixeira, E. I., Johnstone, P., Chakwizira, E., de Ruiter, J., Malcolm, B., Shaw, N., Zyskowski, R., Khaembah, E., Sharp, J., Meenken, E., Fraser, P., Thomas, S., Brown, H., & Curtin, D. (2016). Sources of variability in the effectiveness of winter cover crops for mitigating N leaching. *Agriculture, Ecosystems and Environment*, *220*, 226–235. https://doi.org/10.1016/j.agee.2016.01.019

Thapa, R., Mirsky, S. B., & Tully, K. L. (2018). Cover Crops Reduce Nitrate Leaching in Agroecosystems:A Global Meta-Analysis. *Journal of Environmental Quality*, *47*(6), 1400–1411. https://doi.org/10.2134/jeq2018.03.0107

Thapa, V. R., Ghimire, R., Acosta-Martínez, V., Marsalis, M. A., & Schipanski, M. E. (2021). Cover crop biomass and species composition affect soil microbial community structure and enzyme activities in semiarid cropping systems. *Applied Soil Ecology*, *157*(March 2020), 103735. https://doi.org/10.1016/j.apsoil.2020.103735

Timmusk, S., Nicander, B., Granhall, U., & Tillberg, E. (1999). Cytokinin production by Paenibacillus polymyxa. *Soil Biology and Biochemistry*, *31*(13), 1847–1852. https://doi.org/10.1016/S0038-0717(99)00113-3

Tonitto, C., David, M. B., & Drinkwater, L. E. (2006). Replacing bare fallows with cover crops in fertilizer-intensive cropping systems: A meta-analysis of crop yield and N dynamics. *Agriculture, Ecosystems and Environment*, *112*(1), 58–72. https://doi.org/10.1016/j.agee.2005.07.003

Torsvik, V., & Øvreås, L. (2002). Microbial diversity and function in soil: From genes to ecosystems. *Current Opinion in Microbiology*, *5*(3), 240–245. https://doi.org/10.1016/S1369-5274(02)00324-7

Town, J. R., Gregorich, E. G., Drury, C. F., Lemke, R., Phillips, L. A., & Helgason, B. L. (2022). Diverse crop rotations influence the bacterial and fungal communities in root, rhizosphere and soil and impact soil microbial processes. *Applied Soil Ecology*, *169*(June 2021), 104241. https://doi.org/10.1016/j.apsoil.2021.104241

Turbé, A., Toni, A. De, Benito, P., Lavelle, P., Lavelle, P., Ruiz, N., Putten, W. H. Van der, Labouze, E., Mudgal, S., De Toni, A., Benito, P., Lavelle, P. P., Ruiz, N., Van der Putten, W., Labouze, E., & Mudgal, S. (2010). Soil biodiversity: functions, threaths and tools for policy makers. In *Bio Intelligence Service, IRD, and NIOO, Report for European Commission (DG Environment)* (Issue February).

Van Eerd, L. L. (2018). Nitrogen dynamics and yields of fresh bean and sweet corn with different cover crops and planting dates. *Nutrient Cycling in Agroecosystems*, *111*(1), 33–46. https://doi.org/10.1007/s10705-018-9914-x

Van Staden, J., & Dimalla, G. G. (1976). Cytokinins from Soils. In *Planta (Berl.)* (Vol. 130).

Van Zeijl, A., Op Den Camp, R. H. M., Deinum, E. E., Charnikhova, T., Franssen, H., Op Den Camp, H. J. M., Bouwmeester, H., Kohlen, W., Bisseling, T., & Geurts, R. (2015). Rhizobium Lipo-chitooligosaccharide Signaling Triggers Accumulation of Cytokinins in Medicago truncatula Roots. *Molecular Plant*, *8*(8), 1213–1226. https://doi.org/10.1016/j.molp.2015.03.010

Vargas, L. K., Volpiano, C. G., Lisboa, B. B., Giongo, A., Beneduzi, A., & Passaglia, L. M. P. (2017). Potential of rhizobia as plant growth-promoting rhizobacteria. In *Microbes for Legume Improvement, Second Edition* (pp. 153–174). Springer International Publishing. https://doi.org/10.1007/978-3-319-59174-2_7

Vig, A. P., Rampal, G., Thind, T. S., & Arora, S. (2009). Bio-protective effects of glucosinolates - A review. *LWT - Food Science and Technology*, *42*(10), 1561–1572. https://doi.org/10.1016/j.lwt.2009.05.023

Wallenstein, M. D., McNulty, S., Fernandez, I. J., Boggs, J., & Schlesinger, W. H. (2006). Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. *Forest Ecology and Management*, *222*(1–3), 459–468. https://doi.org/10.1016/j.foreco.2005.11.002

Wang, F., Chen, S., Wang, Y., Zhang, Y., Hu, C., & Liu, B. (2018). Long-term nitrogen fertilization elevates the activity and abundance of nitrifying and denitrifying microbial communities in an upland soil: Implications for nitrogen loss from intensive agricultural

systems. *Frontiers in Microbiology*, *9*(OCT), 1–12. https://doi.org/10.3389/fmicb.2018.02424

Wang, J., Ma, X. M., Kojima, M., Sakakibara, H., & Hou, B. K. (2011). N-glucosyltransferase UGT76C2 is involved in cytokinin homeostasis and cytokinin response in Arabidopsis thaliana. *Plant and Cell Physiology*, *52*(12), 2200–2213. https://doi.org/10.1093/pcp/pcr152

Wang, T. L., Wood, E. A., & Brewin, N. J. (n.d.). *Growth regulators, Rhizobium and nodulation in peas The cytokinin content of a wild-type and a Ti-plasmid-containing strain of R. leguminosarum*.

Wang, Y., Chen, G., Sun, Y., Zhu, K., Jin, Y., Li, B., & Wang, G. (2022). Different agricultural practices specify bacterial community compositions in the soil rhizosphere and root zone. *Soil Ecology Letters*, *4*(1), 18–31. https://doi.org/10.1007/s42832-020-0058-y

Wegner, B. R., Osborne, S. L., Lehman, R. M., & Kumar, S. (2018). *Seven-Year Impact of Cover Crops on Soil Health When Corn Residue Is Removed*. 239–248.

Weisberger, D., Nichols, V., & Liebman, M. (2019). Does diversifying crop rotations suppress weeds? A meta-analysis. *PLoS ONE*, *14*(7), 1–9. https://doi.org/10.1371/journal.pone.0219847

Wu, W., Du, K., Kang, X., & Wei, H. (2021). The diverse roles of cytokinins in regulating leaf development. *Horticulture Research*, *8*(1). https://doi.org/10.1038/s41438-021-00558-3

Xu, W., Ge, Z., & Poudel, D. R. (2015). Application and Optimization of Biolog EcoPlates in Functional Diversity Studies of Soil Microbial Communities. *MATEC Web of Conferences*, *22*, 1–6. https://doi.org/10.1051/matecconf/20152204015

Yang, T., Lupwayi, N., Marc, S. A., Siddique, K. H. M., & Bainard, L. D. (2021). Anthropogenic drivers of soil microbial communities and impacts on soil biological functions in agroecosystems. *Global Ecology and Conservation*, *27*, e01521. https://doi.org/10.1016/j.gecco.2021.e01521

Yang, X. M., Drury, C. F., Reynolds, W. D., & Reeb, M. D. (2019). Legume cover crops provide nitrogen to corn during a three-year transition to organic cropping. *Agronomy Journal*, *111*(6), 3253–3264. https://doi.org/10.2134/agronj2018.10.0652

Yokoyama, A., Yamashino, T., Amano, Y. I., Tajima, Y., Imamura, A., Sakakibara, H., & Mizuno, T. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of Arabidopsis thaliana. *Plant and Cell Physiology*, *48*(1), 84–96. https://doi.org/10.1093/pcp/pcl040

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T., & Sakakibara, H. (2004). Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to cis-zeatin. *Plant Physiology*, *134*(4), 1654–1661. https://doi.org/10.1104/pp.103.037176

Zhang, K., Maltais-Landry, G., & Liao, H. L. (2021). How soil biota regulate C cycling and soil C pools in diversified crop rotations. *Soil Biology and Biochemistry*, *156*. https://doi.org/10.1016/j.soilbio.2021.108219

Zhang, P., Sun, J., Li, L., Wang, X., Li, X., & Qu, J. (2019). Effect of soybean and maize rotation on soil microbial community structure. *Agronomy*, *9*(2), 1–11. https://doi.org/10.3390/agronomy9020042

Zhang, S., Yang, Y., Zhai, W., Tong, Z., Shen, T., Li, Y. C., Zhang, M., Sigua, G. C., Chen, J., & Ding, F. (2019). Controlled-release nitrogen fertilizer improved lodging resistance and

potassium and silicon uptake of direct-seeded rice. *Crop Science*, *59*(6), 2733–2740. https://doi.org/10.2135/cropsci2018.12.0765

Zuber, S. M., Behnke, G. D., Nafziger, E. D., & Villamil, M. B. (2018). Carbon and nitrogen content of soil organic matter and microbial biomass under long-term crop rotation and tillage in Illinois, USA. *Agriculture (Switzerland)*, *8*(3). https://doi.org/10.3390/agriculture8030037

Zukalová, H., & Vašák, J. (2002). The role and effects of glucosinolates of Brassica species - A review. *Rostlinna Vyroba*, *48*(4), 175–180. https://doi.org/10.17221/4217-pse

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*, *61*(4), 533–616. https://doi.org/10.1128/mmbr.61.4.533-616.1997

Chapter 3: N-cycling soil microbial community size and soil health parameters in an intensively managed maize-soybean-winter wheat-cover crop rotation

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Abstract

N-cycling soil microbial communities (SMC) are crucial in mediating soil N-dynamics and are responsible for soil N-losses via nitrate (NO_3^-) leaching and emission of the greenhouse gas N_2O contributing to environmental pollution. These microbial communities respond quickly to agricultural management practices such as crop rotations and N-fertilization. Maize (MZ)-soybean (SB)-winter wheat (WW)-cover crop (CC) rotation is one of the prominent cropping systems, which is managed under high N-fertilization in southern Ontario. However, little is known about the impact of crop phases and N-fertilizers on the N-cycling SMC and potential environmental risk in these areas. To accomplish this, we sampled soils from 12^{th} – 26^{th} November 2020 in fields under different crop phases MZ (n=3), SB-WW (n=4) and CC (n=3) with the same management history and managed by the same farm operator in Ontario. The abundance of total bacterial (*16*S rRNA), nitrifying (*amo*A), and denitrifying (*nir*S and *nir*K) bacterial gene copies were enumerated using quantitative polymerase chain reaction. Furthermore, soil health at different crop phases was assessed by characterizing soil pH, active carbon (AC), soil inorganic carbon, water stable aggregates (WSA), extracellular enzyme activities (EEAs), and soil NO_3^- and ammonium (NH_4^+) levels. Significantly higher AC levels

were found in MZ and CC phases compared to SB-WW phase, while the highest WSA (%) were found in MZ fields ($P<0.05$). Furthermore, our results showed variations in the activity of extracelular enzymes between crop phases, where the abundance of the N-mineralizing enzyme NAG wwas high in both MZ and CC phases compared to WW ($P<0.05$). The ratio of *amo*A:*16S* rRNA genes were significantly elevated in the SB-WW phase, revealing potentially high NO_3^- leaching from these fields, while *nir*K:*16S* rRNA gene ratios were significantly higher in the MZ phase, indicating the potential for N-loss via N_2O emissions. Our results indicate potential to improve N management in MZ-SB-WW-CC rotations to minimize N-losses and optimize plant N-uptake, which contributes to improved crop yields. Furthermore, this work reveals the importance of integrating CC in crop rotations to alleviate the adverse impacts of N-fertilizers and improve soil health.

Keywords: Cover crops, N-fertilizers, soybean-maize rotations, soil health, NO_3^- leaching, N_2O emissions, N-cycling bacterial communities, Ontario

Authors' Contribution: Imesha Perera: Formal analysis, Investigation, Writing – original draft, Writing - review & editing, Visualization. **Anna Kisiala**: Conceptualization, Investigation,Writing - original draft, Writing - review & editing, Visualization. **R. J. Neil Emery:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Karen A. Thompson:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

3.1 Introduction

Soybean (*Glycine max* (L.) Merr.) is an economically important crop rich in protein and fibre, grown for seed production worldwide, especially in temperate and tropical regions (Agomoh et al., 2021; Martinez et al., 2020). Canada is a leading country in SB production, and it is expected to increase yields by 0.5 Mt during the crop year 2022-2023 (AAFC, 2022). Soybean is a leguminous crop which can fulfill its nitrogen (N) requirements through biological N fixation during N-limited conditions (Córdova et al., 2019). This process may account for a relatively high accumulation of N in plant biomass, and as a result, SB residues reach lower C: N ratios. Moreover, they produce relatively low biomass of crop residues, resulting in lower SOC accumulation rates than other crop types (Córdova et al., 2019). This, in turn, results in the reduction of the water holding capacity and nutrient retention in the soil, causing depletion of soil health and reduced crop yields (Córdova et al., 2019). Therefore, in most agricultural fields, SB is grown in rotations with high C returning crops (Studdert and Echeverría, 2000). For instance, in Canada and the midwest USA, SB is grown in 2-3 year rotations with MZ and WW (Agomoh et al., 2021). Crop rotation is an agricultural management practice where multiple crops are grown in succession over time in the same agricultural field (Zhang et al., 2021; Chamberlain et al., 2020; Martinez et al., 2020; Lu et al., 2018; Castellazzi et al., 2008). Numerous studies have reported positive impacts of crop rotation on improving soil health parameters such as SOM (Martinez et al., 2020; Zuber et al., 2018), SOC (Liu et al., 2020; Martinez et al., 2020; Maiga et al., 2019), total carbon (Garcia et al., 2013), soil nutrient content (Zhang et al., 2019), soil structure (Oliveira et al., 2019), and reduced weed pressure (Weisberger et al., 2019); this, in turn, improves overall crop yields. The inclusion of WW or alfalfa in MZ-SB rotations has significantly improved soil health assessment scores compared to monoculture

MZ and simple MZ-SB rotations (Congreves et al., 2015). Furthermore, three-year rotations of SB-MZ-WW in southern Ontario have resulted in large biomass residue returns and SB yields compared to continuous SB (Agomoh et al., 2021).

Along with improving soil physiochemical properties, diversified crop rotations also have an impact on the abundance, diversity, and composition of SMC - the critical drivers of biogeochemical C, N, and phosphorus (P) cycling in agroecosystems (Linton et al., 2020; Mayer et al., 2019; D'Acunto et al., 2018). Nitrogen-cycling SMC are involved in different processes in the N-cycle, including immobilization, mineralization, nitrification, and denitrification, which are widely studied in agroecosystems due to two major reasons (Dai et al., 2020). First, N is an essential macroelement playing a vital role in plant biomass production and physiological functioning (Anas et al., 2020; Rütting et al., 2018) and, therefore, N availability in agroecosystems is a major determinant of crop growth and yields (Ichihashi et al., 2020). Second, higher N inputs through N fertilizers may lead to increased loss of N from agroecosystems via NO_3^- leaching and emission of N_2O, a GHG, which may cause water pollution and depletion of the ozone layer, respectively (Linton et al., 2020; Behnke and Villamil, 2019). Moreover, losses of N cause reductions in crop yields and biomass (Tenuta et al., 2019). The two major processes involved in N_2O emissions are: nitrification - the oxidation of NH_4^+) to NO_3^-, mainly through the action of soil bacterial (*amo*A) and archaeal (*crenamo*A) ammonia oxidizers, and denitrification - the sequential reduction of NO_3^- to N_2O and dinitrogen gas (N_2), which is mediated by soil denitrifying bacterial communities (*nir*S, *nir*K, and *nos*ZI and *nos*ZII) (Gu et al., 2020; Tenuta et al., 2019; Thompson et al., 2016; Snider et al., 2015) and fungal denitrifiers.

The emission of N_2O is a global problem, and in Canada, the agricultural sector accounts for 75% of annual N_2O production (Environment and Climate Change Canada, 2022). Therefore, agricultural management strategies that mitigate N-losses are needed to minimize environmental pollution and improve crop yields by optimizing N uptake by plants. Multiple studies have shown the capacity of diversified crop rotations to reduce N_2O emissions compared to corresponding monocultures (Jiang et al., 2021; Tenuta et al., 2019; Lehman et al., 2017). A reduction in N_2O emissions suggests a shift in the expression of genes nitrifying (*amoA* and *crenamoA*) and denitrifying SMC (*nirS, nirK*, *nos*ZI, and *nos*ZII) activities (Thompson et al., 2016; Snider et al., 2015). Additionally, Paungfoo-Lonhienne et al. (2017) have reported a reduction in the abundance of *amoA* genes in SB-peanut cropping systems compared to SB monoculture. Furthermore, studies have shown the ability of CC, which are living ground covers integrated into crop rotations during the main crop or fallow period (Justes, 2017; Fageria et al., 2005; Hartwig and amp; Ammon, 2002), to alter N-cycling SMC, and thereby impact NO_3^- leaching and N_2O emissions (Muhammad et al., 2019; Rocha et al., 2020). Samaddar et al. (2021) illustrated that the inclusion of a legume crop in a tomato-MZ rotation reduced denitrifying gene abundance and reported the potential for a decrease in N_2O emissions. However, diversified MZ-SB cropping systems with WW and CC have also produced enhanced N_2O fluxes when inorganic N-fertilizers are applied, reflecting the need for changes in N-fertilizer management when WW and CCs are included in crop rotations (Linton et al., 2020; Bayer et al., 2015; Gaudin et al., 2015; Mitchell et al., 2013). Moreover, Linton et al. (2020) reported a positive correlation between *amo*A gene abundance and N_2O emissions after N-fertilizer application emphasizing the impact of N-fertilizers on the size of N-cycling SMC.

These pieces of evidence mirror the importance of studying the N-cycling SMC in relation to agricultural management, especially in fields under intensive N-management. However, little is known about the influence of intensive N-application practices on soil health and N-cycling SMC in MZ-(SB-WW)-CC crop rotations in Ontario. Therefore, in this work, we quantified total bacterial (*16S r*RNA), nitrifying bacterial (*amo*A), and denitrifying bacterial (*nirS*, *nirK*) communities in different phases of a MZ-(SB-WW)-CC rotation to characterize how N-management associated with different crop phases impacts N cycling SMCs. Furthermore, the correlations between soil health parameters and N-cycling bacterial community abundances were studied to understand their associations. We hypothesized that the application of N-fertilizers in the MZ phase and SB-WW phase impact the gene abundances of N-cycling SMC that contribute to N-losses.

3.2 Material and Methods

3.2.1 Study sites and soil sampling

Soils were sampled from operational farms (all managed by the same farm manager) under an MZ-(SB-WW)-CC rotation, located in the Cobourg/Port Hope area in Northumberland County, Southern Ontario, Canada, within the Gages Creek and Cobourg Creek watershed, from November 12th - November 26th, 2020. The study area has a continental climate with a warm summer. Ten fields were selected for sampling based on the three current crop phases: three fields that had been planted to MZ in spring 2020 and had been harvested shortly before sampling, four fields that had been transitioned from SB (spring 2020 planting) to WW (fall 2020 planting), and three fields that had been planted to CC in the spring of 2020 and left unharvested at sampling. Monoammonium phosphate (MAP) fertilizer was applied at MZ

planting and at WW planting (after SB harvesting), with an additional starter NPK (24:6:24) also applied at WW planting.

Soil samples were collected along 125 m long parallel transects (n=4), with 10 samples (12.5 m apart) composited along each transect to 20 cm depth using a 5 cm diameter soil probe. Soils were brought to the lab, air dried, sieved at <2 mm and stored at -20^{o}C until analysis. Soil sampling for molecular analysis was done aseptically, transferred to the lab on ice in a cooler, and stored at -20^{o}C until analysis.

3.2.2 Soil particle-size distribution, wet stable aggregates (WSA), and soil pH

Particle size distribution was assessed for each composited soil per field (n=4). Soil particle-size distribution was carried out using the hydrometer method with a standard buoyancy hydrometer. Soil pH and conductivity were determined using a pre-calibrated pH meter (Mettler-Toledo) in solutions (soil: distilled water ratio of 1:2). To assess the % WSA, 2 g of air-dried sample was placed in aggregate stability- Eijkelkamp wet sieving apparatus and sieved (1mm). Soils were washed with deionized water and dispersing solution of hexametaphosphate, and the soils collected in the cans were oven dried at 115° C until the water evaporated. The weights were recorded, and %WSA were calculated.

3.2.3 Soil Carbon analysis

Soil inorganic carbon (SIC) was determined by igniting soil samples at 850^{o}C for 14 h in a muffle furnace, and the change in masses before and after were used to calculate SIC contents. Active carbon, the labile fraction of SOC, which can be readily oxidized by SMC, was assessed using the $KMnO_4$ method (Weil et al., 2003). The $KMnO_4$ oxidizes simple carbohydrates, amino acids, amines and amides, and C compounds with a hydroxyl group (C-OH), ketone (R2C=O), carboxyl (-COOH), and aliphatic compounds, which are the preferred substrates of SMC. The

absorbance readings of the C oxidized by $KMnO_4$ were obtained at 550 nm with an Epoch™ microplate spectrophotometer (BioTek Instruments, Inc., Germany). The unknown concentrations of AC (permanganate oxidizable C) in the soil samples were calculated using a standard curve prepared with a dilution series of $KMnO_4$ of known concentrations (0.05M, 0.01M, 0.015M and 0.02M) and their absorbance values.

3.2.4 Microbial Extracellular Enzyme Activity (EEA)

The EEA: β-glucosidase (BG), phosphatase (PO), and N-acetylglucosaminidase (NAG) were determined in soil samples using previously described methods (Jackson et al., 2013). The assays were conducted by incubating soil solutions at room temperature (20^oC) with the specific substrates: *p*NP-β-D-glucopyranoside (5mM, 1h), *p*NP-β-N-acetylglucosaminide (2mM, 2h), and *p*NP-phosphate (5 mM, 1h) for the enzymes BG, NAG, and PO, respectively followed by obtaining absorbance readings using Epoch™ microplate spectrophotometer (BioTek Instruments, Inc., Germany) at 410 nm, and the EEAs were calculated using standard curves prepared for each enzyme target.

3.2.5 Quantification of total soil bacterial genes and N-cycling soil bacterial genes

One sample of field moist soil (0.250 g) per composite sample of each transact (n=3) per field were used to extract microbial genomic DNA using the manufacturer's protocol for the DNeasy PowerSoil Pro Kit (Qiagen laboratories, USA). DNA was purified using a Monarch PCR and DNA Cleanup Kit (BioLabs, Canada) and quantified using a NanoDrop (ThermoFisher). Purified DNA was stored at -80^oC until analysis.

Total bacterial communities, nitrifying bacterial communities and denitrifying bacterial communities were quantified using qPCR by targeting *16S*rRNA, *amo*A, *nir*S, and *nir*K genes by using primer pairs 338f/518r, IF/2R, Cd3af/R3Cd, F1aCu/R3Cu, respectively (Thompson et al.,

2016; Snider et al., 2015). Prior to amplification, genomic DNA was diluted to 15 $ng \cdot \mu L^{-1}$ using nuclease-free water to reduce the influence of inhibitors during the reaction. The qPCR reaction mixtures contained 12 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 10 mM each of forward and reverse primers, $ng \cdot \mu L^{-1}$ genomic DNA, and nuclease-free water to a final volume of 20 µL. All amplifications were performed in triplicate on a Quant3 Applied Biosystem (ThermoFisher) alongside a triplicate serial dilution of known standards containing 10^{-1}-10^{-8} copies per reaction of the targeted DNA *16S* rRNA, *amo*A, *nir*S, and *nir*K genes that were cloned from *Clostridium thermocellum* spp., *Nitrosomonas europaea* spp., *Alcaligenes faecalis* spp., and *Pseudomonas aeruginosa* spp., respectively (Thompson et al., 2016). The qPCR reaction conditions consisted of an initial 2 min period at 98ºC, followed by 35 cycles of denaturing at 98ºC for 10 s, annealing at: 55ºC (16S rRNA), 57ºC (*amo*A and *nir*S), or 56ºC (*nir*K) for 30 s, and elongation at 72ºC for 30 s, and the amplicon specificity was observed using the melt curve. The qPCR assays were optimized to obtain reaction efficiencies of 90-110%, and the slope of the standard curve between -3.2 to -3.6 with R^2 values between 0.990 to 1.000. No template controls were included in all qPCR assays.

3.2.6 Statistical analysis

One-way analyses of variance (ANOVA) were conducted to test for differences in soil health parameters, total bacterial gene abundances and N-cycling gene abundances among crop phases in the MZ-(SB-WW)-CC rotation using a generalized linear model in the R platform (R Studio Team (2021). R Studio: Integrated Development Environment for R. R Studio, PBC, Boston, MA).

Within each data set, the independent fixed effects were associated with the crop rotation phase, while the fields and the transects were random effects. Individual treatment means within the

data set were compared using Fishers Least Significant Difference (LSD) post hoc test for all pairwise comparisons. Significant differences among and between least-square means were determined by p values, where the null hypothesis (Ho) was rejected at $P < 0.05$.

Pearson's correlation analysis was conducted to assess the statistical associations between gene abundances and soil health parameters tested. Finally, to visually demonstrate the multiple relationships between soil health parameters, total bacterial gene abundances and N-cycling gene abundances, a principal components analysis (PCA) was carried out in R Studio software. In the PCA, a scree plot was examined for breaks, and PC components with eigenvalues ≥ 1 (PC1 and PC2) were retained in the 2-dimensional plot for visual analysis.

3.3 Results

3.3.1 Soil particle-size distribution, soil pH and soil inorganic carbon (SIC)

According to particle size distribution analysis, two CC-grown fields had sandy loam soils, while the other CC-grown field had loam soil. Two out of 4 WW-grown fields had sandy loam soils, while the other two had loam soil and silt loam soils. All the corn fields (3) had loam soils (**Table 3.1**). Soil pH and SIC showed no significant variation among crop phases ($P<0.05$) **(Table 3.2)**.

3.3.2 Soil active carbon (AC), microbial extracellular enzyme activity (EEA) and wet stable aggregates (WSA)

Active carbon levels were significantly different between crop phases, where the highest AC levels were present in soils under both CC (655±39 ppm) and MZ (610±20 ppm), and the lowest AC levels were found in soils under the WW (487±37 ppm) crop phase **(Table 3.2)**. The EEA showed significant variation between crop phases **(Table 3.2)**. NAG levels were significantly higher in both CC and MZ crop phases than in soils under the SB-WW **(Table 3.2)**. PO levels

were significantly higher in soils under the MZ crop phase, while BG levels were significantly higher in soils under SB-WW than in the other crop phases **(Table 3.2).** Significant differences in the % of WSA at <1mm diameter size were detected between crop phases. Among the three crops, soils under the MZ had a significantly higher % of WSA **(Table 3.2)**.

3.3.3 Levels of soil NH_4^+/NO_3^- ions and the abundance of total bacterial and N-cycling bacterial gene copies

Soil NH_4^+ levels were similar among crop phases, while levels of soil NO_3^- significantly differed, where fields under MZ had higher NO_3^- levels (18.3 ±1.5 ppm) compared to fields under CC (14.1 ±1.4 ppm) and SB-WW (14.4 ±1.7) phases at the time of sampling **(Table 3.2).**

Total bacterial gene abundances (*16*S rRNA) between crop phases were not different ($P<0.05$) **(Table 3.3)**. However, N-cycling SMC abundances (*amo*A and *nir*K) showed variations among fields under different crop phases **(Table 3.3)**. Nitrifying bacterial gene ratios (*amo*A:*16S*rRNA) were markedly different among the three crop phases, where fields under WW had the highest *amo*A:*16S*rRNA gene ratio, while fields under the MZ had the lowest *amo*A:*16S*rRNA ratio **(Table 3.3).** Among the denitrifying bacterial genes tested (*nir*S and *nir*K), only *nir*K:*16Sr*RNA gene ratios significantly varied among crop phases **(Table 3.3)**, where MZ fields had a higher ratio, whilst WW-grown fields had the lowest *nir*K:*16S*rRNA gene ratio compared to other crop phases ($P<0.05$) **(Table 3.3)**.

Nitrifying to denitrifying gene ratios (*amo*A: *nir*K), which represent the potential ability of nitrification compared to denitrification, were significantly higher in both CC (0.86 ±0.01) and WW fields (0.87±0.02), while the lowest ratio was obtained for MZ fields (0.76 ±0.01) **(Table 3.3).**

3.3.4 Correlation between soil health parameters and abundances of total bacterial and N-cycling bacterial genes

Pearson's correlation analysis showed number of correlations between parameters tested (**Supplemetary Fig. 3.1**), therefore, to build upon and visualize the multivariate relationships among soil health parameters (pH, WAS, IC, AC, EEA, NH_4^+ and NO_3^- ions) and the abundances of total (*16S*rRNA), nitrifying (*amo*A), and denitrifying (*nir*S and *nir*K) genes simultaneously, a PCA loading plot was created (**Fig. 3.1**). The first two principal components, PC1 and PC2, accounted for 61.5% and 11.1% of the data set variance, respectively (**Fig. 3.1**). Variables NH_4^+, NO_3^-, WSA, pH, IC, AC and EEA (NAG, PO and BG) loaded significantly on PCA, while *nir*K and *amo*A gene abundances loaded on PC2 (**Fig. 3.1**).

3.4 Discussion

Most agricultural practices, including crop rotations and cover cropping impact soil physical, chemical, and biological attributes, which influence soil health. MZ-SB rotation is one of the most predominant cropping systems in Ontario, and in some operational farms WW and CC are integrated during winter seasons to protect soil from erosion and to enhance subsequent cash crop yields. However, intensive application of N-fertilizers; where MZ fields receive MAP during planting and SB-WW fields receive both MAP and starter N:P: K (24:6:24) before and during WW planting can be seen in these fileds. In this study, we used qPCR to enumerate total (*16S* rRNA) and N-cycling bacterial community (*amo*A, *nir*S, and *nir*K) abundances to studying SMC responses to management to assess whether there are opportunities for reduced N loss in these systems. Supporting our hypothesis, the results showed significantly higher abundances of N-cycling SMC that mediate N-losses in MZ and SB-WW fields compared to CC fields emphasizing the potential N-losses in MZ and SB-WW fields.

3.4.1 Impact of crop rotation phases on soil health parameters

3.4.1.1 Variation of Active Carbon (AC) and Wet Stable Aggregates (WSA) among crop rotation phases

The labile SOC fraction (AC) showed markedly higher levels in both MZ and CC phases (**Table 3.2**), which produce higher biomass compared to WW, which was at early growth stages and had little accumulated biomass at the time of soil sampling. Another likely contributor to the differences we saw in AC levels between rotation phases might be the variations in the composition (Naveed et al., 2017) and quantity (Baumert et al., 2018) of root exudates- a mixture of compounds, including organic acids, sugars, amino acids, carbohydrates, secondary metabolites, hormones etc., released by living roots to their outside environment (reviewed in Preece and Peñuelas, 2020), at different crop phases. As documented in previous studies, differences in WSA (Baumert et al., 2018; Habib et al., 1990), SOM (Denef et al., 2002), AC (Blair & Crocker, 2002), and the activity of SMC (Trivedi et al., 2017) have been observed in soils with different crop types. Furthermore, the deep and intense root system and biomass input of CCs can have a substantial impact on soil C stocks (Thapa et al., 2021; Belfry et al., 2017; Sainju et al., 2002), which aligns with our findings of elevated AC levels (**Table 3.2**). In most studies, CCs have quickly increased AC levels relative to other crops. For example, CCs were grown over 5 eyars in a farmer-led trial conducted in 78 fields across 9 states in the USA, resulting in a significant increase in AC levels compared to non-CC controls (Wood & Bowman, 2021). Furthermore, White et al. (2020) observed a significant increase in AC levels after 8 years of increasing winter CC frequency and addition of compost compared to control in a long-term vegetable cropping system in California. Chahal & Van Eerd (2020) reported a positive impact of CC in improving AC in a tomato cropping system.

Soil aggregate stability defines the strength of the soil structure (Al-Kaisi et al., 2014). Wetting of soil due to runoff and precipitation influences the disintegration of unstable soil aggregates, causing erosion (Barthès & Roose, 2002); therefore, the fraction of WSA is used as one of the major parameters to determine soil health (Al-Kaisi et al., 2014). Furthermore, the stability of soil aggregates is built upon the availability of soil binding compounds such as microbial products, roots and soil organic compounds, and therefore, soil aggregate stability can quickly respond to changes in agricultural management practices, making it a sensitive soil health parameter (Chan and Heenan, 1999). In the current study, we observed a significant difference in the % of WSA among different crop phases in a MZ-(SB-WW)-CC rotation, with % WSA increasing in the order of WW<CC<MZ **(Table 3.2)**.

Similarly, Holeplass et al. (2004) reported significant differences in the % of 0.6-2 mm and <0.6 mm WSA among 3 different crop rotations in southern Norway. Growing barley, pea, and canola in rotation with wheat, Chan and Heenan (1999) observed significant variation in % WSA among crop rotations, with the highest % WSA measured in a wheat rotation diversified with CC-barley compared to other rotations. On the contrary, no significant differences in % WSA were observed between three crop rotations of pearl millet with wheat, chickpea, and mustard in India (Singh et al., 2018) or between SB-WW-SB, MZ-WW-MZ, and SB-WW-MZ rotations in Brazil (Castro Filho et al., 2002). Interestingly, Nouwakpo et al. (2018) demonstrated the ability of MZ residues to improve soil aggregation compared to SB residues, paralleling our results of significantly higher WSA in MZ fields compared to SB-WW fields (**Table 3.2**). Furthermore, Naveed et al. (2018) found a significantly higher ability of MZ root exudates to bind soil particles, improving soil aggregate formation compared to the root exudates of CC-barley. This evidence indicates that significantly high AC levels observed in our MZ fields might have

resulted from a higher accumulation of C from MZ residue returns (Agomoh et al., 2021) and root exudates of MZ (Naveed et al., 2018). The strong correlation between AC levels and WSA (**Supplementary Fig. 3.1**) further reinforces this idea. Interestingly, previous studies also reported a positive correlation of SOC with > 0.25mm (Nouwakpo et al., 2018) and 0.5-0.2 mm and 0.053-0.25 mm % WSA (Al-Kaisi et al., 2014).

3.4.1.2 Variation of soil microbial Extracellular Enzyme Activities (EEA) among crop phases

Microbial extracellular enzymes play a crucial role in soil functioning by catalyzing nutrient cycling reactions, including decomposition of SOM, mineralization reactions, and N-transformation reactions. Extracellular enzyme activities respond rapidly to changes in soil management, including crop rotation, the input of fertilizers, and tillage, and therefore can be considered an early indicator of changes in soil health. In our study, we observed significant variation in the activities of BG, NAG, and PA enzymes among three crop phases in the MZ-(SB-WW)-CC rotation **(Table 3.2)**.

Interestingly, a wide range of previous research highlights the positive relationship between SOM content and EEA (Borase et al., 2020; Martyniuk et al., 2019; Chander et al., 1997). Ekenler & Tabatabai (2003) reported a significant correlation between NAG activity and SOC content. Furthermore, in a double-rice cropping system in southern China, winter CC residue return resulted in significantly higher EEAs of BG and PO compared to no CC fallow fields with no residue return (Tang et al., 2014). Similarly, Hamido & Kpomblekou-A (2009) documented a significant increase in EEA when CCs were grown following tomato in an experimental field in Tuskegee, USA, whilst the same study also indicated a positive correlation between the activities of arylamidase, amidohydrolases, and ureases with SOC. Aligning with the evidence mentioned

above, the significant differences in EEAs we observed among crop phases in the current study might be caused by the variation in the amount and quality of crop residue returns and root exudates, which may be reflected in the SOC and labile C (AC) levels measured at each crop phase (Turner et al., 2002; Singh & Kumar, 2021). The relatively higher levels of biomass return of MZ and CCs compared to other crops (Tang et al., 2014; Hamido & Kpomblekou-A, 2009) may be responsible for the significantly higher AC levels detected in CC and MZ fields **(Table 3.2)**.

The positive associations between EEA and the AC levels (**Fig. 3.1** and **Suppl. Fig. 3.1**) explain the results obtained for NAG and PO activity **(Table 3.2)**. In addition to C content, the availability of N-containing substrates impacts N-mineralizing enzyme activities like NAG (Muruganandam et al., 2009). The CC fields, which had a mixture of leguminous and non-leguminous crops, and SB-WW fields that produce higher N-containing crop residues, likely influenced microbial functioning differently compared to non-leguminous crop fields (MZ). Therefore, it is reasonable to expect higher potential N-mineralization in CC and SB-WW fields compared to MZ fields. Supporting this statement, the CC fields had a significantly higher level of the N-mineralizing enzyme NAG at the time of soil sampling **(Table 3.2)**. Although we expected high potential N-mineralization activity in SB-WW, NAG levels were not significantly increased in these fields **(Table 3.2)**. Winter wheat cultivation is associated with the fall application of N-fertilizers during planting to improve root growth and yields and minimize fungal infections that cause crown rot and take-all root diseases (Huber et al., 1980). According to correspondence with the operating farmer, intensive N-management in the WW fields, where farmers applied MAP and starter N:P:K (24:6:24) during planting of WW may have resulted in a

significantly higher levels of plant available N in the fall, would be one reason for the observed markedly low NAG activities.

EEA and AC levels reflect potential SMC abundance and functioning (Tang et al., 2014; Xue et al., 2006; Turner et al., 2002). Although EEA and AC levels varied among crop phases, the abundance of total bacterial gene copies (*16S* rRNA) is not an absolute quantifier as the number can vary per genome (since one bacterial genome can possess 1-13 gene copies), and we observed similar total bacterial gene abundances between crop phases **(Table 3.3)**. Therefore, perhaps other SMC, for example, soil fungal communities, as mentioned in Muruganandam et al. (2009), might have contributed largely to EEAs observed in the soil samples analyzed in this study.

3.4.2 Impact of crop rotation phases on nitrifying and denitrifying soil bacterial communities

3.4.2.1 Nitrifying bacterial communities: Variation of *amo*A:*16S*rRNA among crop phases

In the current work, we found relatively higher nitrification potential in the WW field, as indicated by *amo*A:*16Sr*RNA, than in other crop fields **(Table 3.3).** One possibility for this might be the occurrence of relatively higher substrates (NH_4^+) for the proliferation of nitrifying bacterial communities (reviewed in Sahrawat, 2008). Since WW was planted soon after SB harvesting, mineralization of high N containing (low C: N) crop residues and N from N-fixing SB might have largely contributed to the N-pool (Baggs, 2000; Tortosa et al., 2015), relative to MZ and CC fields. Therefore, it seems more reasonable to expect significantly high NH_4^+ levels in WW fields, which might have increased nitrifying SMC abundance compared to other crop phases at sampling.

Although we detected significantly higher ratios of *amo*A:16S rRNA genes in WW fields, revealing potentially higher nitrification, the NO_3^- levels were significantly lower in WW fields compared to other crop fields at the time of sampling. This could be due to uptake of NO_3^- by WW roots, or due to NO_3^- entering the denitrification pathway, or otherwise, due to NO_3^- leaching. At the time of sampling, WW was immature (<5cm in height), and therefore had a shallow/weak root system, which likely made WW a poor NO_3^- scavenger. Further, significantly lower denitrification gene copy numbers were observed in WW fields compared to other crop phases, indicating that lower NO_3^- levels are also potentially due to NO_3^- leaching out of the immature root zone. Previous studies have revealed that fall application of N-fertilizers results in loss of N either through nitrification, NO_3^- leaching, or by production of dinitrogen gases, e.g., N_2 and N_2O in the denitrification pathway during the growing season, which also reduces crop growth and yields (Huber et al., 1980). Similar to WW fields, CC fields had significantly higher *amo*A: *nir*K ratios compared to MZ, suggesting a higher potential for nitrification. However, in contrast to WW, the deep and intense root system of CC improve their ability to scavenge NO_3^- and, therefore, the significantly lower NO_3^- levels detected in CC fields compared to MZ in late fall is likely due to plant uptake rather than due to NO_3^- leaching.

Other potential factors that can influence nitrifying SMC are nutrient availability (Mg, Ca) (Raglin et al., 2022) and soil pH (Xue et al., 2006). In our study, since no significant variation in soil pH was observed among crop phases, this factor is unlikely to account for the variations observed in the nitrifying bacterial communities among tested crop phases.

3.4.2.2 Denitrifying bacterial communities: Variation of *nir*K:*16S*rRNA ratios among crop phases

Among the denitrifying bacterial communities tested (*nir*S and *nir*K), the *nir*K gene abundances showed significant variation among different crop phases in the MZ-(SB-WW)-CC rotation (**Table 3.3**). Previous studies have also documented responses of *nir*K gene abundance to N-fertilizer applications and the phase of the crop rotation (Linton et al., 2020; Maul et al., 2019; Coyotzi et al., 2017).

The application of MAP during MZ planting in spring, which inputs NH_4^+ might have enhanced *amo*A gene abundances. The significantly higher NO_3^- levels found in MZ phase at the sampling time (in fall) could have resulted from potential nitrification during the growing season. The significantly higher *nir*K:*16S* rRNA gene ratios observed in MZ fields provide evidence that the markedly higher NO_3^- levels in MZ soils supported denitrifying bacterial communities, likely leading to N-loss via denitrification. The significantly higher substrate availability (NO_3^-) may have co-supported the larger denitrifying bacterial communities (*nir*K) in the MZ fields, potentially promoting N-loss via the emission of N_2O during the growing season. Parkin and Kaspar (2006) observed significantly high N_2O fluxes in the MZ phase in a MZ-SB-CC rotation. However, significantly lower NO_3^- levels and lower ratios of *nir*K:*16S*rRNA in our CC fields suggest growing CCs might scavenge NO_3^- until degradation after CC termination, potentially reducing N-losses via leaching and N_2O emissions during the non-growing season.

3.5 Conclusions

We examined the influence of different phases in a diversified MZ-(SB-WW)-CC crop rotation in southern Ontario on soil health parameters and soil N-dynamics associated with nitrifying (*amo*A) and denitrifying (*nir*S and *nir*K) bacterial communities. During this study, we

discovered significantly higher WSA, AC, NO_3^- levels, N-mineralizing (NAG) and cellulose utilizing (BG) enzyme activities in MZ fields. However, MZ fields that received high amounts of MAP fertilizers during planting had significantly higher denitrification potential vs. nitrification potential, suggesting potential N-losses via N_2O. In contrast to MZ fields, low WSA, AC, NAG and NO_3^- levels were observed in WW fields, potentially due to low biomass production and weak root systems that might have resulted in low SOM and root exudates. Moreover, higher nitrification vs. denitrification potential (*amo*A: *nir*K) was observed in WW fields, suggesting the possibility of N-loss via NO_3^- leaching. Similar to MZ fields, the CC fields also had significantly higher AC levels, which may be due to high residue returns and the quantity and quality of CC root exudates. Significantly higher nitrification potentials (*amo*A:*16S* rRNA) were observed compared to the potential for denitrification (*nir*K:*16S* rRNA) in CC fields. Unlike WW, since CCs have deep and strong root systems, they can scavenge residual NO_3^-, minimizing N-loss via either NO_3^- leaching or denitrification compared to the other two crop phases. Specifically, this study highlights the impacts of intensive use of N-fertilizers during MZ and WW planting in MZ-SB-WW-CC rotations in Southern Ontario on soil nitrifying and denitrifying bacterial communities. Management-induced impacts on these communities may relate to N-losses via N_2O emissions and NO_3^- leaching from MZ and WW phases and may lead reductions in crop yields in these rotations. Furthermore, this study provides evidence for Ontario farmers that integrating CC into cash crop rotations may reduce N-loss during winter seasons and thereby improve future crop yields while improving environmental quality and soil health.

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3.6 Bibliography

AAFC. (2022). Outlook for principal field crops. Agriculture and Agri-Food Canada.

Agomoh, I. V., Drury, C. F., Yang, X., Phillips, L. A., & Reynolds, W. D. (2021). Crop rotation enhances soybean yields and soil health indicators. *Soil Science Society of America Journal*, *85*(4), 1185–1195. https://doi.org/10.1002/saj2.20241

Akiyoshi, D. E., Regier, D. A., & Gordon, M. P. (1987). Cytokinin production by Agrobacterium and Pseudomonas spp. *Journal of Bacteriology*, *169*(9), 4242–4248. https://doi.org/10.1128/jb.169.9.4242-4248.1987

Al-Kaisi, M. M., Douelle, A., & Kwaw-Mensah, D. (2014). Soil microaggregate and macroaggregate decay over, time and soil carbon change as influenced by different tillage systems. *Journal of Soil and Water Conservation*, *69*(6), 574–580. https://doi.org/10.2489/jswc.69.6.574

Anas, M., Liao, F., Verma, K. K., Sarwar, M. A., Mahmood, A., Chen, Z. L., Li, Q., Zeng, X. P., Liu, Y., & Li, Y. R. (2020). Fate of nitrogen in agriculture and environment: agronomic, eco-physiological and molecular approaches to improve nitrogen use efficiency. *Biological Research*, *53*(1), 1–20. https://doi.org/10.1186/s40659-020-00312-4

Andraski, T. W., Bundy, L. G., & Brye, K. R. (2000). Crop Management and Corn Nitrogen Rate Effects on Nitrate Leaching. *Journal of Environmental Quality*, *29*(4), 1095–1103. https://doi.org/10.2134/jeq2000.00472425002900040009x

Baggs, E. M., Rees, R. M., Smith, K.A., and Vinten, A.J.A. (2000). Nitrous oxide emissions from soils after incorporating crop residues. *Soils Use and Management*. 16, 82-87.

Barthès, B., & Roose, E. (2002). Aggregate stability as an indicator of soil susceptibility to runoff and erosion; validation at several levels. *Catena*, *47*(2), 133–149. https://doi.org/10.1016/S0341-8162(01)00180-1

Baumert, V. L., Vasilyeva, N. A., Vladimirov, A. A., Meier, I. C., Kögel-Knabner, I., & Mueller, C. W. (2018). Root exudates induce soil macroaggregation facilitated by fungi in subsoil. *Frontiers in Environmental Science*, *6*(NOV), 1–17. https://doi.org/10.3389/fenvs.2018.00140

Bayer, C., Gomes, J., Zanatta, J. A., Vieira, F. C. B., Piccolo, M. de C., Dieckow, J., & Six, J. (2015). Soil nitrous oxide emissions as affected by long-term tillage, cropping systems and nitrogen fertilization in Southern Brazil. *Soil and Tillage Research*, *146*(PB), 213–222. https://doi.org/10.1016/j.still.2014.10.011

Behnke, G. D., & Villamil, M. B. (2019). Cover crop rotations affect greenhouse gas emissions and crop production in Illinois, USA. *Field Crops Research*, *241*(August), 107580. https://doi.org/10.1016/j.fcr.2019.107580

Belfry, K. D., Trueman, C., Vyn, R. J., Loewen, S. A., & Van Eerd, L. L. (2017). Winter cover crops on processing tomato yield, quality, pest pressure, nitrogen availability, and profit margins. *PLoS ONE*, *12*(7), 1–17. https://doi.org/10.1371/journal.pone.0180500

Blair, N., and Crocker, J. G. (2000). Crop rotation effects on soil carbon and physical fertility of two Australian soils. *Australian Journal of Soil Research*.38, 71-84.

Borase, D. N., Nath, C. P., Hazra, K. K., Senthilkumar, M., Singh, S. S., Praharaj, C. S., Singh, U., & Kumar, N. (2020). Long-term impact of diversified crop rotations and nutrient management practices on soil microbial functions and soil enzymes activity. *Ecological Indicators*, *114*(March), 106322. https://doi.org/10.1016/j.ecolind.2020.106322

Castellazzi, M. S., Wood, G. A., Burgess, P. J., Morris, J., Conrad, K. F., & Perry, J. N. (2008). A systematic representation of crop rotations. *Agricultural Systems*, *97*(1–2), 26–33. https://doi.org/10.1016/j.agsy.2007.10.006

Castro Filho, C., Lourenço, A., Guimarães, M. D. F., & Fonseca, I. C. B. (2002). Aggregate stability under different soil management systems in a red latosol in the state of Parana, Brazil. *Soil and Tillage Research*, *65*(1), 45–51. https://doi.org/10.1016/S0167-1987(01)00275-6

Chahal, I., & Van Eerd, L. L. (2020). Cover crop and crop residue removal effects on temporal dynamics of soil carbon and nitrogen in a temperate, humid climate. *PLoS ONE*, *15*(7), 1–21. https://doi.org/10.1371/journal.pone.0235665

Chamberlain, L. A., Bolton, M. L., Cox, M. S., Suen, G., Conley, S. P., and Ané, J. M. (2020). Crop rotation, but not cover crops, influenced soil bacterial community composition in a corn-soybean system in southern Wisconsin. Applied Soil Ecology, 154(December 2019), 103603. https://doi.org/10.1016/j.apsoil.2020.103603

Chan, K. Y., & Heenan, D. P. (1999). Microbial-induced soil aggregate stability under different crop rotations. *Biology and Fertility of Soils*, *30*(1–2), 29–32. https://doi.org/10.1007/s003740050583

Chander, K., Goyal, S., Mundra, M. C., & Kapoor, K. K. (1997). Organic matter, microbial

biomass and enzyme activity of soils under different crop rotations in the tropics. *Biology and Fertility of Soils*, *24*(3), 306–310. https://doi.org/10.1007/s003740050248Christianson, R. D., & Yuan, Y. (2021). *E c c r w p r a a*. *64*(2), 691–704.

Congreves, K. A., Hayes, A., Verhallen, E. A., & Van Eerd, L. L. (2015). Long-term impact of tillage and crop rotation on soil health at four temperate agroecosystems. *Soil and Tillage Research*, *152*, 17–28. https://doi.org/10.1016/j.still.2015.03.012

Córdova, S. C., Castellano, M. J., Dietzel, R., Licht, M. A., Togliatti, K., Martinez-Feria, R., & Archontoulis, S. V. (2019). Soybean nitrogen fixation dynamics in Iowa, USA. *Field Crops Research*, *236*(November 2018), 165–176. https://doi.org/10.1016/j.fcr.2019.03.018

Coyotzi, S., Doxey, A. C., Clark, I. D., Lapen, D. R., Van Cappellen, P., & Neufeld, J. D. (2017). Agricultural soil denitrifiers possess extensive nitrite reductase gene diversity. *Environmental Microbiology*, *19*(3), 1189–1208. https://doi.org/10.1111/1462-2920.13643

D'Acunto, L., Andrade, J. F., Poggio, S. L., & Semmartin, M. (2018). Diversifying crop rotation increased metabolic soil diversity and activity of the microbial community. *Agriculture, Ecosystems and Environment*, *257*(February), 159–164. https://doi.org/10.1016/j.agee.2018.02.011

Dai, Z., Yu, M., Chen, H., Zhao, H., Huang, Y., Su, W., Xia, F., Chang, S. X., Brookes, P. C., Dahlgren, R. A., & Xu, J. (2020). Elevated temperature shifts soil N cycling from microbial immobilization to enhanced mineralization, nitrification and denitrification across global terrestrial ecosystems. *Global Change Biology*, *26*(9), 5267–5276. https://doi.org/10.1111/gcb.15211

Denef, K., Six, J., Merckx, R., & Paustian, K. (2002). Short-term effects of biological and

physical forces on aggregate formation in soils with different clay mineralogy. *Plant and Soil*, *246*(2), 185–200. https://doi.org/10.1023/A:1020668013524

Environment and Climate Change Canada. (2022). National inventory report : greenhouse gas sources and sinks in Canada : executive summary.

Ekenler, M., & Tabatabai, M. A. (2003). Tillage and residue management effects on β-glucosaminidase activity in soils. *Soil Biology and Biochemistry*, *35*(6), 871–874. https://doi.org/10.1016/S0038-0717(03)00094-4

Fageria, N.,K., Baligar, V. C., Bailey, B. A.(2005). Role of cover crops in improving soil and row cropproductivity. *Communications in Soil Science and Plant Analysis*.

Frąc, M., Oszust, K., & Lipiec, J. (2012). Community level physiological profiles (CLPP), characterization and microbial activity of soil amended with dairy sewage sludge. *Sensors*, *12*(3), 3253–3268. https://doi.org/10.3390/s120303253

Garcia, R. A., Li, Y., & Rosolem, C. A. (2013). Soil Organic Matter and Physical Attributes Affected by Crop Rotation Under No-till. *Soil Science Society of America Journal*, *77*(5), 1724–1731. https://doi.org/10.2136/sssaj2012.0310

Gaudin, A. C. M., Janovicek, K., Deen, B., & Hooker, D. C. (2015). Wheat improves nitrogen use efficiency of maize and soybean-based cropping systems. *Agriculture, Ecosystems and Environment*, *210*, 1–10. https://doi.org/10.1016/j.agee.2015.04.034

Gu, J., Guo, H., & Xiang, H. (2020). The utility of a boundary line approach for simulating denitrification and nitrous oxide emissions from a Regosol under summer maize-winter wheat crop rotation in Southwest China. *Geoderma Regional*, *20*, e00252.

https://doi.org/10.1016/j.geodrs.2020.e00252

Gupta, R., Pizarro, L., Leibman-Markus, M., Marash, I., & Bar, M. (2020). Cytokinin response induces immunity and fungal pathogen resistance, and modulates trafficking of the PRR LeEIX2 in tomato. *Molecular Plant Pathology*, *21*(10), 1287–1306. https://doi.org/10.1111/mpp.12978

Habib, L., Morel, J. L., Guckert, A., Plantureux, S., & Chenu, C. (1990). Influence of root exudates on soil aggregation. *Symbiosis (Rehovot)*, *9*(1–3), 87–91. http://www.epjap.org/10.1051/epjap/2012110475

Hamido, S. A., & Kpomblekou-A, K. (2009). Cover crop and tillage effects on soil enzyme activities following tomato. *Soil and Tillage Research*, *105*(2), 269–274. https://doi.org/10.1016/j.still.2009.09.007

Hartwig, N. L., & Ammon, H. U. (2002). Cover crops and living mulches. *Weed Science*, *50*(6), 688–699. https://doi.org/10.1614/0043-1745(2002)050[0688:aiacca]2.0.co;2

Holeplass, H., Singh, B. R., & Lal, R. (2004). Carbon sequestration in soil aggregates under different crop rotations and nitrogen fertilization in an inceptisol in southeastern Norway. *Nutrient Cycling in Agroecosystems*, *70*(2), 167–177. https://doi.org/10.1023/B:FRES.0000048483.94397.b6

Huber, D. M., Warren, H. L., Nelson, D. W., Tsai, C. Y., & Shaner, G. E. (1980). Response of Winter Wheat to Inhibiting Nitrification of Fall-Applied Nitrogen 1 . *Agronomy Journal*, *72*(4), 632–637. https://doi.org/10.2134/agronj1980.00021962007200040015x

Hussain, A., & Hasnain, S. (2015). *Cytokinin production by some bacteria : its impact on cell*

division in cucumber cotyledons . Afr J Microbiol Res 3 : 704-712 Cytokinin production by some bacteria : Its impact on cell division in cucumber cotyledons. *3*(OCTOBER 2009), 704–712.

Ichihashi, Y., Ichihashi, Y., Date, Y., Date, Y., Shino, A., Shimizu, T., Shibata, A., Kumaishi, K., Funahashi, F., Wakayama, K., Yamazaki, K., Umezawa, A., Sato, T., Kobayashi, M., Kamimura, M., Kusano, M., Kusano, M., Che, F. S., O'Brien, M., … Nihei, N. (2020). Multi-omics analysis on an agroecosystem reveals the significant role of organic nitrogen to increase agricultural crop yield. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(25), 14552–14560. https://doi.org/10.1073/pnas.1917259117

Jackson, C. R., Tyler, H. L., & Millar, J. J. (2013). Determination of microbial extracellular enzyme activity in waters, soils, and sediments using high throughput microplate assays. *Journal of Visualized Experiments : JoVE*, *80*, 1–9. https://doi.org/10.3791/50399

Janečková, H., Husičková, A., Ferretti, U., Prčina, M., Pilařová, E., Plačková, L., Pospíšil, P., Doležal, K., & Špundová, M. (2018). The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. *Plant Cell and Environment*, *41*(8), 1870–1885. https://doi.org/10.1111/pce.13329

Jiang, R., Yang, J. Y., Drury, C. F., He, W., Smith, W. N., Grant, B. B., He, P., & Zhou, W. (2021). Assessing the impacts of diversified crop rotation systems on yields and nitrous oxide emissions in Canada using the DNDC model. *Science of the Total Environment*, *759*, 143433. https://doi.org/10.1016/j.scitotenv.2020.143433

Justes, E. (2017). Cover crops for sustainable farming. In *Cover Crops for Sustainable Farming*. https://doi.org/10.1007/978-94-024-0986-4

Kim, M.-Y., & Lee, K. H. (2022). Electrochemical Sensors for Sustainable Precision Agriculture—A Review. *Frontiers in Chemistry*, *10*(May), 1–14. https://doi.org/10.3389/fchem.2022.848320

Kisiala, A., Laffont, C., Emery, R. J. N., & Frugier, F. (2013). Bioactive cytokinins are selectively secreted by sinorhizobium meliloti nodulating and nonnodulating strains. *Molecular Plant-Microbe Interactions*, *26*(10), 1225–1231. https://doi.org/10.1094/MPMI-02-13-0054-R

Lehman, R. M., Osborne, S. L., & Duke, S. E. (2017). Diversified No-Till Crop Rotation Reduces Nitrous Oxide Emissions, Increases Soybean Yields, and Promotes Soil Carbon Accrual. *Soil Science Society of America Journal*, *81*(1), 76–83. https://doi.org/10.2136/sssaj2016.01.0021

Linton, N. F., Ferrari Machado, P. V., Deen, B., Wagner-Riddle, C., & Dunfield, K. E. (2020). Long-term diverse rotation alters nitrogen cycling bacterial groups and nitrous oxide emissions after nitrogen fertilization. *Soil Biology and Biochemistry*, *149*(April), 107917. https://doi.org/10.1016/j.soilbio.2020.107917

Liu, F., Xing, S., Ma, H., Du, Z., & Ma, B. (2013). Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in Platycladus orientalis container seedlings. *Applied Microbiology and Biotechnology*, *97*(20), 9155–9164. https://doi.org/10.1007/s00253-013-5193-2

Lu, S., Lepo, J. E., Song, H. X., Guan, C. Y., and Zhang, Z. H. (2018). Increased rice yield in long-term crop rotation regimes through improved soil structure, rhizosphere microbial communities, and nutrient bioavailability in paddy soil. Biology and Fertility of Soils,

54(8), 909–923. https://doi.org/10.1007/s00374-018-1315-4

Maiga, A., Alhameid, A., Singh, S., Polat, A., Singh, J., Kumar, S., & Osborne, S. (2019). Responses of soil organic carbon, aggregate stability, carbon and nitrogen fractions to 15 and 24 years of no-till diversified crop rotations. *Soil Research*, *57*(2), 149–157. https://doi.org/10.1071/SR18068

Martinez, J. P., Crespo, C., Sainz Rozas, H., Echeverría, H., Studdert, G., Martinez, F., Cordone, G., & Barbieri, P. (2020). Soil organic carbon in cropping sequences with predominance of soya bean in the argentinean humid Pampas. *Soil Use and Management*, *36*(1), 173–183. https://doi.org/10.1111/sum.12547

Maul, J. E., Cavigelli, M. A., Vinyard, B., & Buyer, J. S. (2019). Cropping system history and crop rotation phase drive the abundance of soil denitrification genes nirK, nirS and nosZ in conventional and organic grain agroecosystems. *Agriculture, Ecosystems and Environment*, *273*(November 2018), 95–106. https://doi.org/10.1016/j.agee.2018.11.022

Mayer, Z., Sasvári, Z., Szentpéteri, V., Rétháti, B. P., Vajna, B., & Posta, K. (2019). Effect of long-term cropping systems on the diversity of the soil bacterial communities. *Agronomy*, *9*(12), 1–10. https://doi.org/10.3390/agronomy9120878

Mitchell, D. C., Castellano, M. J., Sawyer, J. E., & Pantoja, J. (2013). Cover Crop Effects on Nitrous Oxide Emissions: Role of Mineralizable Carbon. *Soil Science Society of America Journal*, *77*(5), 1765–1773. https://doi.org/10.2136/sssaj2013.02.0074

Muhammad, I., Sainju, U. M., Zhao, F., Khan, A., Ghimire, R., Fu, X., & Wang, J. (2019). Regulation of soil CO2 and N2O emissions by cover crops: A meta-analysis. *Soil and Tillage Research*, *192*(November 2018), 103–112.

https://doi.org/10.1016/j.still.2019.04.020

Muruganandam, S., Israel, D. W., & Robarge, W. P. (2009). Activities of Nitrogen-Mineralization Enzymes Associated with Soil Aggregate Size Fractions of Three Tillage Systems. *Soil Science Society of America Journal*, *73*(3), 751–759. https://doi.org/10.2136/sssaj2008.0231

Naveed, M., Brown, L. K., Raffan, A. C., George, T. S., Bengough, A. G., Roose, T., Sinclair, I., Koebernick, N., Cooper, L., Hackett, C. A., & Hallett, P. D. (2017). Plant exudates may stabilize or weaken soil depending on species, origin and time. *European Journal of Soil Science*, *68*(6), 806–816. https://doi.org/10.1111/ejss.12487

Naveed, M., Brown, L. K., Raffan, A. C., George, T. S., Bengough, A. G., Roose, T., Sinclair, I., Koebernick, N., Cooper, L., & Hallett, P. D. (2018). Rhizosphere-Scale Quantification of Hydraulic and Mechanical Properties of Soil Impacted by Root and Seed Exudates. *Vadose Zone Journal*, *17*(1), 170083. https://doi.org/10.2136/vzj2017.04.0083

Nouwakpo, S. K., Song, J., & Gonzalez, J. M. (2018). Soil structural stability assessment with the fluidized bed, aggregate stability, and rainfall simulation on long-term tillage and crop rotation systems. *Soil and Tillage Research*, *178*(March 2017), 65–71. https://doi.org/10.1016/j.still.2017.12.009

Oliveira, M., Barré, P., Trindade, H., & Virto, I. (2019). Different efficiencies of grain legumes in crop rotations to improve soil aggregation and organic carbon in the short-term in a sandy Cambisol. *Soil and Tillage Research*, *186*(September 2018), 23–35. https://doi.org/10.1016/j.still.2018.10.003

Palberg, D., Kisiała, A., Jorge, G. L., & Emery, R. J. N. (2022). A survey of Methylobacterium

species and strains reveals widespread production and varying profiles of cytokinin phytohormones. *BMC Microbiology*, *22*(1), 1–17. https://doi.org/10.1186/s12866-022-02454-9

Parkin, T. B., & Kaspar, T. C. (2006). Nitrous Oxide Emissions from Corn-Soybean Systems in the Midwest. *Journal of Environmental Quality*, *35*(4), 1496–1506. https://doi.org/10.2134/jeq2005.0183

Pasley, H., Nichols, V., Castellano, M., Baum, M., Kladivko, E., Helmers, M., & Archontoulis, S. (2021). Rotating maize reduces the risk and rate of nitrate leaching. *Environmental Research Letters*, *16*(6). https://doi.org/10.1088/1748-9326/abef8f

Paungfoo-Lonhienne, C., Wang, W., Yeoh, Y. K., & Halpin, N. (2017). Legume crop rotation suppressed nitrifying microbial community in a sugarcane cropping soil. *Scientific Reports*, *7*(1), 1–7. https://doi.org/10.1038/s41598-017-17080-z

Pervaiz, Z. H., Iqbal, J., Zhang, Q., Chen, D., Wei, H., & Saleem, M. (2020). Continuous Cropping Alters Multiple Biotic and Abiotic Indicators of Soil Health. *Soil Systems*, *4*(4), 59. https://doi.org/10.3390/soilsystems4040059

Preece, C., & Peñuelas, J. (2020). A Return to the Wild: Root Exudates and Food Security. *Trends in Plant Science*, *25*(1), 14–21. https://doi.org/10.1016/j.tplants.2019.09.010

Raglin, S. S., Soman, C., Ma, Y., & Kent, A. D. (2022). Long Term Influence of Fertility and Rotation on Soil Nitrification Potential and Nitrifier Communities. *Frontiers in Soil Science*, *2*(May). https://doi.org/10.3389/fsoil.2022.838497

Rocha, K. F., Kuramae, E. E., Borges, B. M. F., Leite, M. F. A., & Rosolem, C. A. (2020).

Microbial N-cycling gene abundance is affected by cover crop specie and development stage in an integrated cropping system. *Archives of Microbiology*, *202*(7), 2005–2012. https://doi.org/10.1007/s00203-020-01910-2

Rütting, T., Aronsson, H., & Delin, S. (2018). Efficient use of nitrogen in agriculture. *Nutrient Cycling in Agroecosystems*, *110*(1), 1–5. https://doi.org/10.1007/s10705-017-9900-8

Sahrawat, K. L. (2008). Factors affecting nitrification in soils. *Communications in Soil Science and Plant Analysis*, *39*(9–10), 1436–1446. https://doi.org/10.1080/00103620802004235

Sainju, U. M., Singh, B. P., & Whitehead, W. F. (2002). Long-term effects of tillage, cover crops, and nitrogen fertilization on organic carbon and nitrogen concentrations in sandy loam soils in Georgia, USA. *Soil and Tillage Research*, *63*(3–4), 167–179. https://doi.org/10.1016/S0167-1987(01)00244-6

Sakakibara, H. (2021). Cytokinin biosynthesis and transport for systemic nitrogen signaling. *Plant Journal*, *105*(2), 421–430. https://doi.org/10.1111/tpj.15011

Samaddar, S., Schmidt, R., Tautges, N. E., & Scow, K. (2021). Adding alfalfa to an annual crop rotation shifts the composition and functional responses of tomato rhizosphere microbial communities. *Applied Soil Ecology*, *167*(June), 104102. https://doi.org/10.1016/j.apsoil.2021.104102

Singh, G., Bhattacharyya, R., Das, T. K., Sharma, A. R., Ghosh, A., Das, S., & Jha, P. (2018). Crop rotation and residue management effects on soil enzyme activities, glomalin and aggregate stability under zero tillage in the Indo-Gangetic Plains. *Soil and Tillage Research*, *184*(July), 291–300. https://doi.org/10.1016/j.still.2018.08.006

Singh, J., & Kumar, S. (2021). Seasonal changes of soil carbon fractions and enzyme activities in response to winter cover crops under long-term rotation and tillage systems. *European Journal of Soil Science, 72*(2), 886–899. https://doi.org/10.1111/ejss.13028

Smil, V. (1983). Crop Residues. *Biomass Energies*, 163–236. https://doi.org/10.1007/978-1-4613-3691-4_4

Snider, D., Thompson, K., Wagner-Riddle, C., Spoelstra, J., & Dunfield, K. (2015). Molecular techniques and stable isotope ratios at natural abundance give complementary inferences about N2O production pathways in an agricultural soil following a rainfall event. *Soil Biology and Biochemistry*, *88*, 197–213. https://doi.org/10.1016/j.soilbio.2015.05.021

Studdert, G. A., & Echeverría, H. E. (2000). Crop Rotations and Nitrogen Fertilization to Manage Soil Organic Carbon Dynamics. *Soil Science Society of America Journal*, *64*(4), 1496–1503. https://doi.org/10.2136/sssaj2000.6441496x

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T., & Sakakibara, H. (2004). AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant and Cell Physiology*, *45*(8), 1053–1062. https://doi.org/10.1093/pcp/pch119

Tang, H. M., Xiao, X. P., Tang, W. G., Lin, Y. C., Wang, K., & Yang, G. L. (2014). Effects of winter cover crops residue returning on soil enzyme activities and soil microbial community in double-cropping rice fields. *PLoS ONE, 9*(6), 1–8. https://doi.org/10.1371/journal.pone.0100443

Tenuta, M., Amiro, B. D., Gao, X., Wagner-Riddle, C., & Gervais, M. (2019a). Agricultural management practices and environmental drivers of nitrous oxide emissions over a decade

for an annual and an annual-perennial crop rotation. *Agricultural and Forest Meteorology*, *276–277*(June), 107636. https://doi.org/10.1016/j.agrformet.2019.107636

Tenuta, M., Amiro, B. D., Gao, X., Wagner-Riddle, C., & Gervais, M. (2019b). Agricultural management practices and environmental drivers of nitrous oxide emissions over a decade for an annual and an annual-perennial crop rotation. *Agricultural and Forest Meteorology*, *276–277*(June), 107636. https://doi.org/10.1016/j.agrformet.2019.107636

Thapa, V. R., Ghimire, R., Acosta-Martínez, V., Marsalis, M. A., & Schipanski, M. E. (2021). Cover crop biomass and species composition affect soil microbial community structure and enzyme activities in semiarid cropping systems. *Applied Soil Ecology*, *157*(March 2020), 103735. https://doi.org/10.1016/j.apsoil.2020.103735

Thompson, K. A., Bent, E., Abalos, D., Wagner-Riddle, C., & Dunfield, K. E. (2016). Soil microbial communities as potential regulators of in situ N2O fluxes in annual and perennial cropping systems. *Soil Biology and Biochemistry*, *103*, 262–273. https://doi.org/10.1016/j.soilbio.2016.08.030

Thompson, Karen A., Deen, B., & Dunfield, K. E. (2016). Soil denitrifier community size changes with land use change to perennial bioenergy cropping systems. *SOIL*, *2*(4), 523–535. https://doi.org/10.5194/soil-2-523-2016

Timmusk, S., Nicander, B., Granhall, U., & Tillberg, E. (1999). Cytokinin production by Paenibacillus polymyxa. *Soil Biology and Biochemistry*, *31*(13), 1847–1852. https://doi.org/10.1016/S0038-0717(99)00113-3

Tortosa, G., Hidalgo, A., Salas, A., Bedmar, E. J., Mesa, S., & Delgado, M. J. (2015). Nitrate and flooding induce N2O emissions from soybean nodules. *Symbiosis*, *67*(1–3), 125–133.

https://doi.org/10.1007/s13199-015-0341-3

Trivedi, P., Delgado-Baquerizo, M., Jeffries, T. C., Trivedi, C., Anderson, I. C., Lai, K., McNee, M., Flower, K., Pal Singh, B., Minkey, D., & Singh, B. K. (2017). Soil aggregation and associated microbial communities modify the impact of agricultural management on carbon content. *Environmental Microbiology*, *19*(8), 3070–3086. https://doi.org/10.1111/1462-2920.13779

Turner, B. L., Hopkins, D. W., Haygarth, P. M., & Ostle, N. (2002). B-Glucosidase Activity in Pasture Soils. *Applied Soil Ecology*, *20*(2), 157–162. https://doi.org/10.1016/S0929-1393(02)00020-3

Vachon, K., & Oelbermann, M. (2011). Crop residue input and decomposition in a temperate maize-soybean intercrop system. *Soil Science*, *176*(4), 157–163. https://doi.org/10.1097/SS.0b013e3182104213

Venterea, R. T., Halvorson, A. D., Kitchen, N., Liebig, M. A., Cavigelli, M. A., Del Grosso, S. J., Motavalli, P. P., Nelson, K. A., Spokas, K. A., Singh, B. P., Stewart, C. E., Ranaivoson, A., Strock, J., & Collins, H. (2012). Challenges and opportunities for mitigating nitrous oxide emissions from fertilized cropping systems. *Frontiers in Ecology and the Environment*, *10*(10), 562–570. https://doi.org/10.1890/120062

Weil, R. R., Islam, K. R., Stine, M. A., Gruver, J. B., & Samson-Liebig, S. E. (2003). Estimating active carbon for soil quality assessment: A simplified method for laboratory and field use. *American Journal of Alternative Agriculture*, *18*(1), 3–17. https://doi.org/10.1079/AJAA2003003

Weisberger, D., Nichols, V., & Liebman, M. (2019). Does diversifying crop rotations suppress

weeds? A meta-analysis. *PLoS ONE*, *14*(7), 1–9. https://doi.org/10.1371/journal.pone.0219847

White, K. E., Brennan, E. B., Cavigelli, M. A., & Smith, R. F. (2020). Winter cover crops increase readily decomposable soil carbon, but compost drives total soil carbon during eight years of intensive, organic vegetable production in California. *PLoS ONE*, *15*(2), 1–26. https://doi.org/10.1371/journal.pone.0228677

Wood, S. A., & Bowman, M. (2021). Large-scale farmer-led experiment demonstrates positive impact of cover crops on multiple soil health indicators. *Nature Food*, *2*(2), 97–103. https://doi.org/10.1038/s43016-021-00222-y

Wu, W., Du, K., Kang, X., & Wei, H. (2021). The diverse roles of cytokinins in regulating leaf development. *Horticulture Research*, *8*(1). https://doi.org/10.1038/s41438-021-00558-3

Xue, D., Yao, H., & Huang, C. (2006). Microbial biomass, N mineralization and nitrification, enzyme activities, and microbial community diversity in tea orchard soils. *Plant and Soil*, *288*(1–2), 319–331. https://doi.org/10.1007/s11104-006-9123-2

Zhang, K., Maltais-Landry, G., & Liao, H. L. (2021). How soil biota regulate C cycling and soil C pools in diversified crop rotations. *Soil Biology and Biochemistry*, *156*. https://doi.org/10.1016/j.soilbio.2021.108219

Zhang, P., Sun, J., Li, L., Wang, X., Li, X., & Qu, J. (2019). Effect of soybean and maize rotation on soil microbial community structure. *Agronomy*, *9*(2), 1–11. https://doi.org/10.3390/agronomy9020042

Zuber, S. M., Behnke, G. D., Nafziger, E. D., & Villamil, M. B. (2018). Carbon and nitrogen

content of soil organic matter and microbial biomass under long-term crop rotation and tillage in Illinois, USA. *Agriculture (Switzerland)*, *8*(3). https://doi.org/10.3390/agriculture8030037

Figures and Tables

Table 3.1

Particle size distributions of sand (50µm-2 mm), silt (2µm-50µm), and clay (<2 µm) in fields under different crop phases cover crops (CC), soybean-winter wheat (SB-WW) and maize (MZ).

Crop phase	Soil particle size distributions
CC	Sandy loam soil (60.4-68.0% sand, 24.9-29.0% silt, 7.1-10.6 % clay)
CC	Sandy loam (65.4-70.1% sand, 21.4-26.0% silt, 7.5-8.8 % clay)
CC	Loam (29.9-42.0% sand, 39.6-46.6% silt, 17.6-25.3% clay)
SB-WW	Silt Loam (23.7-26.0% sand, 53.2-56.0% silt, 20.3-20.8% clay)
SB-WW	Sandy loam (59.0-61.2% sand, 27.0-27.6% silt, 11.8-13.3% clay)
SB-WW	Loam (37.4-44.4% sand, 36.3-40.5% silt, 18.6-22.5% clay)
SB-WW	Sandy loam (62.4-70.1% sand, 19.2-28.2% silt, 8.8-10.7% clay)
MZ	Loam (37.1-42.1% sand, 40.7-41.2% silt, 17.1-21.7% clay)
MZ	Loam (34.1-36.5% sand, 38.7-48.1% silt, 20.2-24.9% clay)
MZ	Loam (36.5-42.5% sand, 38.0-43.5% silt, 18.7-20.0% clay)

Table 3.2.

Comparisons of soil parameters among fields with different crop phases cover crops (CC), winter wheat (WW), and maize (MZ) in a MZ-(SB-WW)-CC rotation in an operational field in southern Ontario (November 2020). Parameters are pH, % wet stable aggregates (WAS), active carbon (AC) ppm, inorganic carbon (IC) %, nitrates (NO_3^-) ppm, and ammonium ions (NH_4^+) ppm, and extra cellular enzyme activities (EEA) of β-glucosidase (BG), N-acetylglucosaminidase (NAG),

phosphatase (PO) (µM hr/g dry soil). The comparisons were done using one-way ANOVA and the data are means (n=3) ± SE; parameters indicated as NS are not significantly different (LSD test, $p < 0.05$).

Crop phase	**pH** (NS)	**WSA**	**IC** (NS)	**AC**	**BG**	**NAG**	**PO**	**NO_3^-**	**NH_4^+** (NS)
CC	6.8 ±0.1	27.8^{b}±2.5	0.99 ±0.02	655^{a} ±39	0.08^{b} ±0.01	0.11^{a}±0.01	0.46^{b}±0.03	14.1^{b} ±1.4	4.6 ±0.3
SB-WW	6.5 ±0.2	24.5^{b} ±1.3	1.01 ±0.02	487^{b} ±37	0.10^{a} ±0.01	0.10^{b}±0.01	0.57^{b}±0.05	14.4^{b} ±1.7	4.3 ±0.3
MZ	6.7±0.1	33.5^{a} ±1.4	1.01 ±0.01	610^{a} ±20	0.08^{ab}±0.01	0.11^{a}±0.01	0.73^{a}±0.05	18.3^{a} ±1.5	4.3±0.2
Df	2	2	2	2	2	2	2	2	2
F-value	0.87	6.65	0.71	6.75	3.04	7.71	7.09	2.16	0.27

Table 3.3

Comparison of the abundances of soil microbial groups among different crop phases cover crops (CC), winter wheat (WW), and maize (MZ) in a corn-soybean-winter wheat-cover crop rotation in an operational field in southern Ontario (November 2020). Abundance was detected by (log10 copies per g dry soil) of total bacterial (*16S* rRNA), nitrifying (*amo*A), and denitrifying (*nir*S and *nir*K) gene abundances, and *amo*A:*16S* rRNA, *nir*S:*16S* rRNA, *nir*K:*16S* rRNA *and amo*A:*nir*K gene ratios The comparisons were done using one-way ANOVA and the data are means (n=3) ± SE; parameters indicated as NS are not significantly different (LSD test, $p < 0.05$).

Crop phase	***16S* rRNA** (NS)	***amo*A**	***nir*S** (NS)	***nir*K**	***amo*A: *16S* rRNA**	***nir*S: *16S* rRNA** (NS)	***nir*K: *16S* rRNA**	***amo*A: *nir*K**
CC	8.9±0.02	6.5^{b} ±0.1	7.5 ±0.2	7.6^{b} ±0.1	0.73^{b} ±0.01	0.82 ±0.01	0.85^{b} ±0.01	0.86^{a} ±0.01
SB-WW	8.9±0.02	6.7^{a} ±0.1	7.4 ±0.1	7.8^{b} ±0.1	0.75^{a} ±0.01	0.81 ±0.01	0.87^{b} ±0.01	0.87^{a} ±0.02
MZ	9.0±0.03	6.2^{c} ±0.0	7.2 ±0.2	8.2^{a} ±0.1	0.69^{c} ±0.01	0.80 ±0.02	0.91^{a} ±0.01	0.76^{b} ±0.01
Df	2	2	2	2	2	2	2	2
F-value	1.91	20.3	0.55	6.25	16.4	0.37	7.69	16.7

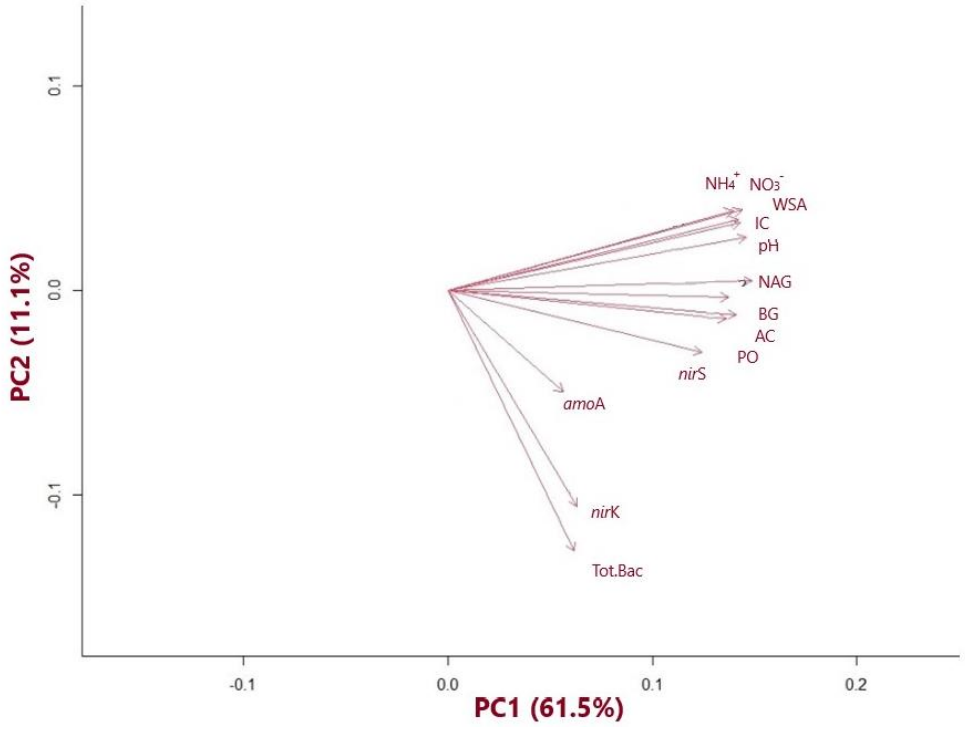
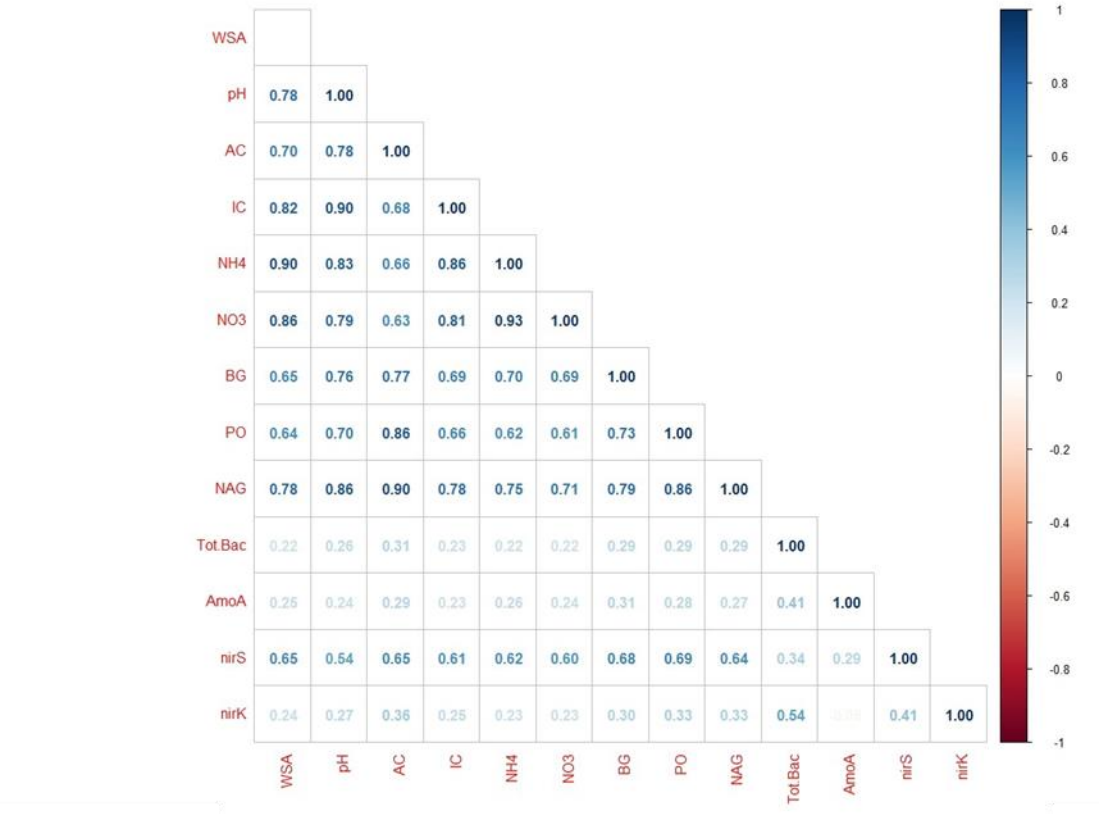


Fig. 3.1. A principal component analysis (PCA) of soil parameters taken from samples under the different crop phases maize (MZ), winter wheat (WW) and cover crops (CC) in a MZ-SB-WW-CC rotation in an operational field in southern Ontario (November 2020). Variables included were soil pH, wet stable aggregates (WSA), inorganic carbon (IC), nitrate ions (NO_3^-), ammonium ions (NH_4^+), and enzyme activities of β-glucosidase (BG), Phosphatase (PO), N-acetylglucosaminidase (NAG), total bacterial (*16Sr*RNA), ammonia oxidizing bacterial (*amo*A), nitrifying (*nir*S and *nir*K) gene abundances The first two principal components (PC1 and PC2) are shown.



Supplementary Fig. 3.1. Pearson correlation results that representing the correlations between soil health parameters- soil pH, wet stable aggregates (WSA), inorganic carbon (IC), nitrate ions (NO_3^-), ammonium ions (NH_4^+), and enzyme activities of β-glucosidase (BG), Phosphatase (PO), N-acetylglucosaminidase (NAG), and the total bacterial (*16Sr*RNA), ammonia oxidizing bacterial (*amo*A), nitrifying (*nir*S and *nir*K) gene abundances in soils samples under the different crop phases maize (MZ), winter wheat (WW) and cover crops (CC) in a MZ-(SB-WW)-CC rotation in an operational field in southern Ontario (November 2020).

Chapter 4: Cytokinin profiling as a new, dynamic indicator of soil health: assessment in cover cropped soils

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Abstract

The impact of agricultural management strategies on soil health is assessed using traditional physicochemical and biological analyses; however, the effect of agricultural management on soil hormone profiles, which are crucial factors in plant growth and development, is not well studied. In the current work, we investigated the impact of cover crops (CC) on soil health parameters such as soil pH, soil organic matter (SOM), soil labile or active carbon (AC), soil microbial community (SMC) diversity, microbial community level physiological profiling (CLPP), and extracellular enzyme activities (EEA) of β-glucosidase (BG), N-acetylglucosaminidase (NAG), and phosphatase (PO). In addition, the impact of CCs on soil cytokinins (CKs), which are essential chemical signals that promote crop growth and yield formation, was also explored. A greenhouse experiment was carried out by growing two CCs: tillage radish (*Raphanus sativus*) and cereal rye (*Secale cereale*) in custom-designed rhizo-pots to easily separate rhizosphere and bulk soils. The presence of CCs significantly increased SOM estimates, soil AC levels, SMC diversity and NAG activity compared to uncultivated, control soils. Furthermore, there were significant differences in AC, SMC diversity, carbon use as determined by CLPP, and NAG

activity between cover cropped rhizosphere and bulk soils. To discover the impact of CCs on soil CK profiles, CKs in soil were purified using solid phase extraction and analyzed using High-Resolution Mass Spectrometry (HRMS) in parallel reaction monitoring mode (PRM). We detected free base-CKs (FB-CKs - trans-zeatin (tZ), isopentenyladenine (iP)), riboside-CKs (RB-CKs -cis-zeatin riboside (cZR), isopentenyladenosine (iPR) and methylthiolated CKs (2MeS-CKs – 2-methylthio-Zeatin (MeSZ), 2-methylthio-Zeatin ribosides (MeSZR), 2-methylthio-isopentenyladenine (MeSiP), 2-methylthio-isopentenyladenine riboside (MeSiPR)) in the soil samples, and the observed CK levels considerably increased when CCs were grown compared to the control soil. This work presents the first comprehensive analysis of soil CK profiles affected by cover-cropping, a sustainable management strategy used to improve soil health and crop yield. Furthermore, the relationship between soil health parameters and soil CK profiles was also established. Namely, total CKs showed a strong correlation with AC and SMC diversity, suggesting the potential use of soil CK profiles as a novel tool in soil health assessment.

Keywords: Cover crops (CC), Cereal ryegrass, Tillage radish, Soil microbial communities (SMC), cytokinins (CK), soil organic carbon (SOC), active carbon (AC), extracellular enzyme activity (EEA)

Authors' Contribution: Imesha Perera: Formal analysis, Investigation, Writing – original draft, Writing - review & editing, Visualization. **Anna Kisiala**: Conceptualization, Methodology, Investigation,Writing - original draft, Writing - review & editing, Visualization. **Karen A. Thompson:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **R. J. Neil Emery:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition.

4.1 Introduction

Soil is a natural, non-renewable, multicomponent, and dynamic ecosystem. It is composed of inorganic matter (rock and mineral particles), organic matter (plant residues and microbial decomposed products), living organisms, water, and gas (Dazzi and Lo Papa, 2022; Pulleman et al., 2012). Soils are considered the largest reservoir of biodiversity on earth that acts as a substrate for plants and a wide range of soil-dwelling organisms such as microbes, nematodes, earthworms, and insects (Yang et al., 2018; reviewed in Doran and Zeiss, 2000). Soil-dwelling microorganisms are collectively described as soil microbial communities (SMC). They play an essential role in nutrient cycling in the ecosystem via the secretion of extracellular enzymes that are involved in the decomposition of soil organic matter (SOM), nutrient mineralization, nitrogen (N) fixation, N-transformation reactions, and others (Tregubova et al., 2021; Grzadziel et al., 2018; Stockdale and Watson, 2009; reviewed in Doran and Zeiss, 2000).

Soil is a principal feature in most ecosystems, including agricultural fields (reviewed in Doran and Zeiss, 2000; reviewed in Schoonover and Crim, 2015; Stockdale and Watson, 2009), and therefore, maintaining good soil health is crucial for the well-being of plants and animals, long term sustainability of agriculture, and a healthy environment (Grzadziel et al., 2018). Soil health can be defined as the ability of a soil to act as a living ecosystem to maintain environmental quality and the health of plants, animals, and humans (Bonfante et al., 2020; Lehmann et al., 2020; Doran and Zeiss, 2000; Doran and Parkin, 1994). It is assessed using a combined set of measurable physiochemical and biological attributes known as soil health parameters (Prabha et al., 2020). Examples of commonly characterized soil health parameters include soil texture, soil aggregation, bulk density and water holding capacity, as well as chemical parameters including soil pH, SOM, soil organic carbon (SOC) and active carbon (AC), nutrient levels, abundance and

diversity of SMC, or microbial extracellular enzyme activity (EEA) (Bonfante et al., 2020; Lehmann et al., 2020).

Soil health decline due to intensive agriculture is a major problem farmers face worldwide (OFA, 2021). Depletion of SOM increased soil erosion, and increased weeds and pests are common in agricultural fields. One of the recommended management strategies to improve soil health is the adoption of cover crops (CC) in cropping systems (Cover Crops Canada, 2018). Cover crops are living ground covers integrated into crop rotations during the main crop or fallow period. If not winter-killed or used for grazing or feed, CCs are often terminated using manual or chemical methods before the next main crop phase (Justes, 2017; Fageria et al., 2005; Hartwig and Ammon, 2002). Cover crop species provide a variety of ecosystem services, including soil protection from erosion, reduction of leaching, suppression of weeds and soil borne-pathogens, biomass production contributing to residue returns, and if legumes are included, increased atmospheric nitrogen (N_2) fixation (Belfry and Van Eerd, 2016). Integration of CCs in cash crop rotations results in enhanced soil health as illustrated by increases in SOM, SOC, total N content, AC, hydraulic conductivity, infiltration rates, and soil aggregation (Ghimire et al., 2019; Nouri et al., 2019; Zeynep et al., 2019; Chahal and Van Eerd, 2018). Soil health improvement by CC is of critical significance for plant growth promotion and increased crop yields (Belfry et al., 2017; Congreves et al., 2017).

Phytohormones are small signalling molecules involved in growth, development and other physiological processes in plants throughout their life cycle. They are key regulators that promote plant growth and yield formation (Smith et al., 2017; Jameson and Song, 2016). Originally termed as plant growth regulators, phytohormones are produced not only by living plants but are also found in soil inhabitants such as microorganisms, insects, nematodes, and

earthworms, among others (reviewed in Wong et al., 2015; reviewed in Stirk and van Staden, 2010). Plants and microorganisms can release phytohormones to their immediate environment, such as soil (reviewed in Wong et al., 2015; reviewed in Stirk and van Staden, 2010; Timmusk et al., 1999; Phillips and Torrey, 1972), which may have positive implications for soil health and plant growth. However, few studies have examined phytohormones as potentially important soil health parameters (Murofushi et al., 1983).

Cytokinins (CKs) are a potent group of phytohormones responsible for plant growth promotion and improvement of crop yields (High et al., 2019; Jameson and Song, 2016; Stirk and Staden, 2010). The chemical structure of CK forms contains an adenine derivative with a side chain at N^6 position. Based on the configuration of the isoprenoid side chain, CKs can be either active or serve as inactive precursors of conjugates. Free base CKs (FB-CKs), including isopentenyladenine (iP), trans-zeatin (tZ), cis-zeatin (cZ) and dihydrozeatin (DHZ) are the most biologically active CKs (Kisiala et al., 2019). The most common CK conjugates are glucosides (GLUC-CKs), ribosides (RB-CKs), and nucleotides (NT-CKs). Another, less known CK group, the methylthiolated CKs (2MeS-CKs), are derivatives of Z and iP with a thiol group (–SH) at position 2 of the adenine ring and are derived through the tRNA degradation pathway (reviewed in Gibb et al., 2020).

In plants, CKs are involved in the regulation of a wide spectrum of developmental processes, including shoot and root growth, control of shoot apical dominance and branching, leaf expansion, development of chloroplasts and chlorophyll production, delaying of leaf senescence, and reproductive development (Kieber and Schaller 2014; reviewed in Stirk and Staden 2010). Furthermore, CKs mediate plant responses to abiotic environmental factors such as nutrient availability, osmotic potential, heat, salinity, and drought (reviewed by Hai et al., 2020; Jorge et

al., 2019; Pavlů et al., 2018) in addition to improving plant immunity against pathogen infections (Gupta et al., 2020).

Cytokinins are evolutionarily conserved among distinct groups of organisms, and their presence has been detected in representatives of all kingdoms of life (Aoki et al., 2019; Spíchal, 2012). A wide range of studies have reported the ability of living organisms to release endogenously produced CKs to the outside environment (Palberg et al., 2022; Bean et al., 2021; Kisiala et al., 2013; reviewed in Stirk and van Staden, 2010). Plants can release significant levels of CKs into their growth environment. Plant roots are highly extrusive sites of CK biosynthesis, and the high surface area of the root system facilitates the exchange of matter between plants and soil (Stirk and van Staden, 2010). Furthermore, the presence of active CKs has been reported in *in vitro* cultures of diverse microorganisms. Kisiala et al. (2013) identified the ability of N_2-fixing, symbiotic rhizobia strains to produce 25 forms of CKs, including bioactive forms. Early studies also reported the ability of *Rhizobium japonicum* and *Rhizobium leguminosarum* to release zeatin (Z)-like CKs (Phillips and Torrey, 1972), and the Rhizobacterium *Paenibacillus polymyxa* to release iP-type CKs (Timmusk et al., 1999). Other potential sources of CKs in soil are decomposing plant matter and other CK-producing, soil-dwelling organisms such as earthworms, nematodes and insects (Andreas et al., 2020; Wong et al., 2015; Stirk and van Staden, 2010).

Despite the extensive knowledge of the occurrence of CK production by non-plant organisms, their biological roles and potential benefits are largely undefined in organisms outside of the plant kingdom. However, CKs have ample opportunity to feedback and impact plant or non-plant organisms alike once they are in the rhizosphere. Studies on soil CK profiles are limited due to the poor understanding of the potential role of soil CK functioning in the soil ecosystem. To date, very few studies have reported on the occurrence of CKs in soil. Exceptions include High et al.

(2019), who identified iP and tZ-type CKs in soils and described the positive impact of earthworms on soil CK content. Furthermore, in an early study, Van Staden and Dimalla (1976) showed the combined effect of living roots and rhizosphere microorganisms, including *Rhizobium* sp. and mycorrhizal fungi (*Lycoperdon* sp. and *Scleroderma* sp.), to enhance levels of Z and zeatin riboside (ZR)-like activity in soil using an indirect bioassay, where they used soybean callus to check the CK-like activity in soil samples.

In the current study, we carried out a greenhouse experiment with two non-leguminous CC species: cereal rye (*Secale cereale* L.) and tillage radish (*Raphanus sativus* L. var. *longipinnatus*), to identify the impact of CCs on soil CK profiles and to study the relationships between established soil health parameters (pH, SOM, AC, microbial EEAs, soil microbial community-level physiological profiling (CLPP), SMC diversity) and soil CK forms and levels. The soil health parameters and soil CK profiles were assessed in two soil zones (rhizosphere and bulk soil) and compared between CC species. We purified soil CKs using solid phase extraction and successfully identified and quantified picomolar levels of FB-CKs (iP, tZ), RB-CKs (cZR, iPR), and 2MeS-CKs (2MeSZR, 2MeSiPR) present in the soil matrix using High-Resolution Accurate-Mass QExactive Orbitrap tandem mass spectrometry. As such, this study presents the most comprehensive analysis of CK profiles in soil. Our study revealed that CCs improve soil health parameters, such as SOM, AC, SMC diversity, and EEAs, while at the same time, they impact soil CK profiles. Furthermore, soil CK levels were positively correlated with select soil health parameters, such as AC, SMC diversity, and EEAs, demonstrating that characterization of soil CK profiles may be a useful potential new soil health indicator in sustainable agriculture.

4.2. Material and Methods

4.2.1. Soil and plant materials

Soils were collected for the greenhouse pot experiment from two different locations at the Trent Experimental Farm (TEF) in Peterborough, Ontario, Canada (44°21'45.9"N 78°16'55.1"W). According to the particle-size distribution of clay, silt and sand measured with the hydrometer method using a standard buoyancy hydrometer, the two soils had two different soil textures: loam (L) (12% clay, 48% silt, 40% sand), and sandy loam (SL) (12% clay, 24% silt, 64% sand). Soils were air-dried and passed through a 2-mm sieve to remove stones and plant debris before potting.

Two CC species were used in the experiment: tillage radish (*Raphanus sativus* L. var. *longipinnatus*) and cereal rye (*Secale cereale* L.). These are non-leguminous CCs that fall under the category of *Brassica* sp. (tillage radish) and grass sp. (cereal rye). These two CC types have different root structures, whereby tillage radish has a tuber with a long tap root, and cereal rye has a shallow fibrous root system.

4.2.2. Greenhouse pot experiment

The greenhouse trial was established at Trent University to analyze the impacts of the two CCs on soil health parameters and soil CK levels in rhizosphere and bulk (external to the rhizosphere) soils separately. Customized rhizo-pots were designed to easily separate rhizosphere soil and bulk soil at the time of plant harvest. A single pot was comprised of a small pot called the ‘rhizo-pot’ that was built using geotextile fabric to allow movement of water, nutrients, and microbes between soil zones while restricting root growth from the rhizosphere zone to the bulk zone. The rhizo-pots were placed into the larger, ‘outer pots’ **(Fig. 4.1)**. Combined pots were filled with soil and placed in the greenhouse in a randomized block design. Eight pots were filled with L

soil and used to tillage radish (4 pots) and cereal rye (4 pots) (**Table 4.1**). Only 3 pots of tillage radish were grown in SL soil due to limitations in the amount of soil collected during the previous Fall. After emergence, tillage radish seedlings were thinned to 4 plants per pot, and cereal rye seedlings were thinned to 6 plants per pot. Plants were watered daily with tap water and harvested after three months. Following harvest, soil zones were collected separately and stored at -20^{o}C until analysis.

4.2.3. Analysis of soil health characteristics

4.2.3.1. Soil pH and soil carbon analysis

For soil pH, soil solutions were prepared by mixing soil and distilled water in a ratio of 1:2, and the measurements were taken with a pre-calibrated pH meter (Mettler-Toledo) (Hendershot et al. 2008). The SOM content was measured in 5 technical replicates of soils (5g) from each soil zone/CC by loss on ignition (LOI) at 550^{o}C for 4h in a muffle furnace. Masses of the soil before and after ignition were recorded, and the percentage difference in weight was taken as the SOM content [%]. The AC, the labile fraction of SOC which is readily oxidized by SMC, was assessed using the $KMnO_4$ method (Weil et al., 2003). The $KMnO_4$ oxidizes simple carbohydrates, amino acids, amines and amides, and C compounds with a hydroxyl group (C-OH), ketone (R2C=O), carboxyl (-COOH), and aliphatic compounds, which are the preferred substrates of SMC. The absorbance readings of C oxidized by $KMnO_4$ were obtained at 550 nm with an Epoch™ microplate spectrophotometer (BioTek Instruments, Inc., Germany). A standard curve of $KMnO_4$ with a dilution series with the known concentrations (0.05M, 0.01M, 0.015M and 0.02M) was prepared and plated on the same microplate to determine the AC concentrations in unknown samples.

4.2.3.2. Community-level physiological profiling (CLPP) and SMC diversity

To assess the patterns of C source utilization by SMCs in rhizosphere and bulk soils grown under each of the CCs/soil types, community-level physiological profiling (CLPP) was carried out (Weber and Legge, 2010). Biolog EcoPlates™- 96 well plates consisting of 31 different C sources in triplicate with a blank (water) in triplicate were used for the assay. The assay was conducted by inoculating each well with 150 µL aliquot of the soil solution (10 g of pre-incubated soil in 90 mL of 0.85% NaCl solution) and incubating the plates in the dark at room temperature (20^{o}C). The absorbance readings were obtained with an Epoch™ microplate spectrophotometer (BioTek Instruments, Inc., Germany) at 590 nm every 24 h for 168 h. Since maximum absorbance readings (OD_{590}) were obtained at 168 h, a single time point absorbance at 168h was used in the data analysis. In addition, the absorbance readings obtained by CLPP (at 168h) were used to calculate the SMC diversity using the Shannon diversity index.

4.2.3.3. Microbial extracellular enzyme activities (EEAs)

The microbial EEA: β-glucosidase (BG), phosphatase (PO), and N-acetylglucosaminidase (NAG), were determined in rhizosphere and bulk soils under CCs (Jackson et al., 2013). The assays were conducted by incubating 150 µL of soil solutions (5 g of soil in 5 mL of 50 mM acetate buffer) at room temperature (20^{o}C) with 150 µL of the specific substrates: *p*NP-β-D-glucopyranoside (5mM, 1h), *p*NP-β-N-acetylglucosaminide (2mM, 2h), and *p*NP-phosphate (5 mM, 1h), respectively for the enzymes: BG, NAG, and PO, followed by absorbance readings using an Epoch™ microplate spectrophotometer (BioTek Instruments, Inc., Germany) at 410 nm and the unknown EEA were calculated using standard curves prepared for each enzyme target.

4.2.4. Analysis of soil CK profiles

The CK analyses were conducted to determine the diversity and abundance of soil CK profiles in the rhizosphere and bulk soils under each CC/soil type. Thirty-two CK forms were investigated, and any individual CKs detected were assessed separately and as part of their functional group categories (free base CKs (FB-CKs), riboside CKs (RB-CKs), and methylthiolated CKs (2MeS-CKs)) and also grouped together as soil total CKs (which adds together all active CKs with inactive precursors or conjugates). Prior to the analysis, dry soil samples (1.5 g) were suspended in ice-cold 50% acetonitrile (ACN) solution, spiked with the isotopically labelled internal CK standards (10 ng): [$^2H_5$]ZR, [$^2H_3$]DZR, [$^2H_6$] iPR, [$^2H_3$]DZ, [$^2H_6$]iP, [$^2H_6$]2MeSZ, [$^2H_6$]2MeSZR, [$^2H_6$]2MeSiP and [$^2H_6$]2MeSiPR (OlChemIm Ltd., Olomouc, Czech Republic), and ground using a Retsch MM400 ball mill (Retsch, Haan, Germany) with two zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada). The homogenized soil samples were purified using HLB cartridges (Canadian Life Sciences, Peterborough, Canada) according to a modified, previously published protocol (Šimura et al., 2018). Quantification of CKs was carried out in Parallel Reaction Monitoring (PRM) mode using Orbitrap QExactive - High-Resolution Mass Spectrometry (HRMS) (Thermo Scientific, San Jose, USA) coupled with Dionex Ultimate3000 High-Performance Liquid Chromatography system (Kisiala et al., 2019). Obtained phytohormone data were quantified using Xcalibur 3.0.63 software (Thermo Scientific, San Jose, USA) using the isotope dilution method based on the recovery of endogenous compounds and recovery of internal standards.

4.2.5. Statistical analysis

All the statistical analyses were conducted using the R Studio software (R Studio: Integrated Development Environment for R. R Studio, PBC, Boston, MA). Soil health parameters and CK

content were compared between the two non-plant control soil types (L and SL soils) collected from TEF using student t-tests. One-way analysis of variance (ANOVA) was conducted to test for differences in soil CK levels, soil health parameters (pH, SOM, AC, EEA), and SMC diversity between control soil and cover cropped soils (L soils – TR, TB, CR, CB; SL soils - TR and TB) (**Supplementary Table 4.1**) with a generalized linear model. Mean separation between control soil and cover cropped soils was determined using the Fisher's Least Significant Difference test (LSD), with significance declared at $p \leq 0.05$. Data normality was checked using a Shapiro-Wilks test, and homoscedastic assumptions were checked using Bartlett's test. Significant differences among and between least-square means and two-sample t-tests were determined by p-values, with Ho rejected at $p<0.05$ unless otherwise stated.

For CLPP, the data were corrected prior to statistical analysis by subtracting OD values of blanks from the mean OD value of C substrates, and the negative values were set to zero (Feigl et al., 2017). Next, the data were normalized by dividing OD values by average well colour development (AWCD) within a single Ecoplate. Normalized data at one time-point (168 h) were used to determine the similarities and differences in SMC C-source utilization patterns and SMC diversity between soil zones and between CCs and soil types. Since the number of variables (P= 31 different C sources, **Supplementary Table 4.2)** were greater than the number of samples (N= 22 soil samples) (P>N), we conducted a sparse principal component analysis (sPCA), which reduces the dimensionality of the data set by adding sparsity to the input variables, to explain the variance among the many variables' relationships and to build a 2-dimensional plot for visual cluster analyses using "sparse principal component analysis HJ biplot" package in R software. Furthermore, the CLPP data were used to calculate SMC diversity using the Shannon-Weaver diversity index in R Studio software with the package "vegan."

To visually demonstrate the multiple relationships of soil health parameters and the CK profiles between CC type and soil zones in cover cropped L soils, a PCA analysis was carried out. The matrix used for the PCA analysis consisted of a total of 16 data points/samples (N) which included cover cropped L soil samples (N= L soil: TR (4) + TB (4) + CR (4) + CB (4)), and 6 variables (P) (P = AC + SMC diversity + NAG activity + BG activity + PO activity + Total CK). In the PCA, a scree plot was examined for breaks, and PC components with eigen values ≥ 1 (PC1 and PC2) were retained for the build-up of the 2-dimensional plot for visual analysis.

4.3. Results

4.3.1. Analysis of soil health characteristics in control soil

The SL control soil had significantly higher SOM and AC contents compared to L control soil **(Table 4.1)**. No significant differences were observed in soil pH, SMC diversity and total soil CK levels between the two control soil types **(Table 4.1)**.

4.3.2. Analysis of soil health characteristics in cover cropped soil

4.3.2.1. Soil pH and soil carbon

No significant differences in pH were observed between the cover cropped soils and both control soil types (data not shown). Compared to control soil, significantly higher SOM levels were observed only in L-CR soils. No differences in SOM were found between SL cover cropped soils and the respective control soil **(Fig. 4.2A)**. Cover cropped L soils had higher levels of AC compared to control soils, while SL cover cropped soils had higher AC levels than control soils only in the rhizosphere zone ($P<0.05$) **(Fig. 4.2B).** In both L and SL cover cropped soils, AC levels were significantly higher in rhizosphere zones compared to their paired respective bulk soils **(Fig. 4.2B)**.

4.3.2.2. Diversity of Soil Microbial Community (SMC) and Community Level Physiological Profiling (CLPP)

In sandy loam soils, the diversity of C-source utilizing SMC was significantly different in TR compared to control soil, while no significant difference was observed in any type of cover cropped soils compared to the control in L soils **(Fig. 4.3)**.

For the sparse principal component analysis (sPCA) which was carried out to visualize the C-source utilization ability of SMC, CLPP data points of both soil zones of L soil under tillage radish (n=4 (TR) + 4 (TB)) and cereal rye (n= 4 (CR) + 4 (CB)), SL under tillage radish (n=3 (TR) + 3 (TB)), and total soil samples (L, n =16 + SL, n= 6) were taken to create 3 matrixes (P × n) separately that represent utilization of 31 C sources in cover cropped L soil (31 × 16), SL soil (31 × 6), and total soil samples (31 × 22), and generated 3 sPCA biplots. After visualization of the sPCA biplots, SL cover cropped soils separated out on the biplot (**Fig. 4.4**). The PC1 axis accounted for 33.3 % of the dataset variance demonstrated mainly the SMC utilization of polymers (P2 and P3), carboxylic acids (CA4, CA5, CA6, CA7 and CA8), amino acids (AA1, AA2, AA3, AA4, AA5), and all amines and amides (A1 and A2) were significant loading factors on PC1, while PC2 axis accounted for 9.49% of the dataset variance, and SMC utilization of different carbohydrate types (C1, C2, C3, C4, C6, C7, C9, C10; **Suppl. Table 4.2**, **Fig. 4.4**) were significant loading factors.

4.3.2.3. Microbial Extracellular Enzyme activity (EEA)

In L soils, NAG activity was significantly different between the two CCs (LSD test, $p < 0.05$). The highest NAG activity was found in bulk soil under cereal rye **(Table 4.2)**. No significant differences were observed in BG and PO activities among any type of cover cropped L soils, and no significant differences in any EEAs were found among cover cropped SL soils **(Table 4.2).**

4.3.3. Soil cytokinin (CK) profiles

The presence of both CCs significantly increased total CK levels compared to control soils in both rhizosphere and bulk zones of L and SL soils **(Fig. 4.5)**. Rhizosphere soil under tillage radish had significantly higher levels of total CKs compared to their bulk soil counterpart **(Fig. 4.5).** Among 32 CK types scanned for on the LC-MS, 4 CK types were detected in L soils while 8 CK types were detected in SL soils (**Fig. 4.6 A-H**). Free-base tZ and iP CKs were detected only in SL soils. Levels of iP were 10-fold higher in SL-TR soil compared to SL control soils, and in rhizosphere soils, it was even more dramatically elevated (27.7-fold over control soil) (**Fig. 4.6 A and 4.6 B**). Furthermore, the most active CK form, tZ, was found only in SL-TR, and no tZ was detected in SL-TB or SL control soils (**Fig. 4.6 A**).

Riboside CKs (iPR and cZR) were consistently present in both soil types (L and SL). Notably, cZR was the most abundant CK form detected in this study. Both cZR and iPR were significantly elevated in cover cropped soils compared to control soils (**Fig. 4.6 C** and **D**). The higher levels of CK-RBs in cover cropped soils were considerably different between two soil types (L and SL), where they showed over 5-fold higher levels in SL soils compared L soil samples (**Fig. 4.6 C** and **4.6 D**).

Four types of MeS-CKs (MeSZ, MeSZR, MeSiP, and MeSiPR) were found in SL soils, and two types (MeSZR and MeSiPR) in L soils (**Fig. 4.6 E-H**). In general, profiles of MeS-CKs resembled those of their CK-FB and CK-RB conjugates both in patterns and abundance. In tillage radish SL soils, MeSZ and MeSZR levels were significantly different between soil zones, where rhizosphere soils had higher MeSZ and MeSZR levels compared to their bulk soils (**Fig. 4.6 E and G**). In cereal rye L soils, no MeSZ was detected, while MeSZR levels were not significantly different between soil zones. Similar to the patterns observed for CK-FBs and CK-

RBs, MeSiP was found only in SL soils, while MeSiPR was detected in both soil types (**Fig 4.6 F** and **H**). When detected, MeS-CKs levels were significantly higher in both soil zones compared to the control soils.

4.3.4. Relationship between AC, SMC diversity, EEA, and total CKs in cover cropped soils

A PCA was conducted to visualize the multivariate relationships among typically measured soil health parameters (SMC diversity, AC content, EEA) and total CK levels in different soil types/zones/CC (**Fig. 4.7**). The first two principal components, PC1 and PC2 accounted for 87.27% cumulative variance. Active carbon content and SMC diversity loaded on PC1 strongly correlated with total CK abundance, while EEAs loaded on PC2.

4.4 Discussion

Cover cropping is a management strategy used to protect soil from erosion and improve soil health during the fallow period. Cover cropping is directly linked with the increase in SOM levels, and it can be used as a management strategy to restore SOC lost from agricultural lands (Kaspar and Singer, 2015; Olson et al., 2010; Lal, 2004). Rehabilitation of SOM improves soil health via retaining water and nutrients, reducing soil erosion, improving soil structure, facilitating good drainage and aeration in soil, and improving crop yields (Oldfield et al., 2019; Musinguzi et al., 2015; Olson et al., 2010; Lal, 2006). In our study, a significant increase in estimated SOM levels were observed in the CR samples collected from the loam (L) soil compared to control soil (**Fig. 2A**). Moreover, we found significantly higher levels of AC in each type of cover cropped L soils as well as in the TR samples collected from the sandy loam (SL) soil (**Fig. 2B**), revealing the positive impact of living roots on soil health. Furthermore, our results clearly indicate the positive influence of the proximity of living roots on AC levels, as we observed higher levels of AC in the rhizosphere compared to the bulk soils (**Fig. 2B**). This is

likely reflective of increased C inputs from rhizodeposition and root exudation (Mutegi et al., 2011).

Active carbon, which is the labile fraction of SOC, represents substrates for SMC, and is one of the major limiting factors for SMC growth (Hargreaves and Hofmockel, 2014). Therefore, C added via living roots improves substrate availability for the growth of SMC (Hargreaves and Hofmockel, 2014). The increasing trend in SMC diversity **(Fig. 4.3 C)**, observed in the rhizosphere soils compared to the bulk soils, is most likely due to higher inputs of plant-derived C in rhizosphere soil. The composition, quantity and quality of C entering the soil via root exudates and plant detritus matter depend on the plant species, plant productivity and fluctuation in abiotic factors; in turn, the quality and quantity of these inputs impact the diversity, composition, and abundance of SMC (Hooper et al., 2000). Although previous studies reported changes in SMC diversity with plant species, no significant variation in SMC diversity was observed between the soils under the two CC species in our work (**Fig. 4.3**). Heterotrophic SMC largely acquire their energy through the decomposition of organic material, which involves the conversion of complex organic matter to plant available nutrients via the microbial EEAs, which therefore play a major role in the cycling of C through the ecosystem (Six et al., 2006). Therefore, the utilization of C-sources by SMCs represents a critical process in the cycling of C in the ecosystem (Lladó and Baldrian, 2017). CLPP analyses revealed the functioning of SMC in the ecosystem based on the utilization of substrate (C sources) (Lladó and Baldrian, 2017). Our CLPP data showed a divergent trend in C-source utilization of SMC between soil zones, reflecting the impact of living roots and their associated root exudates on the metabolic functioning of SMCs in the rhizosphere **(Fig. 4.4)**.

Microbial EEAs are an important soil health parameter that reflect the functioning of the soil microbiome. Microbial EEAs are responsible for the degradation of complex SOC to simple C compounds, which heterotrophic SMCs use to fulfill their energy needs. The type and the quantity of enzymes released by SMC depend on the composition and the abundance of substrates available in the environment (Six et al., 2006). In our study, the observed variation of NAG activity between CC types indicates that different crops may affect the composition and abundance of C-sources available for microbial degradation. Overall, the analysis of the traditional soil health parameters in our study provided further evidence of the important role of living roots in improving soil health.

Plants can alter their environment via the secretion of root exudates (Huang et al., 2014). Root exudates are rich in C-based organic compounds, including amino acids, organic acids, sugars, phenolics, proteins, and secondary metabolites, as well as inorganic ions, inorganic acids, water, and oxygen (Reviewed in Badri and Vivanco, 2009). In addition to these compounds, a few early studies suggested the presence of CK phytohormones (FB-CKs (iP, cZ, tZ), RB-CKs (cZR, tZR), and conjugated-tZ) in rice root exudates (Soejima et al., 1992; Murofushi et al., 1983). This provided the compelling suggestion that plants can release endogenous CKs to the outside rhizosphere environment. Aligning with this claim, we detected significantly higher levels of total CKs in both zones of soils grown under both tillage radish and cereal rye compared to the control soils (**Fig. 4.5**). Furthermore, CK-profiles were higher in rhizosphere soils compared to bulk soils, emphasizing the fact that living roots, and perhaps associated microorganisms that live around them, are probable sources of CKs in soil.

A wide range of previous studies reported the presence of FB-CKs and RB-CKs in the roots of plants such as beans (*Phaseolus vulgaris*) (Allee and Republic, 1981), pea (*Pisum sativum* L.)

(Short and Torrey, 2000), maize (*Zea mays* L.) (Zalabák et al., 2014; Takei et al., 2001), wheat (*Triticum aestivum* L.) (Kudoyarova et al., 2014), soybean (*Glycine max*) (Prudent et al., 2016), ryegrass (*Lolium multifolorum*) (Guo et al., 2020; Wang et al., 2012), tomato (Glanz-Idan et al., 2020), potatoes (*Solanum tuberosum* L.) (Raspor et al., 2020), and in other plant parts such as seeds of annual ryegrass (*Lolium rigidum*) (Goggin et al., 2015) and kernels of barley (*Hordeum vulgare* L.) (Powell et al., 2013). Hewett and Wareing (1973) showed the presence of high CK levels in senescing leaves, emphasizing the ability of degrading plant matter to secrete CKs into soil matrix. Although previous studies have detected only FB- CKs (iP, tZ,cZ), RB-CKs (cZR, tZR, iPR) and conjugated Z in root exudates (Soejima et al., 1992; Murofushi et al., 1983), the majority of work that found CKs in roots have suggested roots secrete CKs accumulated and synthesized inside the roots to the soil environment via root exudation. The evidence indicates that plants may significantly contribute to pools of CKs in soil. These findings also support the claim that at least some of the CKs detected in our work, namely tZ, iP, cZR and iPR, are likely of plant origin. In our study, 2MeS-CKs were among the most predominant forms of CKs in the soil samples **(Fig 4.7)**. It is possible that the 2MeS-CKs detected in rhizosphere soils could be of microbial origin. The methylthiolated CKs are thought to be synthesized in microorganisms in higher abundances compared to other life forms, including protists, bacteria, plants (Jorge et al., 2019), mammals and other animals (Reviewed in Gibb et al., 2020). The rhizosphere is rich in the abundance and diversity of SMCs (estimated at 10^{11} microbial cells/g of plant roots) and is even considered by some to represent the plants' second genome (Berendsen et al., 2012; Egamberdieva et al., 2008).

While the classical measures of soil health (SOM, AC, SMC diversity) responded to cover cropping in our trials as predicted, soil CK profiles showed more dynamic and indicative

changes among all conditions and situations for which greater soil health would be expected. This perhaps reflects the combined contributions of both plants and SMCs to overall soil health. For example, the cover-cropped soils had considerably higher CK levels compared to control soils. This may reflect a useful "half-life" of CK existence in soil whereby they are produced by living organisms in healthy soil but rapidly disappear, under deteriorating soils, when a few remaining organisms utilize remaining CKs whilst they stop producing them. The short persistence of CKs in soil may be explained, at least partially, because of the energy and nutrient-rich nature of CK molecules, which microbes could quickly take up and use for growth or in the purine salvage pathway (Ashihara et al., 2018). As such, the expected duration of soil CKs would be relatively short and partially rely on the presence of additional organisms associated with plants that are capable of producing and releasing new CKs to the soil (e.g., bacteria, fungi, archaea, protists, nematodes, insects) (Andreas et al., 2020; Kisiala et al., 2013).

Beyond control soils, even more subtle distinctions were observed among treatments involving CCs. For example, rhizosphere zones generally had higher CK levels than bulk soil zones for both tillage radish and cereal rye. Known as the most active CK, tZ, was only detected in TR, while another active FB-CK form, iP, was more widely detected and correlated very strongly in the order of TR > TB > control soil. Riboside-CK profiles can be used as a proxy for what is happening with more active FB-CKs since they are often detected in higher abundances – yet their activity level is still a matter of debate (Nguyen et al., 2021). According to cZR and iPR detected in our work, they were more commonly found in the analyzed samples and more variable among CCs and soil zones (**Fig. 4.6C and 4.6D**). As such, they are potentially a strong barometer for soil health. In a similar manner, 2MeS-CKs were consistently detected at relatively high levels in soils **(Fig. 4.6 E-H)**. The challenge with 2MeS-CKs is that their biological

functions in plants or any other microorganisms are still largely unknown (Gibb et al 2020). However, to a large extent, 2MeS-CKs seemed to mirror FB-CKs and RB-CKs profiles and fluctuate in correspondence with soil health.

As CKs are one of the critical growth promoting and yield enhancing agents, our CK results create a firm starting point for the potential use of soil CK profiles as new, sensitive parameters to assess the impact of agricultural management strategies on soil health. The positive associations observed in the conducted PCA analysis indicate strong connections between soil total CK profiles and AC and SMC diversity **(Fig. 4.7)**, which further strengthens the aforementioned claim.

Single or combined measures of different CK forms should be further explored to develop CKs as a new novel soil health parameter for enabling accurate assessments of the impact of agricultural management strategies on soil health. One such powerful new tool could be electrochemical sensors for CK detection, which could be adapted from the currently available approaches (Kim and Lee, 2022) to measurements of liquid soil suspension. This would allow for real-time, non-destructive CK measurements for sensitive and rapid characterization of soil health.

4.5. Conclusions

Tillage radish and cereal rye cover crops resulted in a significant increase in soil health parameters, including SOM, AC, and SMC diversity, compared to soils without cover crops. In addition to evaluating these parameters, we purified CKs from the complex soil matrix using solid phase extraction and quantified them using HRMS (PRM), which allowed identification with high specificity and sensitivity of over 30 CK forms. We observed strong associations

between soil health indicators and total CKs (tZ, iP, cZR, iPR, MeSZ, MeSZR, MeSiP, MeSiPR). The assessed soil CK profiles increased significantly in the cover cropped soils, suggesting the potential application of CK analysis as a tool to evaluate soil health as influenced by agricultural management strategies. Interestingly, total soil CK profiles showed a positive correlation with AC and SMC diversity, further supporting our hypothesis. This is the first study reporting comprehensive soil CK analysis and the potential role of soil CK profiles as a novel, reliable soil health parameter.

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4.6 Bibliography

Aoki, M. M., Seegobin, M., Kisiala, A., Noble, A., Brunetti, C., & Emery, R. J. N. (2019). Phytohormone metabolism in human cells: Cytokinins are taken up and interconverted in HeLa cell culture. *FASEB BioAdvances*, *1*(5), 320–331. https://doi.org/10.1096/fba.2018-00032

Andreas, P., Kisiala, A., Neil Emery, R. J., De Clerck-Floate, R., Tooker, J. F., Price, P. W., Miller, D. G., Chen, M. S., & Connor, E. F. (2020). Cytokinins are abundant and widespread among insect species. *Plants*, *9*(2). https://doi.org/10.3390/plants9020208

Ashihara, H., Stasolla, C., Fujimura, T., and Crozier, A. (2018). Purine salvage in plants. Phytochemistry, 147, 89–124. https://doi.org/10.1016/j.phytochem.2017.12.008

Badri, D. V., & Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant, Cell and Environment*, *32*(6), 666–681. https://doi.org/10.1111/j.1365-3040.2009.01926.x

Bean, K. M., Kisiala, A. B., Morrison, E. N., and Emery, R. J. N. (2021). Trichoderma Synthesizes Cytokinins and Alters Cytokinin Dynamics of Inoculated Arabidopsis Seedlings. *Journal of Plant Growth Regulation*, *0123456789*. https://doi.org/10.1007/s00344-021-10466-4

Belfry, K. D., Trueman, C., Vyn, R. J., Loewen, S. A., and Van Eerd, L. L. (2017). Winter cover crops on processing tomato yield, quality, pest pressure, nitrogen availability, and profit margins. *PLoS ONE*, *12*(7), 1–17. https://doi.org/10.1371/journal.pone.0180500

Berendsen, R. L., Pieterse, C. M. J., & Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science*, *17*(8), 478–486. https://doi.org/10.1016/j.tplants.2012.04.001

Bonfante, A., Basile, A., and Bouma, J. (2020). Targeting the soil quality and soil health concepts when aiming for the United Nations Sustainable Development Goals and the EU Green Deal. *Soil*, *6*(2), 453–466. https://doi.org/10.5194/soil-6-453-2020

Chahal, I., & Van Eerd, L. L. (2020). Cover crop and crop residue removal effects on temporal dynamics of soil carbon and nitrogen in a temperate, humid climate. *PLoS ONE*, *15*(7), 1–21. https://doi.org/10.1371/journal.pone.0235665

Congreves, K. A., Hayes, A., Verhallen, E. A., & Van Eerd, L. L. (2015). Long-term impact of tillage and crop rotation on soil health at four temperate agroecosystems. *Soil and Tillage Research*, *152*, 17–28. https://doi.org/10.1016/j.still.2015.03.012

Cover crops Canada. (2018). Building soil health and increasing yields with cover crops. https://covercrops.ca/

Dazzi, C., & Lo Papa, G. (2022). A new definition of soil to promote soil awareness, sustainability, security and governance. *International Soil and Water Conservation Research*, *10*(1), 99–108. https://doi.org/10.1016/j.iswcr.2021.07.001

Doran, J. W., & Parkin, T. B. (1994). Defining soil quality for a sustainable environment. SSSA Spec. Pub. No. 35. In *Defining and assessing soil quality*. http://dx.doi.org/10.2136/sssaspecpub35.c1

Doran, J. W., & Zeiss, M. R. (2000). DigitalCommons @ University of Nebraska - Lincoln Soil health and sustainability : managing the biotic component of soil quality Soil health and sustainability : managing the biotic component of soil quality. *Applied Soil Ecology*, *June*.

Egamberdieva, D., Kamilova, F., Validov, S., Gafurova, L., Kucharova, Z., & Lugtenberg, B. (2008). High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. *Environmental Microbiology*, *10*(1), 1–9. https://doi.org/10.1111/j.1462-2920.2007.01424.x

Fageria, N.K., Baligar, V.C., Bailey, B.A. (2005). Role of cover crops in improving soil and row crop productivity. *Communications in Soil Science*.

Gibb, M., Kisiala, A. B., Morrison, E. N., and Emery, R. J. N. (2020). The Origins and Roles of Methylthiolated Cytokinins: Evidence From Among Life Kingdoms. *Frontiers in Cell and Developmental Biology*, *8*(November). https://doi.org/10.3389/fcell.2020.605672

Ghimire, R., Ghimire, B., Mesbah, A. O., Sainju, U. M., & Idowu, O. J. (2019). Soil health response of cover crops in winter wheat–fallow system. *Agronomy Journal*, *111*(4), 2108–2115. https://doi.org/10.2134/agronj2018.08.0492

Glanz-Idan, N., Tarkowski, P., Turečková, V., & Wolf, S. (2020). Root-shoot communication in tomato plants: Cytokinin as a signal molecule modulating leaf photosynthetic activity. *Journal of Experimental Botany*, *71*(1), 247–257. https://doi.org/10.1093/jxb/erz399

Goggin, D. E., Emery, R. J. N., Kurepin, L. V., & Powles, S. B. (2015). A potential role for endogenous microflora in dormancy release, cytokinin metabolism and the response to fluridone in Lolium rigidum seeds. *Annals of Botany*, *115*(2), 293–301. https://doi.org/10.1093/aob/mcu231

Grzadziel, J., Furtak, K., & Galazka, A. (2018). Community-level physiological profiles of microorganisms from different types of soil that are characteristic to poland-a long-term microplot experiment. *Sustainability (Switzerland)*, *11*(1). https://doi.org/10.3390/su11010056

Guo, Y. D., Wang, X. L., Qi, L., Shi, J., Liu, Y. H., Li, X. L., Zhang, L. X., Qin, D. H., & Song, P. (2020). Effects of rhizosphere soil nitrification on the compensatory growth of Italian ryegrass (Lolium multiflorum Lam) based on root-produced cytokinin. *Grassland Science*, *September 2019*, 306–314. https://doi.org/10.1111/grs.12317

Gupta, R., Pizarro, L., Leibman-Markus, M., Marash, I., & Bar, M. (2020). Cytokinin response induces immunity and fungal pathogen resistance, and modulates trafficking of the PRR LeEIX2 in tomato. *Molecular Plant Pathology*, *21*(10), 1287–1306. https://doi.org/10.1111/mpp.12978

Hargreaves, S. K., & Hofmockel, K. S. (2014). Physiological shifts in the microbial community drive changes in enzyme activity in a perennial agroecosystem. *Biogeochemistry*, *117*(1), 67–79. https://doi.org/10.1007/s10533-013-9893-6

Hartwig, N. L., & Ammon, H. U. (2002). Cover crops and living mulches. *Weed Science*, *50*(6), 688–699. https://doi.org/10.1614/0043-1745(2002)050[0688:aiacca]2.0.co;2

Hewwett, E. W., & Wareing, P. F. (1973). Cytokinins in Populus×robusta: Qualitative Changes during Development. *Physiologia Plantarum*, *29*(3), 386–389. https://doi.org/10.1111/j.1399-3054.1973.tb04835.x

High, K. E., Ashton, P. D., Nelson, M., Rylott, E. L., Thomas-Oates, J. E., & Hodson, M. E. (2019). New approaches using mass spectrometry to investigate changes to cytokinin and abscisic acid (ABA) concentrations in soil. *Soil Biology and Biochemistry*, *135*(April), 108–116. https://doi.org/10.1016/j.soilbio.2019.04.017

Hooper, D. U., Bignell, D. E., Brown, V. K., Brussaard, L., Dangerfield, J. M., Wall, D. H., Wardle, D. a, Coleman, D. C., Giller, K. E., Lavelle, P., Van Der Putten, W. H., De Ruiter, P. C., Rusek, J., Silver, W. L., Tiedje, J. M., & Wolters, V. (2000). Aboveground and Belowground Biodiversity in Terrestrial Ecosystems : Patterns , Mechanisms , and. *BioScience*, *50*(12), 1049–1061. http://www.bioone.org/doi/abs/10.1641/0006-3568(2000)050[1049:IBAABB]2.0.CO;2

Huang, X. F., Chaparro, J. M., Reardon, K. F., Zhang, R., Shen, Q., & Vivanco, J. M. (2014).

Rhizosphere interactions: Root exudates, microbes, and microbial communities1. *Botany*, *92*(4), 267–275. https://doi.org/10.1139/cjb-2013-0225

Jameson, P. E., & Song, J. (2015). Cytokinin: A key driver of seed yield. Journal of Experimental Botany, 67(3), 593–606. https://doi.org/10.1093/jxb/erv461

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., and Emery, R. J. N. (2019). Endosymbiotic Methylobacterium oryzae mitigates the impact of limited water availability in lentil (Lens culinaris Medik.) by increasing plant cytokinin levels. *Environmental and Experimental Botany*, *162*(June), 525–540. https://doi.org/10.1016/j.envexpbot.2019.03.028

Justes, E., (2017). Cover crop for sustainable farming. Cover crops for sustainable farming. 1-91.

Kaspar, T. C., and Singer, J. W. (2015). The Use of Cover Crops to Manage Soil. *Soil Management: Building a Stable Base for Agriculture*, 321–337. https://doi.org/10.2136/2011.soilmanagement.c21

Kieber, J. J., & Schaller, G. E. (2014). Cytokinins. *The Arabidopsis Book*, *12*(February 2015), e0168. https://doi.org/10.1199/tab.0168

Kim, M.-Y., & Lee, K. H. (2022). Electrochemical Sensors for Sustainable Precision Agriculture—A Review. *Frontiers in Chemistry*, *10*(May), 1–14. https://doi.org/10.3389/fchem.2022.848320

Kisiala, A., Kambhampati, S., Stock, N. L., Aoki, M., and Emery, R. J. N. (2019). Quantification of Cytokinins Using High-Resolution Accurate-Mass Orbitrap Mass Spectrometry and Parallel Reaction Monitoring (PRM). *Analytical Chemistry*, *91*(23), 15049–15056. https://doi.org/10.1021/acs.analchem.9b03728

Kisiala, A., Laffont, C., Emery, R. J. N., and Frugier, F. (2013). Bioactive cytokinins are selectively secreted by sinorhizobium meliloti nodulating and nonnodulating strains. Molecular Plant-Microbe Interactions, 26(10), 1225–1231.https://doi.org/10.1094/MPMI-02-13-0054-R

Kudoyarova, G. R., Korobova, A. V., Akhiyarova, G. R., Arkhipova, T. N., Zaytsev, D. Y., Prinsen, E., Egutkin, N. L., Medvedev, S. S., and Veselov, S. Y. (2014). Accumulation of cytokinins in roots and their export to the shoots of durum wheat plants treated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP). *Journal of Experimental Botany*, *65*(9), 2287–2294. https://doi.org/10.1093/jxb/eru113

Lal, R. (2006). Enhancing crop yields in the developing countries through restoration of the soil organic carbon pool in agricultural lands. *Land Degradation and Development*, *17*(2), 197–209. https://doi.org/10.1002/ldr.696

Lal, R. (2006).Soil carbon sequesteration impacts on global climate chane and food security. *Science*.

Lehman, R. M., Osborne, S. L., & Duke, S. E. (2017). Diversified No-Till Crop Rotation Reduces Nitrous Oxide Emissions, Increases Soybean Yields, and Promotes Soil Carbon

Accrual. *Soil Science Society of America Journal*, *81*(1), 76–83.

Lladó, S., & Baldrian, P. (2017). Community-level physiological profiling analyses show potential to identify the copiotrophic bacteria present in soil environments. *PLoS ONE*, *12*(2). https://doi.org/10.1371/journal.pone.0171638

Murofushi, N., Inoue, A., Watanabe, N., Ota, Y., & Takahashi, N. (1983). Identification of cytokinins in root exudate of the rice plant. *Plant and Cell Physiology*, *24*(1), 87–92. https://doi.org/10.1093/oxfordjournals.pcp.a076517

Musinguzi, P., Ebanyat, P., Tenywa, J. S., Basamba, T. A., Tenywa, M. M., & Mubiru, D. (2015). Precision of farmer-based fertility ratings and soil organic carbon for crop production on a Ferralsol. *Solid Earth*, *6*(3), 1063–1073. https://doi.org/10.5194/se-6-1063-2015

Mutegi, J. K., Petersen, B. M., Munkholm, L. J., & Hansen, E. M. (2011). Belowground carbon input and translocation potential of fodder radish cover-crop. *Plant and Soil*, *344*(1), 159–175. https://doi.org/10.1007/s11104-011-0737-7

Nouri, A., Lee, J., Yin, X., Tyler, D. D., & Saxton, A. M. (2019). Thirty-four years of no-tillage and cover crops improve soil quality and increase cotton yield in Alfisols, Southeastern USA. *Geoderma*, *337*, 998–1008. https://doi.org/10.1016/j.geoderma.2018.10.016

Nguyen, H. N., Nguyen, T. Q., Kisiala, A. B., & Emery, R. J. N. (2021). Beyond transport: cytokinin ribosides are translocated and active in regulating the development and environmental responses of plants. *Planta*, *254*(3), 1–17. https://doi.org/10.1007/s00425-021-03693-2

Oldfield, E. E., Bradford, M. A., & Wood, S. A. (2019). Global meta-analysis of the relationship between soil organic matter and crop yields. *Soil*, *5*(1), 15–32. https://doi.org/10.5194/soil-5-15-2019

Olson, K. R., Ebelhar, S. A., & Lang, J. M. (2010). Cover crop effects on crop yields and soil organic carbon content. *Soil Science*, *175*(2), 89–98. https://doi.org/10.1097/SS.0b013e3181cf7959

Palberg, D., Kisiała, A., Jorge, G. L., & Emery, R. J. N. (2022). A survey of Methylobacterium species and strains reveals widespread production and varying profiles of cytokinin phytohormones. *BMC Microbiology*, *22*(1), 1–17. https://doi.org/10.1186/s12866-022-02454-9

Pavlů, J., Novák, J., Koukalová, V., Luklová, M., Brzobohatý, B., & Černý, M. (2018). Cytokinin at the crossroads of abiotic stress signalling pathways. *International Journal of Molecular Sciences*, *19*(8), 1–36. https://doi.org/10.3390/ijms19082450

Phillips, D. A., & Torrey, J. G. (1970). Cytokinin Production by Rhizobium japonicum. *Physiologia Plantarum*, *23*(6), 1057–1063. https://doi.org/10.1111/j.1399-3054.1970.tb08880.

Powell, A. F., Paleczny, A. R., Olechowski, H., & Emery, R. J. N. (2013). Changes in cytokinin form and concentration in developing kernels correspond with variation in yield among field-grown barley cultivars. *Plant Physiology and Biochemistry, 64*, 33–40. https://doi.org/10.1016/j.plaphy.2012.12.010

Prudent, M., Salon, C., Smith, D. L., and Emery, R. J. N. (2016). Nod factor supply under water stress conditions modulates cytokinin biosynthesis and enhances nodule formation and N nutrition in soybean. *Plant Signaling and Behavior*, *11*(9). https://doi.org/10.1080/15592324.2016.1212799

Pulleman, M., Creamer, R., Hamer, U., Helder, J., Pelosi, C., Pérès, G., & Rutgers, M. (2012). Soil biodiversity, biological indicators and soil ecosystem services-an overview of European approaches. *Current Opinion in Environmental Sustainability*, *4*(5), 529–538. https://doi.org/10.1016/j.cosust.2012.10.009

Raspor, M., Motyka, V., Ninković, S., Dobrev, P. I., Malbeck, J., Ćosić, T., Cingel, A., Savić, J., Tadić, V., and Dragićević, I. (2020). Endogenous levels of cytokinins, indole-3-acetic acid and abscisic acid in in vitro grown potato: A contribution to potato hormonomics. *Scientific Reports*, *10*(1), 1–13. https://doi.org/10.1038/s41598-020-60412-9

Schoonover, J. E., & Crim, J. F. (2015). An Introduction to Soil Concepts and the Role of Soils in Watershed Management. *Journal of Contemporary Water Research & Education*, *154*(1), 21–47. https://doi.org/10.1111/j.1936-704x.2015.03186.x

Short, K. C., & Torrey, J. G. (2000). *Cytokinins*. *1972*, 155–160

Šimura, J., Antoniadi, I., Široká, J., Tarkowská, D., Strnad, M., Ljung, K., & Novák, O. (2018). Plant hormonomics: Multiple phytohormone profiling by targeted metabolomics. *Plant Physiology*, *177*(2), 476–489. https://doi.org/10.1104/pp.18.00293

Six, J., Frey, S. D., Thiet, R. K., & Batten, K. M. (2006). Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. *Soil Science Society of America Journal*, *70*(2), 555–569. https://doi.org/10.2136/sssaj2004.0347

Smith, S. M., Li, C., & Li, J. (2017). Hormone function in plants. In *Hormone Metabolism and Signaling in Plants*. Jiayang Li, Chuanyou Li and Steven Smith. https://doi.org/10.1016/B978-0-12-811562-6.00001-3

Soejima, H., Sugiyama, T., & Ishihara, K. (1992). Changes in cytokinin activities and mass spectrometric analysis of cytokinins in root exudates of rice plant (Oryza sativa L.): Comparison between cultivars Nipponbare and Akenohoshi. *Plant Physiology*, *100*(4), 1724–1729. https://doi.org/10.1104/pp.100.4.1724

Spíchal, L. (2012). Cytokinins - Recent news and views of evolutionally old molecules. *Functional Plant Biology*, *39*(4), 267–284. https://doi.org/10.1071/FP11276

Stirk, W. A., & van Staden, J. (2010). Flow of cytokinins through the environment. *Plant Growth Regulation*, *62*(2), 101–116. https://doi.org/10.1007/s10725-010-9481-x

Stockdale, E. A., & Watson, C. A. (2009). Biological indicators of soil quality in organic farming systems. *Renewable Agriculture and Food Systems*, *24*(4), 308–318. https://doi.org/10.1017/S1742170509990172

Takei, K., Sakakibara, H., Taniguchi, M., and Sugiyama, T. (2001). Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: Implication of cytokinin species that induces gene expression of maize response regulator. *Plant and Cell Physiology*, *42*(1), 85–93. https://doi.org/10.1093/pcp/pce009

Timmusk, S., Nicander, B., Granhall, U., & Tillberg, E. (1999). Cytokinin production by Paenibacillus polymyxa. *Soil Biology and Biochemistry*, *31*(13), 1847–1852. https://doi.org/10.1016/S0038-0717(99)00113-3

Tregubova, P., Koptsik, G., Stepanov, A., Koptsik, S., & Spiers, G. (2021). Organic amendments potentially stabilize metals in smelter contaminated Arctic soils: An incubation study. *Heliyon*, *7*(1), e06022. https://doi.org/10.1016/j.heliyon.2021.e06022

Van Staden, J., and Dimalla, G.,G. (1976). Cytokinins from Soils. *Planta*. 130,85-87

Wang, X. L., Liu, D., & Li, Z. Q. (2012). Effects of the coordination mechanism between roots and leaves induced by root-breaking and exogenous cytokinin spraying on the grazing tolerance of ryegrass. *Journal of Plant Research*, *125*(3), 407–416. https://doi.org/10.1007/s10265-011-0442-x

Weber, K. P., & Legge, R. L. (2010). Community-level physiological profiling. *Methods in Molecular Biology (Clifton, N.J.)*, *599*(October), 263–281. https://doi.org/10.1007/978-1-60761-439-5_16

Weil, R. R., Islam, K. R., Stine, M. A., Gruver, J. B., & Samson-Liebig, S. E. (2003). Estimating active carbon for soil quality assessment: A simplified method for laboratory and field use. *American Journal of Alternative Agriculture*, *18*(1), 3–17. https://doi.org/10.1079/AJAA2003003

Wong, W., Tan, S., and Ge, L. (2015). The Importance of Phytohormones and Microbes in Biofertilizers. https://doi.org/10.1007/978-3-319-24654-3_6

Yang, G., Wagg, C., Veresoglou, S. D., Hempel, S., and Rillig, M. C. (2018). How Soil Biota Drive Ecosystem Stability. *Trends in Plant Science*, *23*(12), 1057–1067. https://doi.org/10.1016/j.tplants.2018.09.007

Zalabák, D., Galuszka, P., Mrízová, K., Podlešáková, K., Gu, R., & Frébortová, J. (2014). Biochemical characterization of the maize cytokinin dehydrogenase family and cytokinin profiling in developing maize plantlets in relation to the expression of cytokinin dehydrogenase genes. *Plant Physiology and Biochemistry*, *74*, 283–293. https://doi.org/10.1016/j.plaphy.2013.11.020

Zeynep, D., Nihat, T., and Işık, D. (2019). Effects of Different Cover Crops on Soil Quality Parameters and Yield in an Apricot Orchard. *International Journal of Agriculture and*

Biology, *21*(1), 25–33. https://doi.org/10.17957/IJAB/15.0000

	Loam soil	Sandy loam soil
pH	7.67 ± 0.20	7.45 ± 0.11
SOM	5.14 ± 0.05	5.85* ± 0.04
AC	316.19 ± 32.03	674.27* ± 2.89
SMC diversity	1.82 ± 0.42	2.15 ± 0.08
Total CK	0.09 ± 0.01	0.16 ± 0.07

Figures and Tables

Table 4.1

A comparison of characteristics of loam and sandy loam soils collected from the Trent Experimental Farm, Peterborough, ON, in 2019. Data (Mean ± SE) were tested by a two-sample t-test for pH, soil organic carbon (%) (SOM), active carbon (AC) (ppm), soil microbial community (SMC) diversity (Shannon diversity index), and total CK (pmol g^{-1} dry soil). Means (n=3) followed by an asterisk (*) in a row are significantly different between soil types ($p < 0.05$).

Table 4.2

Enzyme activities (μM hr/g dry soil) of β-glucosidase (BG), N-acetylglucosaminidase (NAG), phosphatase (PO) in rhizosphere (TR) and bulk (TB) soils under tillage radish (loam and sandy loam) and rhizosphere (CR) and bulk (CB) soils under cereal rye (loam soil). The comparisons between treatments in loam soil and sandy loam soils were done separately using one-way ANOVA and a two-sample t-test, respectively. Data are means (n=3) ± SE; values with the same letters in a row are not significantly different (LSD test, $p < 0.05$).

	Loam Soil				**Sandy loam soil**	
	TR	**TB**	**CR**	**CB**	**TR**	**TB**
BG	$0.28^{a} \pm 0.04$	$0.28^{a} \pm 0.02$	$0.25^{a} \pm 0.04$	$0.18^{a} \pm 0.01$	$0.18^{a} \pm 0.03$	$0.23^{a} \pm 0.02$
NAG	$0.14^{b} \pm 0.01$	$0.11^{b} \pm 0.01$	$0.14^{ab} \pm 0.01$	$0.19^{a} \pm 0.01$	$0.10^{a} \pm 0.01$	$0.11^{a} \pm 0.01$
PO	$0.74^{a} \pm 0.07$	$0.60^{a} \pm 0.08$	$0.60^{a} \pm 0.02$	$0.47^{a} \pm 0.02$	$0.43^{a} \pm 0.01$	$0.60^{a} \pm 0.12$

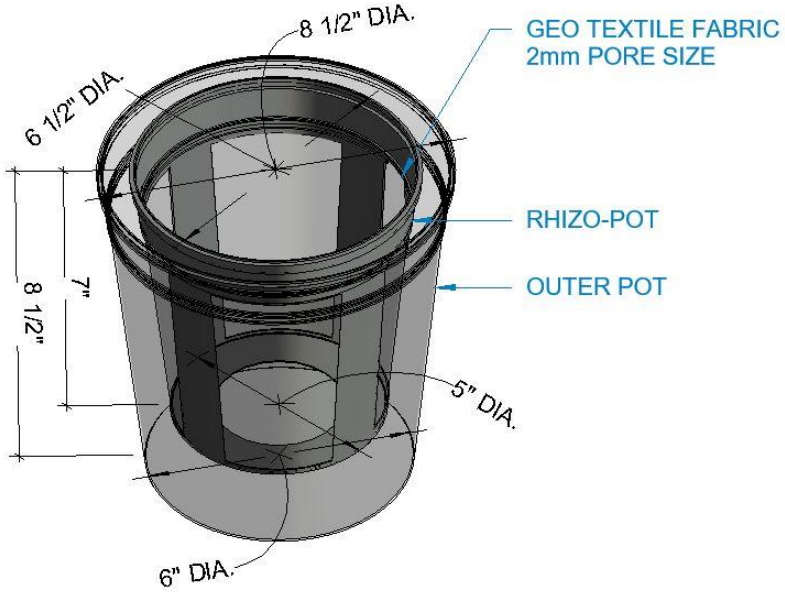

Fig. 4.1. A schematic diagram of the pots used in the greenhouse experiment. A single pot was fitted with a small pot inside and called 'rhizo-pots'. The rhizo-pots were custom designed using geotextile fabric with a 2mm pore size to allow water, nutrients, and microbes to pass between rhizosphere and bulk soil zones, while restricting root growth from the rhizosphere to bulk zones.

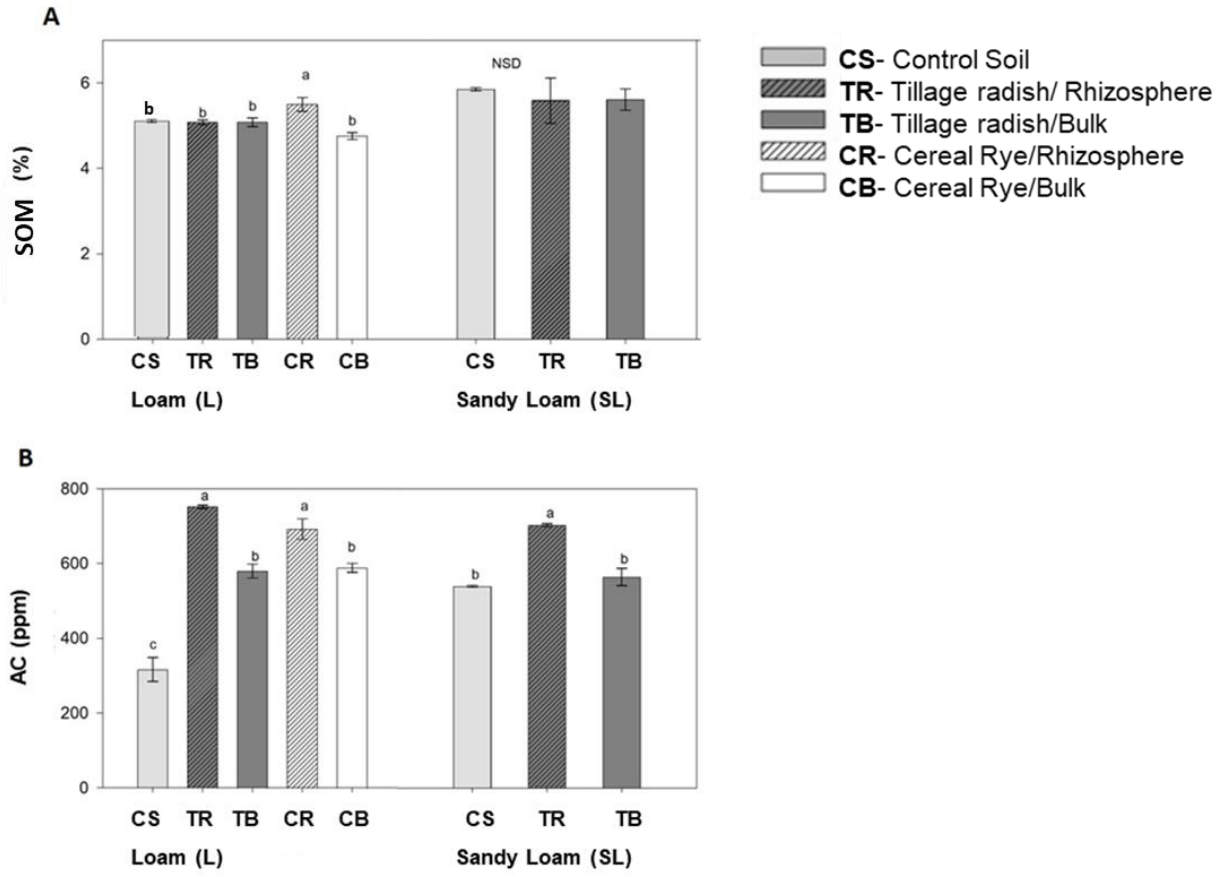

Fig. 4.2. The effect of cover crops on **A.** soil organic matter (SOM) levels, **B.** active carbon (AC) levels, and **C.** soil microbial community (SMC) diversity, compared to control soils. ANOVA was used to compare metrics between crops and soil zones within soil and sandy loam soils. Values are means ± SE (n= 3), different letters above columns indicate significant differences among treatments (Fisher's least significant difference test, $p \leq 0.05$). Abbreviations: CS- control soil, TR -rhizosphere soils under tillage radish; TB - bulk soils under tillage radish; CR - rhizosphere soils under cereal rye; CB - bulk soils under cereal rye.

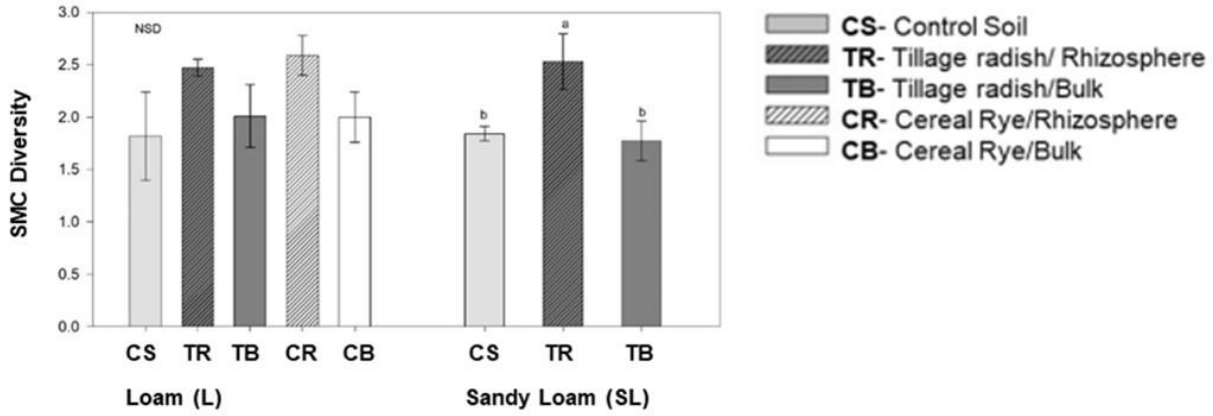

Fig. 4.3. The effect of cover crops on soil microbial community (SMC) diversity, compared to control soils. ANOVA was used to compare treatments separately in loam and sandy loam soils. Values are means ± SE (n= 3), different letters above columns indicate significant differences among treatments (Fisher's least significant difference test, $p \leq 0.05$). Abbreviations: CS- control soil, TR -rhizosphere soils under tillage radish; TB - bulk soils under tillage radish; CR - rhizosphere soils under cereal rye; CB - bulk soils under cereal rye.

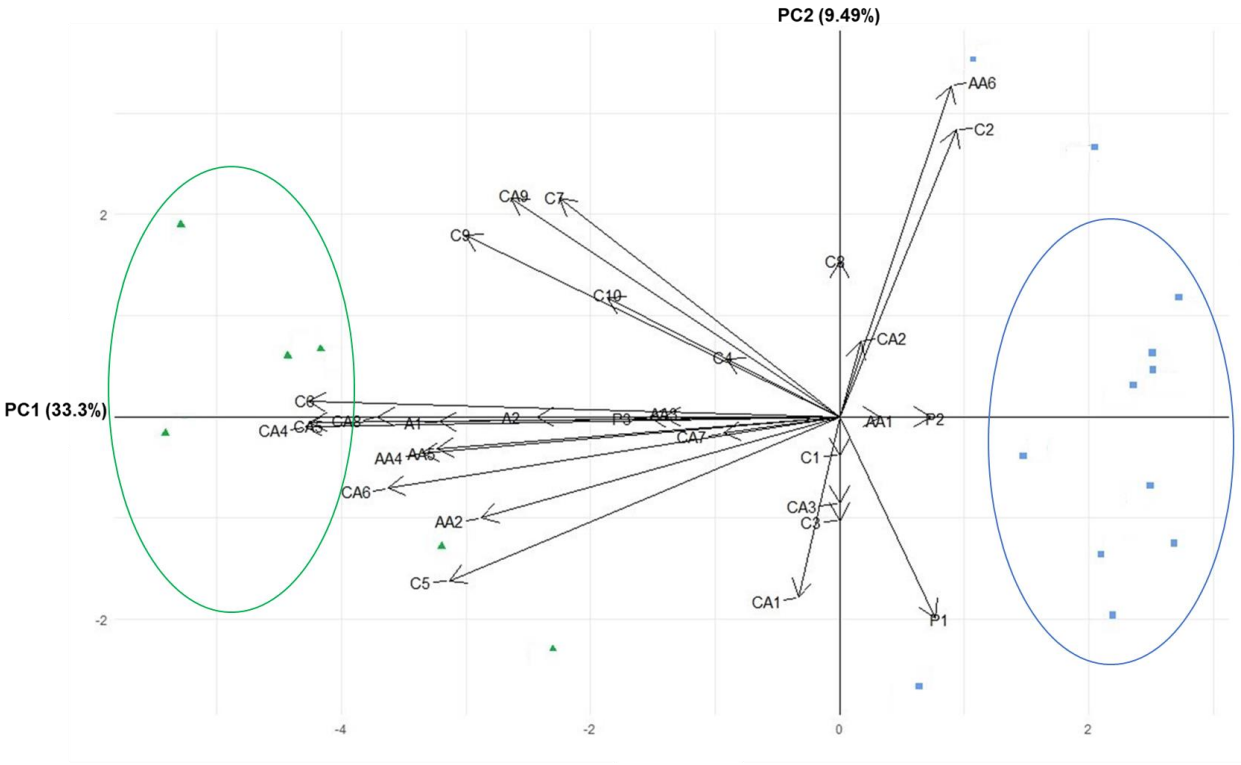

Fig. 4.4. 2D representation of the first two principal components (PC1 and PC2) derived from sparse PCA analysis of the normalized absorbance readings (590 nm) of BiologEcoPlates obtained as a result of substrate (30 carbon sources) utilization by soil microbial communities present in sandy loam rhizosphere and bulk soils grown under tillage radish. The blue squares indicate the scores for rhizosphere soil samples while green triangles indicate the scores for bulk soil samples. The vector loadings indicate the 31 different carbon sources: Carbohydrates (C1-C10), Carboxylic acids (CA1-CA9), amines and amides (A1 and A2), amino acids (AA1-AA6), and polymers (P1-P4). The two elliptical circles represent the two clusters, namely, the green circle illustrates the cluster of bulk soil samples, and the blue circle illustrates the cluster of rhizosphere soil samples.

Abbreviations: A1- Phenylethylamine; A2- Putrescine; AA1- L-Arginine; AA2- L-Asparagine; AA3- L-Phenylalanine; AA4- L-Serine; AA5- L-Threonine; AA6- Glycyl-L-Glutamic Acid; C1- Pyruvic Acid Methy Ester; C2- D-Cellobiose; C3- Alpha-D-Lactose; C4- Beta-Methyl-D-Glucoside; C5- D-Xylose; C6- i-Erythritol; C7- D-Mannitol; C8- N-Acetyl-D-Glucosamine; C9- Glusose-1-Phosphate; C10- D,L-a-Glycerol Phosphate; CA1- D-Glucosaminic Acid; CA2- D-Galactonic Acid y- Lactone; CA3- D-Galacturonic Acid; CA4- 2-Hydroxy Benzoic Acid; CA5- 4-Hydroxy Benzoic Acid; CA6- Gamma-Amino Butyric Acid; CA7- Itaconic Acid; CA8- alpha-Keto Butyric Acid; CA9- alpha-Keto Butyric Acid; P1- Tween 40; P2- Tween 80; P3- Alpha-Cyclodextrin

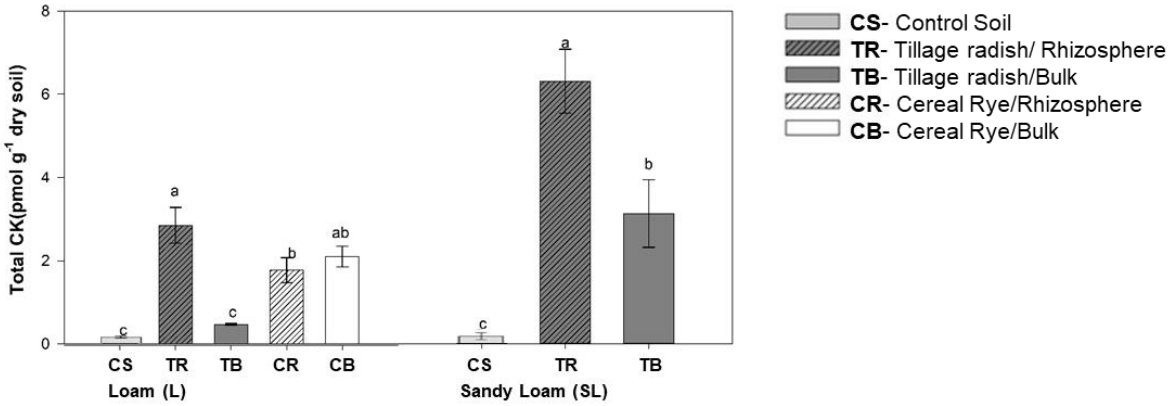

Fig. 4.5. Comparison of total CK concentrations (pmol g^{-1} dry weight) between control soil (CS) and in cover cropped soils. Values are means ± SE (n= 3), different letters above columns indicate significant difference among treatments (Fisher's least significant difference test, $p \leq 0.05$). Abbreviations: CS- control soil, TR -rhizosphere soils under tillage radish; TB - bulk soils under tillage radish; CR - rhizosphere soils under cereal rye; CB - bulk soils under cereal rye

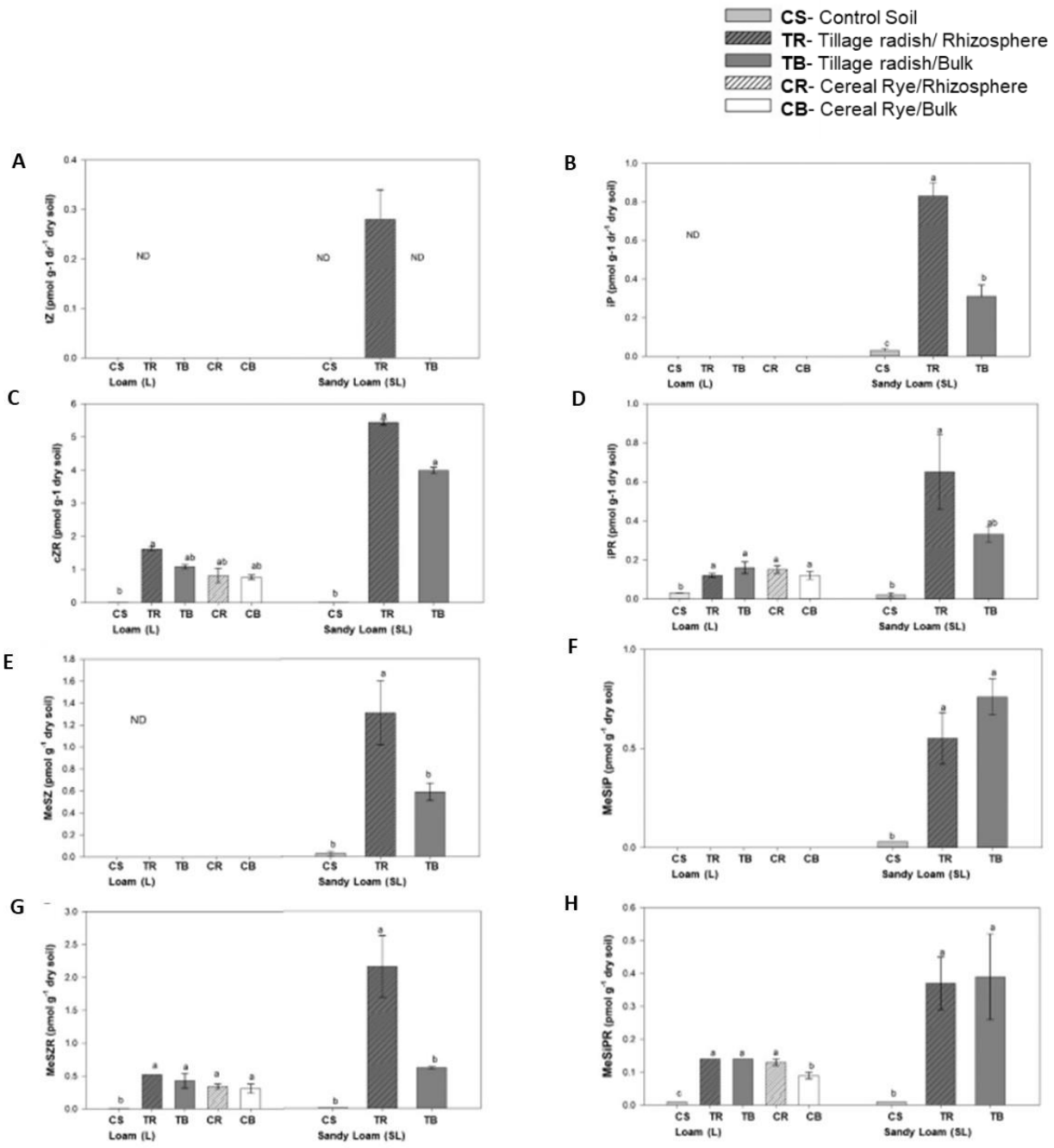

Fig. 4.6. The effect of cover crops on free base CKs **A.** tZ levels, **B.** iP levels; riboside-CKs **C.** cZR levels, **D.** iPR levels; Methylthiol cytokinins **E.** MeSZ levels, **F.** MeSiP levels, **G.** MeSZR levels, **H.** MeSiPR levels, compared to control soils. ANOVA was used to compare metrics between treatments separately in loam soil and sandy loam soils. Values are means ± SE (n= 3), different letters above columns indicate significant difference among treatments (Fisher's least significant difference test, $p \leq 0.05$). Abbreviations: CS- control soil, TR -rhizosphere soils under tillage radish; TB - bulk soils under tillage radish; CR - rhizosphere soils under cereal rye; CB - bulk soils under cereal rye

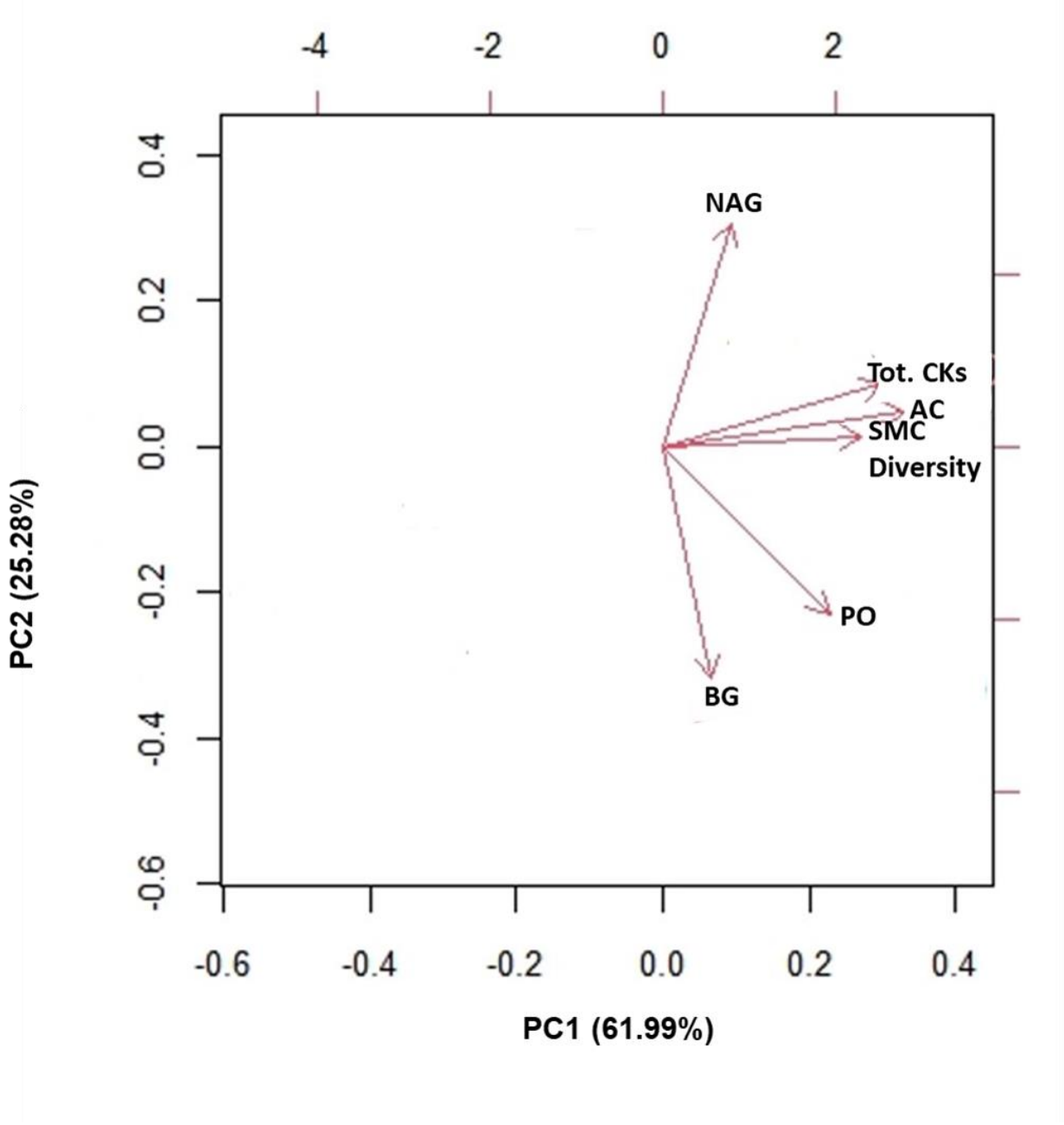

Fig. 4.7. 2D representation of the first two principal components (PC1 and PC2) derived from the principal component analysis (PCA) of the data set total cytokinins (Tot. CK), active carbon (AC), soil microbial community (SMC) diversity, and enzyme activities of β-glucosidase (BG), Phosphatase (PO), N-acetylglucosaminidase (NAG) in loam rhizosphere and bulk soils samples under the cover crops tillage radish and cereal rye.

Supplementary Table 4.1.

Abbreviations used to denote different treatments and soil types

Abbreviations	Description
L	Loam soil
SL	Sandy loam soil
TR	Tillage radish- Rhizosphere
TB	Tillage radish- Bulk
CR	Cereal rye- Rhizosphere
CB	Cereal rye- Bulk

Supplementary Table 4.2

The names of 31 different carbon sources inside the wells of Biolog®EcoPlate™ and the guilds (Frąc et al., 2012)

Guilds	Abbreviations	Name of the C-source
Amines & Amides	A1	Phenylethylamine
	A2	Putrescine
Amino Acids	AA1	L-Arginine
	AA2	L-Asparagine
	AA3	L-Phenylalanine
	AA4	L-Serine
	AA5	L-Threonine
	AA6	Glycyl-L-Glutamic Acid
Carbohydrates	C1	Pyruvic Acid Methy Ester
	C2	D-Cellobiose
	C3	Alpha-D-Lactose
	C4	Beta-Methyl-D-Glucoside
	C5	D-Xylose
	C6	i-Erythritol
	C7	D-Mannitol
	C8	N-Acetyl-D-Glucosamine
	C9	Glusose-1-Phosphate
	C10	D,L-a-Glycerol Phosphate
Carboxylic & Acetic Acids	CA1	D-Glucosaminic Acid
	CA2	D-Galactonic Acid y-Lactone
	CA3	D-Galacturonic Acid
	CA4	2-Hydroxy Benzoic Acid
	CA5	4-Hydroxy Benzoic Acid
	CA6	Gamma-Amino Butyric Acid
	CA7	Itaconic Acid
	CA8	alpha-Keto Butyric Acid
	CA9	D-Malic Acid
Polymers	P1	Tween 40
	P2	Tween 80
	P3	Alpha-Cyclodextrin

Chapter 5: General Discussion and Conclusions

Agricultural management practices are implemented to increase cash crop yields by improving soil health by enhancing SOM, SOC, water holding capacity, and SMC abundance, diversity, and functioning (Liu et al., 2020; Chamberlain et al., 2020; Zhang et al., 2019; D'Acunto et al., 2018; Hartwig & Ammon, 2002). However, intensive management practices in operational fields have resulted in environmental pollution via NO_3^- leaching and emission of the GHG, N_2O. SB is a widely grown crop in Ontario, and it is grown in rotation with MZ and WW and sometimes, farmers incorporate CC mixtures into these rotations (Agomoh et al., 2021; Córdova et al., 2019). Growing CCs during fallow and winter seasons helps protect soil from erosion and reduce nutrient leaching and N losses via nitrification and denitrification (Muhammad et al., 2019; Rocha et al., 2020). Although a wide range of ongoing research is focused on the benefits of CCs in improving soil health and environmental quality, their impact on soil cytokinin profiles and the relationships between microbial N cycling, soil health parameters, and CK profiles are not yet known.

In this thesis, I investigated the impacts of an intensively managed crop rotation of MZ-(SB-WW)-CC on soil health parameters and N-cycling bacterial community abundances in different crop rotation phases to understand the potential for N-losses and environmental risk due to N leaching or emission of greenhouse gases at each crop phase. In addition, I examined the impact of management strategies, specifically cover cropping, on soil CK hormone levels to determine the potential downstream applications of using CCs during fallow periods. I also studied the relationships between soil CK profiles and traditional soil health parameters.

In **Chapter 3**, I assessed soil health parameters in soil samples collected from operating farms with a MZ-(SB-WW)-CC rotation to determine the impact of each crop phase on soil health. AC

and WSA were significantly higher in MZ and CC fields than in WW fields. I also found that MZ and CC fields had higher N-mineralizing abilities based on the activity of the N-mineralizing enzyme NAG. However, SB-WW fields that would have high N-containing crop residues had lower NAG activity. The application of N-fertilizers MAP and starter N:P:K before and during the planting of WW - that would result in high NO_3^-/NH_4^+ inputs might be the reason for decreasing activity of NAG in WW fields. In addition, I used qPCR techniques to enumerate the total soil bacterial communities (*16S* rRNA), nitrifying bacterial communities (*amo*A), and denitrifying bacterial communities (*nir*S and *nir*K) at different crop rotation phases. I found higher *amo*A abundances in SB-WW fields, revealing potentially higher nitrification that could result in higher soil NO_3^- levels over the non-growing season. However, the NO_3^- levels in WW fields were significantly lower, which may be due to leaching or denitrification rather than plant uptake, since WW had a small (immature) root system at the time of sampling.

Furthermore, the denitrifying community size, based on *nir*K gene copies, was significantly lower in the SB-WW phase, revealing a possible lower denitrification potential. Therefore, the significantly lower NO_3^- levels in SB-WW fields might be due to NO_3^- leaching. By contrast, the lower NO_3^- levels detected in CC fields may be due to higher plant uptake, since CCs act as scavengers with a strong and deep root system and appear to have a lower potential for denitrification based on *nir*K gene abundance. MZ fields, where fertilizer (MAP) was added during planting in spring, might have supported nitrification during the growing season, potentially contributing to higher NO_3^- levels observed during sampling in fall, that may have supported the denitrifying bacterial communities (*nir*K and *nir*S). This may potentially lead to N-loss via emission of GHG, N_2O.

In **chapter 4**, I carried out a greenhouse trial to identify the impact of CC on soil CK profiles. I purified soil CKs from cover cropped and non-plant control soil samples and characterized and quantified them using LC-HRMS. The CC-grown soil had significantly higher CK levels than control soils, indicating the positive impact of growing CCs/living roots in improving plant growth by increasing hormones in soil. In addition, I detected significantly higher AC, EEA, and SMC diversity in CC-grown soils compared to control soils, suggesting living roots enhance soil health parameters. Interestingly, AC levels showed a strong correlation with total CK levels, revealing the potential use of CK analyses as a novel tool in assessing soil health, with possible future application in soil carbon monitoring approaches.

Overall, this study reveals the importance of CCs on the improvement of soil health and reduction of potential N-loss via denitrification and NO_3^- leaching. In addition, the growing of CCs improve soil hormone profiles, specifically CK hormones, which act as a plant growth promoter, and could benefit subsequent cash crop growth and yields. This study highlights the linkage between soil CKs and soil health, further providing evidence CK profiles may represent a novel soil health parameter. Further studies on the relationship between soil health parameters and soil CK levels in agricultural fields would be warranted in developing soil-CK profiling as an indicator of soil health.

5.1 Bibliography

Agomoh, I. V., Drury, C. F., Yang, X., Phillips, L. A., & Reynolds, W. D. (2021). Crop rotation enhances soybean yields and soil health indicators. Soil Science Society of America Journal, 85(4), 1185–1195. https://doi.org/10.1002/saj2.20241

Córdova, S. C., Castellano, M. J., Dietzel, R., Licht, M. A., Togliatti, K., Martinez-Feria, R., & Archontoulis, S. V. (2019). Soybean nitrogen fixation dynamics in Iowa, USA. *Field Crops Research*, *236*(November 2018), 165–176. https://doi.org/10.1016/j.fcr.2019.03.018

Chamberlain, L. A., Bolton, M. L., Cox, M. S., Suen, G., Conley, S. P., & Ané, J. M. (2020). Crop rotation, but not cover crops, influenced soil bacterial community composition in a corn-soybean system in southern Wisconsin. *Applied Soil Ecology*, *154*(December 2019), 103603. https://doi.org/10.1016/j.apsoil.2020.103603

D'Acunto, L., Andrade, J. F., Poggio, S. L., & Semmartin, M. (2018). Diversifying crop rotation increased metabolic soil diversity and activity of the microbial community. *Agriculture, Ecosystems and Environment*, *257*(August 2017), 159–164. https://doi.org/10.1016/j.agee.2018.02.011

Hartwig, N. L., & Ammon, H. U. (2002). Cover crops and living mulches. *Weed Science*, *50*(6), 688–699. https://doi.org/10.1614/0043-1745(2002)050[0688:aiacca]2.0.co;2

Liu, K., Bandara, M., Hamel, C., Knight, J. D., & Gan, Y. (2020). Intensifying crop rotations with pulse crops enhances system productivity and soil organic carbon in semi-arid environments. *Field Crops Research*, *248*(October 2019), 107657. https://doi.org/10.1016/j.fcr.2019.107657

Muhammad, I., Sainju, U. M., Zhao, F., Khan, A., Ghimire, R., Fu, X., & Wang, J. (2019). Regulation of soil CO2 and N2O emissions by cover crops: A meta-analysis. *Soil and Tillage Research*, *192*(November 2018), 103–112. https://doi.org/10.1016/j.still.2019.04.020

Palberg, D., Kisiała, A., Jorge, G. L., & Emery, R. J. N. (2022). A survey of Methylobacterium species and strains reveals widespread production and varying profiles of cytokinin phytohormones. *BMC Microbiology*, *22*(1), 1–17. https://doi.org/10.1186/s12866-022-02454-9

Rocha, K. F., Kuramae, E. E., Borges, B. M. F., Leite, M. F. A., & Rosolem, C. A. (2020). Microbial N-cycling gene abundance is affected by cover crop specie and development stage in an integrated cropping system. *Archives of Microbiology*, *202*(7), 2005–2012. https://doi.org/10.1007/s00203-020-01910-2

Zhang, K., Maltais-Landry, G., & Liao, H. L. (2021). How soil biota regulate C cycling and soil C pools in diversified crop rotations. *Soil Biology and Biochemistry*, *156*. https://doi.org/10.1016/j.soilbio.2021.108219