Siderophore-producing *Pseudomonas* spp.: a potential mechanism of plant-growth promotion by black soldier fly frass

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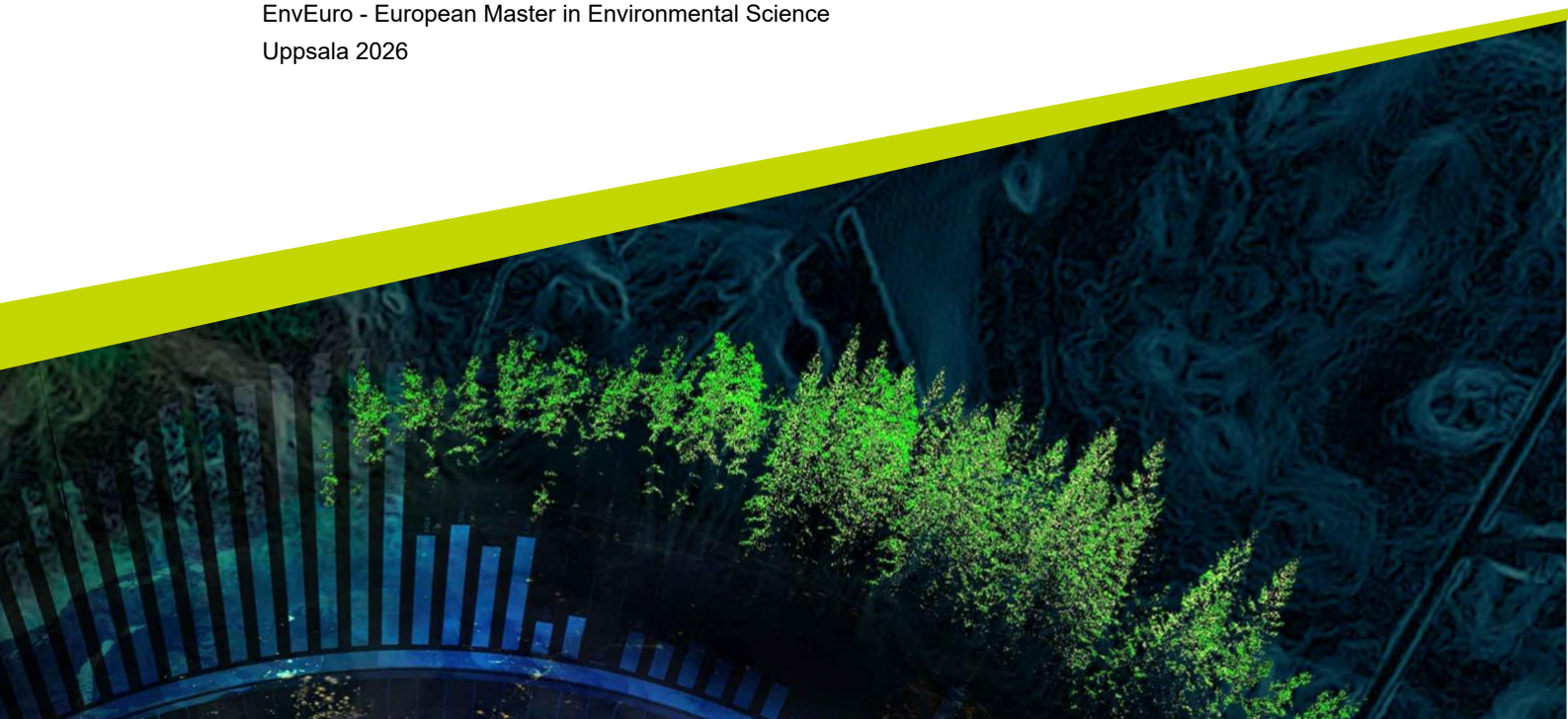

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Abstract

Black soldier fly larvae (BSFL) bioconversion has emerged as a sustainable technology for addressing both food security and biowaste management challenges. A valuable by-product of this process, frass, has been increasingly recognized as a biofertilizer with plant growth-promoting and biostimulant properties. However, the microbial mechanisms underlying these effects remain poorly understood. This study aimed to investigate whether siderophore-producing *Pseudomonas* spp. contribute to the documented biostimulant properties of frass. It was hypothesized that plant growth promotion by frass is, in part, mediated by frass-associated microorganisms, and specifically that siderophore-producing *Pseudomonas* spp. in frass enhance plant iron nutrition. Iron nutrition is essential for various plant metabolic processes but can be limited by low bioavailability of iron in the soil. The study was conducted in two phases. In Phase 1, pig manure was bioconverted by BSFL, and the resulting frass was characterized and tested for phytotoxicity. Frass was also screened microbiologically for *Pseudomonas* spp. abundance, and isolates were tested for siderophore production. In Phase 2, a greenhouse experiment was conducted using four treatments: sterile soil (control, C), sterile soil with sterile frass (X), sterile soil with fresh frass (Y), and fresh soil with fresh frass (Z). After 38 days, plant growth parameters were assessed, and rhizosphere samples were analyzed for *Pseudomonas* spp. abundance and isolates were tested for siderophore production. The results showed that frass contained *Pseudomonas* spp. of which 59 % of screened isolates were positive for siderophore production. Soil amendment with BSFL frass promoted kale growth by up to 195 % increase in above-ground biomass compared to the negative control, although effects varied depending on whether frass and soil were sterile or fresh. Up to 93.3 % of the screened *Pseudomonas* spp. isolates from the rhizosphere soil were siderophore producers. These findings support the hypothesis that plant growth promotion by frass is, in part, mediated by frass-associated microorganisms. However, the specific role of siderophore-producing *Pseudomonas* spp. in enhancing plant iron nutrition remains unresolved based on the current data. To the authors' knowledge, this is the first study to examine the potential role of siderophore-producing *Pseudomonas* spp. from BSFL frass in plant growth promotion. If confirmed in future studies, this mechanism may be particularly relevant for the application of frass-based biofertilizers in iron-limited agricultural soils.

Keywords: *Hermetia illucens*, frass, biostimulants, siderophore-producing *Pseudomonas* spp., iron bioavailability

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Abbreviations

Abbreviation	Description
BCE	Bioconversion efficiency
BSFL	Larvae of the black soldier fly (*Hermetia illucens*)
CAS	Chrome azurol S
CFU	Colony-forming units
GI	Germination index
HDTMA	Hexadecyltrimethylammoniumbromide
IAA	Indole-3-acetic acid
MSW	Municipal solid waste
PSA	Pseudomonas selective agar
SLU	Swedish University of Agricultural Sciences
Spp.	Species pluralis
TS	Total solids
VS	Volatile solids

1. Introduction

The global human population is predicted to reach 8.5 billion people by 2030 and surpass 9 billion people by 2050 (United Nations 2024). The growth in human population will exert unprecedented demand on global resources, especially food. Additionally, the increasing rate of crop pest infestations and disease outbreaks, declining soil health, and intensifying geopolitical conflict could push many of the Sustainable Development Goals, e.g. SDG 2 (Zero Hunger), seemingly out of reach (Allen 2025).

Besides the growing demand for food, population growth will generate enormous amounts of municipal solid waste (MSW), especially biowaste (the biodegradable fraction) (Cook et al. 2026). About 2.1 billion tonnes of MSW are generated annually worldwide (UNEP 2024). In 2020, 38% of global MSW was disposed of uncontrolled by dumping and open burning and 30% was disposed of in landfills (UNEP 2024). Approximately half of the MSW consisted of biowaste, including food waste and agricultural residues (UNEP 2024). Inadequately managed biowaste causes human health and environmental problems, such as disease outbreaks, methane emissions, as well as water and ecosystem pollution (Yatoo et al. 2024). Therefore, innovative approaches are needed to meet the global food demand and manage the biowaste generated in society. This requires sustainable technologies that can be used to manage current environmental problems and adopt sustainable processes for feed and food production (Sarangi et al. 2024).

The treatment of biowaste using larvae of the black soldier fly (*Hermetia illucens*) (BSFL) presents a promising opportunity to address the challenges mentioned. BSFL are able to break down a wide variety of organic material and convert it into protein-rich biomass, thereby reducing biowaste volume. The two main products of the bioconversion process are nutrient-rich larvae that can be used as feed for livestock (Lalander et al. 2019), and nutrient-rich frass used mainly as a biofertilizer (Tanga et al. 2022).

Field and pot studies have shown that frass can promote plant and soil health (Lopes et al. 2022). Plant-growth promotion by frass has been demonstrated for various crops, including cereals such as maize (*Zea mays*) (Beesigamukama et al. 2020), vegetables such as kale (*Brassica oleracea*) (Anyega et al. 2021) and forage crops such as ryegrass (*Lolium multiflorum*) (Menino et al. 2021). Besides plant growth promotion, soil amendment with frass has been shown to exhibit antifungal and nematicidal effects, as demonstrated in sugar beets (*Beta vulgaris*) (Escobar Rodríguez et al. 2025) and spinach (*Spinacia oleracea*) (Kisaakye et al. 2024). Accordingly, there is growing interest in the utilization of frass to increase the productivity of crop production systems. However, despite rapid and recent progress in the elucidation of frass composition and its plant-growth potential (Agustiyani et al. 2021), there are major gaps regarding the mechanisms of frass-mediated plant-growth promotion (Lopes et al. 2022).

Plant-growth promotion and suppression of crop pests and pathogens have been linked to the composition of frass (Kisaakye et al. 2024). Frass is rich in plant-essential nutrients, including macronutrients (N, P, K) and micronutrients (e.g. Fe, Cu, Mn) (Menino et al. 2021). Frass is also rich in substances that have

been demonstrated to have biostimulant properties, such as chitin, humic substances and beneficial microorganisms (Gold et al. 2020; Escobar Rodríguez et al. 2025). BSFL bioconversion may alter the microbial community (Wang et al. 2024) and harbor a core gut microbiome that includes beneficial microorganisms (Wynants et al. 2019; Green 2023). However, the role of frass-associated microbial communities in plant growth promotion remains poorly understood.

Most studies on frass-amended soils have focused on the impact of frass on soil nutrient concentrations, pH changes, and crop yields (Tan et al. 2021). Additionally, microbiome analyses using culture-independent techniques such as 16S rRNA gene sequencing have revealed that frass application can alter the abundance and structure of microbial communities in frass-amended soils (Fuhrmann et al. 2022). Frass contains many communities of microorganisms, including beneficial bacteria, collectively referred to as plant growth-promoting (rhizo)bacteria (Green 2023; Wang et al. 2025; Jiang et al. 2026). Plant growth-promoting rhizobacteria promote plant health via various mechanisms, including the production of plant hormones (e.g. IAA, gibberellin and ethylene), nitrogen fixation and nutrient acquisition (Pérez-Montaño et al. 2014). For example, plant growth-promoting rhizobacteria such as *Bacillus* spp., *Enterobacter* spp. and *Pseudomonas* spp. have been shown to promote plant health, for example by enhancing the iron nutrition of plants (Jha & Saraf 2015).

Iron is essential for plant growth and plays a role in various biological processes such as respiration, DNA replication, chlorophyll biosynthesis and photosynthesis (Zuluaga et al. 2023). Although iron is abundant in soil, its bioavailability is limited (10^{-7}-10^{-9} M), which is four orders of magnitude lower than the optimal requirement for plants and microorganisms (Zuluaga et al. 2023). Under iron-limited conditions, some microorganisms synthesize low molecular weight (400 - 1500 Da) secondary metabolites called siderophores, which sequester iron (Jha & Saraf 2015). Siderophores bind iron with high affinity, making it available to the microorganism (Jha & Saraf 2015). Among the plant growth-promoting rhizobacteria, *Pseudomonas* spp. have been widely investigated for their plant-growth promotion. *Pseudomonas* is a large genus with diverse species such as *P. fluorescens, P. aeruginosa* and *P. putida*, which are frequently found in various environments, including soil, water and compost (Sah et al. 2021).

Pseudomonas spp. can promote plant growth via diverse mechanisms, particularly by increasing the bioavailability of micronutrients such as iron. Plant inoculations with siderophore-producing *Pseudomonas* spp. have been reported to enhance the iron nutrition of plants and thereby enhance plant growth of various crops such as tomatoes and sugar beet (Sah et al. 2021). *Pseudomonas* spp. have also been detected in frass using culture-independent approaches (Green 2023; Wang et al. 2025; Jiang et al. 2026). However, it is largely unknown whether siderophore-producing *Pseudomonas* spp. in frass could play a role in plant-growth promotion by frass.

1.1 Study aim, objectives, and guiding hypotheses

1.1.1 Specific objectives

The aim of this study was to investigate whether siderophore-producing *Pseudomonas* spp. contribute to the previously reported biostimulant properties of frass. This question was addressed through three sub-objectives.

i) Determine the abundance of siderophore-producing *Pseudomonas* spp. in BSFL frass.
ii) Assess the effect of BSFL frass on the growth of kale (*Brassica oleracea*).
iii) Determine the abundance of siderophore-producing *Pseudomonas* spp. in the rhizosphere of BSFL frass-amended soil.

1.1.2 Research hypotheses

This thesis was guided by the hypothesis that plant growth promotion by frass is in part mediated by frass-associated microorganisms. Additionally, it was hypothesized that siderophore-producing *Pseudomonas* spp. in frass promote plant growth by enhancing the iron nutrition of plants.

2. Literature overview

The treatment of biowaste using larvae of the black soldier fly presents a promising opportunity to address global challenges such as the predicted rise in food demand due to a growing population (FAO et al. 2025) and large amounts of inadequately treated biowaste (UNEP 2024).

The black soldier fly (*Hermetia illucens*) is a member of the *Stratiomydiae* family, which is distributed in tropical and temperate regions (Sheppard et al. 2002). Under optimal conditions, the life cycle lasts between 40 and 43 days (Tomberlin et al. 2002) and can be divided into four stages: egg, larva, pupa, and fly (Liu et al. 2017). The fly stage serves the purpose of reproduction, and during this phase, the flies rely entirely on the nutrient reserves they previously acquired during the larval stage (Sheppard et al. 2002). During the larval stage, which lasts 22–24 days (Tomberlin et al. 2002), the saprophagous larvae feed on a broad range of decaying organic material, such as vegetables, fruits or animal manure (Diener et al. 2015). In various studies, for example, larvae were fed a substrate consisting of pig manure (Liu et al. 2019; Parodi et al. 2021; Veldkamp et al. 2021; Naser El Deen et al. 2023). The amount of organic matter is thereby reduced by up to 70 % material degradation, and the biomass of the larvae is increased through the final larval stage (prepupa) (Diener et al. 2015). The biomass in the larval and pre-pupal stage has a high protein and fat content, making it a valuable alternative for animal feed and biofuel, as identified in numerous studies summarized by Surendra et al. (2020).

The use of this bioconversion technology for biowaste management and animal-feed production also results in a compost residue that is suitable for use as plant fertilizer (Lalander et al. 2015). This residue, called frass, is classified under EU Regulation 2021/1925 as “a mixture of excrements derived from farmed insects, the feeding substrate, parts of farmed insects, dead eggs, and with a content of dead farmed insects not exceeding 5% in volume and 3% in weight” (Commission Regulation (EU) 2021/1925, 2021).

The physicochemical and biological composition of the frass varies depending on various factors of the bioconversion process, with the substrate used playing a central role (Wynants et al. 2019; Kreft et al. 2026). This influence of different substrates on the composition of the frass has already been demonstrated for physicochemical properties, such as pH and EC (Klammsteiner et al. 2020), as well as for the diversity and abundance of microorganisms (Wynants et al. 2019; Kreft et al. 2026; Lomonaco et al. 2026). Summaries of various frass analyses from different substrates revealed an average pH of about 7.5 and a C/N ratio of about 15 (Lopes et al. 2022; Lomonaco et al. 2024). In their summary, Lopes et al. (2022) noted that, despite the different substrates used, the total C and N contents in the frass (around 37% and 3%, respectively) did not differ greatly. In contrast, levels of phosphorus, potassium, and micronutrients seemed to depend on the substrate used (Lopes et al. 2022).

Due to its composition, frass has already been identified as a high-value organic fertilizer by several previous studies, and plant-growth promotion by frass has been demonstrated for various crops (Chiam et al. 2021; Chirere et al. 2021; Abiya et al. 2022; Lomonaco et al. 2024; Gurung et al. 2025). For example, the

addition of frass derived from BSFL bioconversion of brewer's spent grains resulted in similar or higher yields in comparison to commercial organic fertilizer in the cultivation of kale (*Brassica oleracea*), tomatoes (*Solanum lycopersicum*), and French beans (*Phaseolus vulgaris*) (Anyega et al. 2021), as well as in maize (Zea mays) (Beesigamukama et al. 2020). With increasing application rates of frass derived from food waste and agricultural by-products, significant increases in biomass were observed in tomato plants (Salomon et al. 2025) and ryegrass (*Lolium multiflorum*) (Menino et al. 2021), respectively. Agustiyani et al. (2021) found significantly higher canopy and root weight and number of leaves for Pakchoi (*Brassica rapa*) that was cultivated with BSFL frass addition in comparison to the unamended negative control and positive control with chemical fertilizer. Watson et al. (2021) found that 5% BSFL frass stimulated soil microbial biomass growth. Additionally, nematicidal effects of the frass have been demonstrated in spinach (*Spinacia oleracea*) (Kisaakye et al. 2024). The addition of frass derived from pig manure also resulted in improved seed germination compared to the negative control (Liu et al. 2019).

Seed germination is a sensitive process in which the metabolism shifts from utilizing nutrients stored within the seed (heterotrophic) to nutrient uptake from the soil (autotrophic) (Bewley 1997; Ha et al. 2017a; Gómez-Álvarez et al. 2023). The seed germination process is influenced by several factors such as nutrient supply (Zhang et al. 2017), as well as physical factors, such as soil compaction and penetration resistance (Karssen 1980; Stibbe & Terpstra 1982; Letey 1985). These physical soil properties, such as penetration resistance, can be improved by adding compost (Aggelides & Londra 2000; Celik et al. 2004; Evanylo et al. 2016; Dolit et al. 2022; Noor et al. 2023). At the same time, seeds can also influence the soil, for example, through their native seed microbiome (Tyc et al. 2020; Shao et al. 2021). Principles of soil microbial ecology, such as community coalescence and priority effects, play a role in this context (Rillig et al. 2015; Chen et al. 2025). Priority effects refer to the fact that the timing and order of colonization of a vacant habitat determine the composition of the microbial community (Fukami 2015). In addition, social dynamics can also play a role in microbial communities, such as the production and cheating of shareable compounds (“public goods”) like siderophores (Butaitė et al. 2017).

The frass-mediated effects on plant growth have been attributed to different underlying mechanisms. A factor that has already been identified by previous studies is the provision of plant-essential macro- and micronutrients through frass amendments (Beesigamukama et al. 2020; Salomon et al. 2025). Frass contains concentrations of macronutrients (C, N, P, and K) that are similarly high or higher compared to manure (Gärttling & Schulz 2022) and to other composts (Salomon et al. 2025). The frass’ micronutrient contents vary greatly depending on the substrate used for bioconversion (Lopes et al. 2022). Beesigamukama et al. (2020) assumed that the increased nutrient availability resulting from the application of frass was the reason for the observed greater plant height, nutrient uptake, and chlorophyll concentration in frass-amended maize. With increasing N application rates from BSF frass, positive effects on the growth of tomato plants (Salomon et al. 2025) and ryegrass (Menino et al. 2021) were detected.

In addition to its nutrient composition, it is assumed that bioactive compounds also contribute to the potential of the frass as an organic fertilizer (Lopes et al.

2022). Bioactive compounds are substances that influence plant physiology without being nutritious and include representatives such as humic substances and phytohormones (Wong et al. 2020). Lopes et al. (2026) identified a significant concentration of phytohormones in BSFL frass derived from food waste. Green (2023) also analyzed BSFL frass from kitchen waste and detected phytohormones. In frass derived from bean and pea seed waste, Kaczor et al. (2026) also identified various phytohormones, thereby highlighting the importance of biostimulants in the frass for increased stress tolerance and performance of crops.

Microorganisms with plant growth-promoting properties can also act as biostimulants by alleviating stresses in plants (Mishra et al. 2022). By analyzing different frass microbiomes, members belonging to the plant growth-promoting bacteria group have been identified (Fuhrmann et al. 2022; Green 2023; Lomonaco et al. 2026). In a similar type of bioconversion using yellow mealworms (*Tenebrio molitor*), insect frass was produced, which also contained plant growth-promoting bacteria (Poveda et al. 2019). Green (2023) demonstrated the role of plant growth-promoting activity of *Enterococci* from BSFL frass, which were presumably transferred from the BSFL gut into the frass. Previous studies identified certain bacteria, such as *Enterococcus* spp. and *Pseudomonas* spp. in the guts of BSFL, despite differences in experimental setup, and therefore hypothesized these bacteria to be members of a possible core gut microbiota of BSFL (Wynants et al. 2019; Gold et al. 2020; Tanga et al. 2021). Lomonaco et al. (2026) identified bacteria from genera such as *Enterobacter* and *Bacillus* and their plant growth-promoting properties in frass derived from different substrates and found evidence that plant growth-promoting bacteria from BSFL frass contribute to the reported beneficial effects on plant growth. *Pseudomonas* spp. is another bacterial genus, which has already been identified in BSFL frass by various studies and which is known for its plant growth-promoting properties (Shumo et al. 2021; Praeg & Klammsteiner 2023).

Plant growth-promoting rhizobacteria are microorganisms that colonize the roots of plants and enhance plant growth by a wide range of mechanisms in the rhizosphere. The term ‘plant growth-promoting rhizobacteria’ was developed by Kloepper and Schroth after they inoculated seeds of crops (radish, sugar beet, potato) with plant growth-promoting *Pseudomonas* strains and detected yield increases up to 144% (Kloepper et al. 1980). Plant growth-promoting rhizobacteria are found in the soil as a component of the extensive soil microbiome and are particularly associated with roots of different plants (Glick et al. 1999). In addition, plant growth-promoting rhizobacteria were analyzed in different types of organic fertilizers, such as compost (de Brito et al. 1995) and pig manure (Wang et al. 2017). Bacteria with plant growth-promoting traits can enter the soil not only through the addition of organic fertilizers, such as pig manure (Zhang et al. 2011), but also as part of the seed microbiome (Johnston-Monje & Raizada 2011; Simonin et al. 2022).

Plant growth-promoting bacteria are able to support plants through direct and indirect mechanisms (Glick et al. 1999). Direct mechanisms include the production of plant hormones (e.g. indole-3-acetic acid, gibberellin), nitrogen fixation, and nutrient acquisition (Pérez-Montaño et al. 2014). For example, plant growth-promoting rhizobacteria such as *Bacillus* spp., *Enterobacter* spp. and *Pseudomonas* spp. have been shown to promote plant health, for example by

enhancing the nutrition of plants (Jha & Saraf 2015). Plant growth-promoting rhizobacteria indirectly promote plant growth by functioning a s biocontrol agents with mechanisms to decrease pathogenic effects on plants (de Brito et al. 1995; Glick et al. 1999).

Species of the genus *Pseudomonas* are frequently studied representatives of plant growth-promoting rhizobacteria. Among the *Pseudomonas* spp. that exhibit plant growth-promoting effects are species such as *P. fluorescens*, *P. putida* (Noumavo et al. 2013), *P. protegens* (Wang et al. 2020) and *P. aeruginosa* (Khan et al. 2022). *Pseudomonas* spp. are known for various mechanisms through which they can exhibit plant growth-promoting and antifungal activity, such as phosphate solubilization, Indole-3-acetic acid (IAA) production, and siderophore production (Qessaoui et al. 2019; Khan et al. 2022). Under iron-limited conditions, various *Pseudomonas* spp. can produce and release siderophores.

Siderophores are low molecular weight (400 - 1500 Da) secondary metabolites (Neilands 1995) that bind Fe (III) with high affinity. The ability of *Pseudomonas* spp. to produce siderophores can be detected, for example, using the chrome azurol S assay (Schwyn & Neilands 1987). Based on their different functional groups that bind to Fe (III), siderophores are divided into four classes: hydroxamates, catecholates, carboxylates, and the mixed type, which contains two or three of these functional groups (Timofeeva et al. 2022). The structures of approximately 270 of more than 500 different known types of siderophores have been characterized (Ahmed & Holmström 2014). The various siderophore types differ in their high iron affinities by a range of 30 orders of magnitude (Miethke & Marahiel 2007). For example, microbial siderophores such as rhizobactin (carboxylate type) have a stability constant of $Kf = 10^{19}$, pyoverdin (mixed type) $Kf = 10^{24}$ and enterobactin (catecholate type) $Kf = 10^{52}$ (Ahmed & Holmström 2014).

This high iron affinity is responsible for the plant growth-promoting and antifungal properties mediated by siderophores. The siderophores released by microorganisms bind Fe (III) with high affinity and form soluble iron complexes that increase iron bioavailability and thereby improve plant growth (Timofeeva et al. 2022). The antifungal properties of siderophores result from their higher affinity for iron in comparison to fungi, which increases competition for iron and thereby inhibits the growth of pathogens (Kloepper et al. 1980). Because of these properties, siderophores, such as pyoverdine produced by *Pseudomonas* spp., have been extensively studied.

Iron is an essential micronutrient for plants, which is required for various key processes in plant metabolism, such as chlorophyll synthesis, respiration and the electron transport chain during photosynthesis (Marschner 2011). Although iron is the fourth most abundant element in the Earth's crust, it is often present in the soil in its oxidized form as ferric iron (Fe III), which is not bioavailable due to its low solubility. The solubility of iron in the soil is influenced by the pH level and is lowest between pH 7.5 and 8.5 (Lindsay 1991). Depending on the pH, the iron concentration in soil ranges from (10^{-7}- 10^{-9} M), which is four orders of magnitude lower than the optimal requirement for plants and microorganisms (Zuluaga et al. 2023).

Since iron is mainly present in the soil as Fe (III), plants have two different uptake strategies for the iron they need. In plants that utilize iron uptake

Strategy I, Fe (III) is reduced by an enzyme at the plasma membrane before being transported across the cell membrane, and the solubility of Fe (III) is additionally increased by acidifying the rhizosphere using the plants' proton pumps. Plants of the *Poaceae* family use iron uptake Strategy II, in which the plants produce and release phytosiderophores which chelate Fe (III) with high affinity and are subsequently reabsorbed by the plant (Connorton et al. 2017).

Under iron-limited conditions, some *Pseudomonas* species can produce and secrete siderophores, thereby increasing the bioavailability of iron to plants and thus promoting plant growth. Plant growth promotion under Fe- limited conditions (soil pH = 8.1) was detected in apple rootstocks that were inoculated with a pyoverdine-producing *Pseudomonas* spp. The inoculated apple rootstocks exhibited improved iron uptake as well as increased root development and biomass, which was associated with the ability of siderophores to make iron available to plants under iron-limited conditions (Ha et al. 2017a).

3. Materials and Methods

3.1 Materials

3.1.1 Materials for bioconversion

Substrate for bioconversion

The substrate used for the bioconversion was pig (*Sus scrofa*) manure. Pig manure was obtained from specific pathogen-free pigs of the Swedish University of Agricultural Sciences (SLU) pig research centre (Lövsta, Sweden) and stored at 4 °C until further use. For more detailed information on the substrate, see Appendix 1.

Black soldier fly larvae

The larvae used in this study were obtained from the black soldier fly population at SLU, which has been running continuously since 2015. The larvae used were about 4 days old and measured about 0.2 cm at the start of the experiment.

3.1.2 Materials for greenhouse experiment

Frass

The frass produced during the bioconversion (phase 1) was pooled from the three replicates and mixed into a single 50-L plastic bag. The homogenised frass was divided into two equal portions of about 5 kg each. One portion (hereafter referred to as fresh frass) was kept in a dark plastic bag at 8 °C in the cold room. The remaining frass was sterilised by autoclaving, as described for the sterile soil.

Soil

The soil used in this study was collected from a mixed topsoil-subsoil pile that had been excavated from a grass-covered area on the SLU campus (Uppsala, Sweden). The soil from the pile was dug using a shovel and was placed into several plastic containers (60 L × 40 W × 12 H cm, Eurobox, Auer, Germany) and kept at room temperature to enable thawing. The thawed soil was air-dried at room temperature for a period of six days. During this period, the soil was mixed by hand once per day to accelerate drying. After six days, the air-dried soil from the different plastic containers was pooled and mixed. The pooled soil was sieved through a 6 mm custom-made sieve to remove debris, including stones and plant parts, to achieve a uniform texture.

For the greenhouse experiment, the soil was divided into two portions. One portion (about 10 kg; hereafter referred to as fresh soil) was transferred into a dark plastic bag and stored for three weeks at room temperature until use. The remaining soil was split into two 15-kg aliquots in autoclave bags and sterilised by autoclaving at 120 °C for 20 min. After the initial autoclaving, the soil was

allowed to cool overnight and then autoclaved twice more under the same conditions to eliminate the resident soil microbiome (both spore-forming and non-spore-forming microorganisms) and weed seeds. The autoclaved soil (hereafter referred to as sterile soil) was transferred to the greenhouse until use.

Seeds

Seeds of kale *(Brassica oleracea convar capitata*) were obtained from Magic Garden Seeds (Regensburg, Germany).

3.1.3 Materials for microbiological analysis

Chemicals

Isolation and enumeration of *Pseudomonas* spp. were performed using *Pseudomonas* selective agar (PSA) medium and peptone buffer (Merck, Darmstadt, Germany). To prepare the peptone buffer, Tween-80 (0.1 % v/v; Sigma-Aldrich, St Louis, MO USA) was added.

The following chemicals were used for the CAS assay:
Chrome azurol S (dye content 45 %), Iron (III) chloride hexahydrate (≥ 98 %), Hexadecyltrimethylammoniumbromide (HDTMA; ≥ 98 %), 8-hydroxyquinoline (98,5 %) and PIPES (≥ 99.5 %) were obtained from Sigma-Aldrich (St Louis, MO USA). The Potassium phosphate (KH_2PO_4; 99 %), Sodium chloride (NaCl; ≥ 99 %), Ammonium chloride (NH_4Cl; ≥ 98 %) and Sodium hydroxide (NaOH; ≥ 97 %) were purchased from VWR (Leuven, Belgium) and Bacto™ Casamino Acid (Thermo Fisher Scientific, Waltham, MA, USA).

Chrome azurol S (CAS) agar plates

CAS agar plates were prepared according to Schwyn and Neilands (1987) following the protocol of Louden et al. (2011).
First, all glassware was cleaned with 6 M HCl to remove trace elements, then rinsed with ddH_2O. Thereafter, the blue dye (see Table 1) and the mixture solution (see Table 2) were prepared.

Table 1. Preparation of blue dye according to Louden et al. (2011).

Solution	Ingredients
Solution 1	Prepared by dissolving 0.06 g of CAS in 50 mL of ddH2O (Milli Q water)
Solution 2	Prepared by dissolving 0.0027 g of FeCl3×6 H2O in 10 mL of 10 mM HCl.
Solution 3	Dissolve 0.073 g of HDTMA in 40 ml of ddH2O.
Final Solution	Mix Solution 1 with 9 ml of Solution 2. Then mix with Solution 3. (Solution should now be a blue color. Autoclave and store in a plastic container/bottle.)

Table 2. Preparation of mixture solution according to Louden et al (2011).

Solution	Ingredients
Minimal Media 9 (MM9) Salt Solution Stock	Dissolve 15 g KH_2PO_4, 25 g NaCl, and 50 g NH_4Cl in 500 ml of ddH_2O.
20% Glucose Stock	Dissolve 20 g glucose in 100 ml of ddH_2O.
NaOH Stock	Dissolve 25 g of NaOH in 150 ml ddH_2O; pH should be ~12.
Casamino Acid Solution	Dissolve 3 g of Casamino acid in 27 ml of ddH_2O. Extract with 3% 8-hydroxyquinoline in chloroform to remove any trace iron. Filter sterilize.

The final product was mixed together in a 1000 mL laboratory bottle (borosilicate glass bottle, polypropylene cap, Duran®), and during all additions, the bottle was placed on a magnetic stirrer, and the medium was stirred continuously. To prepare the final solution, 100 mL of MM9 salt solution was mixed into 750 mL of ddH_2O. Then, 32.24 g of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) was dissolved. Because PIPES does not dissolve at pH < 5, the pH was increased to 6, PIPES was added, and the pH was adjusted to 6.8. After that, 15 g of Bacto agar was added. After the Bacto agar visually dissolved, the bottle was removed from the magnetic stirrer and capped. The mixture was autoclaved. After autoclaving, the bottle was cooled to 50°C at room temperature. Between the following additions, the bottle was gently stirred by hand. First, 30 mL of sterile Casamino acid solution and 10 mL of sterile 20% glucose were mixed in. Then, 100 mL of the blue dye was introduced. The dye was poured along the glass wall, and the final product was thoroughly mixed until all components were visually in solution. After the medium was well mixed, the plates were poured aseptically.

The chrome azurol S assay (CAS) was developed to determine whether bacterial isolates are capable of producing siderophores (Schwyn & Neilands 1987). In the CAS assay, halos form when iron (Fe III) is removed from the dye complex by a chelator with a higher affinity for iron, such as a siderophore, causing the dye's color to change from blue to orange (Schwyn & Neilands 1987). The size of the halos is influenced by several factors, such as the type and binding constant of the siderophore, as well as the growth rate of the tested isolate (Schwyn & Neilands 1987) and the quantity of secreted siderophores (Shin et al. 2001). For this reason, the CAS assay is classified as a purely qualitative method in various studies (Milagres et al. 1999; Arora & Verma 2017).

Pseudomonas *selective agar plates and peptone buffer*

The PSA medium was prepared following the instructions provided by the manufacturer (Merck). Upon autoclaving, the medium was allowed to cool and aseptically poured into 90 mm Ø plates (Sarstedt, Nümbrecht, Germany) that were stored at 4 °C until use. Similarly, the peptone buffer (pH 7.4) was prepared following the instructions provided by the manufacturer (Merck). Before autoclaving, Tween-80 (0.1 % v/v; Sigma-Aldrich, St Louis, MO USA) was

added to the buffer and stirred using a magnetic stirrer (IKA-Werke Staufen, Germany) to dissolve the ingredients. The buffer was then autoclaved at 121 °C for 30 min. After cooling, the buffer was stored at 4 °C until use.

3.2 Experimental outline

The structure of this study can be divided into two phases (see Figure 1).

In Phase 1, pig manure was bioconverted by BSFL. After 15 days, the larvae were separated from the frass. Subsequently, parameters of the resulting frass, such as volatile solids (VS) and total solids (TS), were analyzed and the frass was tested for phytotoxicity. The frass was microbiologically examined for the number of *Pseudomonas* bacteria present, and the detected *Pseudomonas* spp. isolates were screened for their ability to produce siderophores.

In Phase 2, the produced frass was tested in a greenhouse experiment for its effect on the growth of kale (*Brassica oleracea*). For this purpose, four treatments were prepared: sterile soil (C) as a negative control, sterile soil mixed with sterile frass (X), sterile soil mixed with fresh frass (Y), and fresh soil mixed with fresh frass (Z). After 38 days, the experiment was terminated, and the plant growth parameters were determined. In addition, rhizosphere samples from the four treatments were analyzed microbiologically to determine the number of *Pseudomonas* spp. bacteria present. Finally, *Pseudomonas* isolates were screened for their ability to produce siderophores.

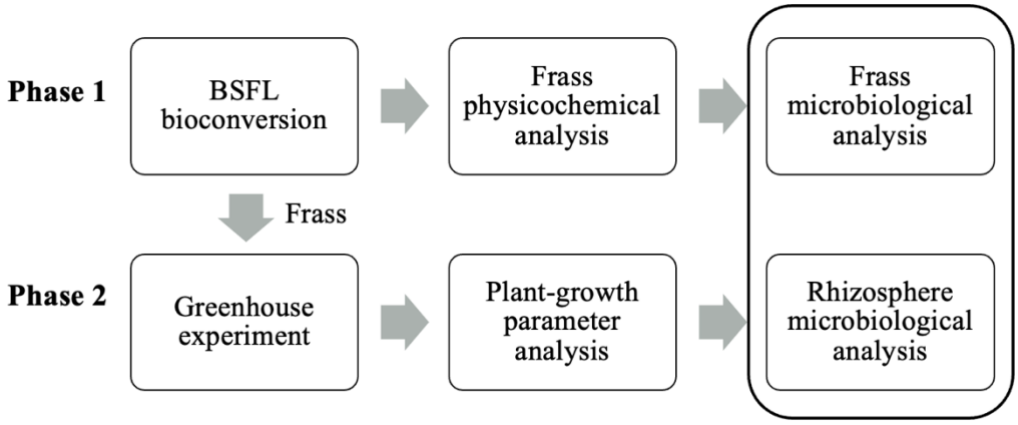


Figure 1. Schematic overview of the experimental design, illustrating the two main phases and their key components. The circle connecting "frass microbiological analysis" and "rhizosphere microbiological analysis" indicates that the same method was applied in both analyses.

3.2.1 Phase 1: Bioconversion of pig manure by BSFL

Bioconversion was performed in triplicate using dark plastic containers (60 L × 40 W × 12 H cm, Eurobox, Auer, Germany). The substrate (4 kg) was added to each box, upon which seed larvae were added to give a larval density of 5 larvae cm^{-2}. Each larva received a feed dose of 0.2 g VS $larva^{-1}$, spread over three feeding events. The feeding was done at the start of the bioconversion, as

well as on day 3 and day 6. The three boxes were stacked on top of each other in a bakery trolley, with a spacing of about 4 cm between them, and incubated in a temperature-controlled room. The boxes were rearranged every other day to account for possible temperature variations depending on the height of the box in the room. The temperature and humidity in the room were monitored using Tinytag Plus 2 data loggers (Gemini, Chichester, UK). The average room temperature and humidity during BSFL bioconversion were 24.1 ± 2.6 °C and 31.7 ± 1.7 %, respectively. The bioconversion process was terminated after 15 days by sieving the larvae from the frass using sieves with a mesh size of 5 mm and 2 mm. The prepared frass was packaged and stored at 8°C, and the larvae were stored in the freezer until further use.

The frass and the larvae were subsequently analyzed for TS and VS, and the bioconversion process parameters, bioconversion efficiency (BCE) and material reduction (TS-based) were calculated from these values. For more detailed information on the bioconversion process parameters, see Appendix 1.

The pH level of the fresh frass was analyzed by an external accredited laboratory (Eurofins Agro Testing). A seed germination test was conducted to evaluate the phytotoxicity of the frass. Finally, the frass was microbiologically analyzed by isolating and enumerating *Pseudomonas* spp. and screening for siderophore-producing *Pseudomonas* isolates using the CAS assay.

3.2.2 Phase 2: Greenhouse experiment with BSFL frass amendments

The greenhouse experiment was conducted at the Ecology Department, SLU (Uppsala, Sweden) from 09th March to 16th April 2026. The frass obtained from the bioconversion experiment (Section 3.2.1) was used. In preparation for the treatments, both a part of the soil and a part of the frass were sterilized by autoclaving.

The greenhouse experiment consisted of four treatments. Each treatment was conducted in ten replicates. The treatments were: sterile soil (C) as a negative control, sterile soil mixed with sterile frass (X), sterile soil mixed with fresh frass (Y), and fresh soil mixed with fresh frass (Z). For treatments X, Y and Z, frass was added at 10 % w/w. The arrangement of the pots in the greenhouse was randomized (see Appendix 2 for an overview).

Mixtures were prepared by weighing and pooling the soil or the soil with frass for each treatment into 50-L bags. This approach was performed to ensure homogeneity of the mixtures. Afterwards, for each replicate, 700 g of the sterile soil for the negative control or soil-frass mixture from the respective pool was added into 1 L designated pots. Using these mixtures, an external laboratory (Eurofins Agro Testing, Jena, Germany) conducted a soil analysis for each prepared treatment to determine the pH level and nutrient content, including the macronutrients potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), as well as the micronutrients boron (B), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) (see Appendix 3).

For planting, three holes were prepared in the centre of the soil in each pot, arranged in a triangle with a 2 cm spacing between them. One seed of kale was

placed in each of these holes at a 0.5 cm depth and then covered with the soil or soil-frass mixture and watered until the soil surface was wet. Each pot was placed on a separate plastic tray to retain water and prevent cross-contamination from the effluent. Pots were watered before planting and thereafter every other day during the course of the experiment. Watering was performed manually until drainage occurred, which was checked visually at the pot base. The conditions in the climate-controlled greenhouse were set to a temperature of 25/20 °C (day/night) for the first 24 h, then to 17/15 °C (day/night) for the rest of the experiment. The artificial lamps (BX120 Solray 385, Valoya, Helsinki, Finland) provided 12 h of light per day at an intensity of 250 µmol m^{-2} s^{-1}. Humidity and temperatures were monitored throughout the entire experiment using TinyTag sensors.

Ten days after sowing, the germination rate was determined for each pot. Thinning was done 24 days after planting (at which point the first pair of true leaves appeared) to reduce the number of plants to a single vigorous plant per pot. The experiment was terminated after 38 days. At the end of the experiment, the plant growth parameters of the kale were analyzed. In addition, the rhizosphere soils from the four treatments were subjected to microbiological analysis. This included both the isolation and enumeration of *Pseudomonas* spp. in the rhizosphere and the screening for siderophore-producing *Pseudomonas* spp. isolates using the CAS assay.

3.3 Sampling

To analyze the TS and VS of frass and larvae, samples were taken in triplicate from the thoroughly mixed material. For each sample, 10 g of fresh frass and 15 g of larvae were used.

In preparation for the microbial analysis of the frass, the frass was thoroughly mixed, and a sample of approximately 150 g was taken, transferred into plastic sampling bags, and stored at 4 °C until further processing.

To analyze plant growth across the different treatments, the height of each plant and the number of developed leaves were measured. Eight out of ten plants from each greenhouse treatment were randomly selected for a more comprehensive analysis. Of these, five were used for each treatment to determine the above-ground biomass. The remaining three plants in each treatment were used for the leaf area analysis and rhizosphere soil analysis.

The rhizosphere soil samples were collected for three plants per treatment. To do this, the root mass with attached soil was removed from the pot and gently shaken to remove bulk soil. The roots and attached soil (rhizosphere soil) were transferred into plastic sampling bags and kept in a cool box with ice packs. After sampling, the rhizosphere soil samples were stored at 4°C for 7 days until microbiological analysis.

For the microbiological analysis to enumerate *Pseudomonas* spp., 5 g of the material under investigation (frass or rhizosphere soil) was used. For each frass or rhizosphere sample, five *Pseudomonas* spp. isolation agar plates were prepared. From each of these plates, 12 colonies were selected randomly and screened for siderophore production, resulting in 60 isolates per replicate and a total of 180 isolates for the frass analysis. For the rhizosphere soil analysis, due to the extensive analysis, 8 colonies were selected randomly from each of the

Pseudomonas isolation agar plates. This resulted in 40 colonies for each of three replicates per treatment and a total of 480 isolates screened for siderophore production.

3.4 Analyses

3.4.1 Physico-chemical analyses

Total solids and volatile solids

To determine TS, pre-weighed samples in aluminium weighing boats (Eldorado, Stockholm, Sweden) were dried at 65 °C for 48 h in a convection-type hot-air oven (Electrolux, Stockholm, Sweden). To determine the VS, the dried samples were burned at 250 °C for 2 h followed by 550 °C for 4 h in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany).

Assessment of frass phytotoxicity

The phytotoxicity of frass was assessed using a seed germination assay (Yang et al. 2021). Frass samples obtained from each bioconversion box were used in the assay. Triplicates were prepared for each sample. First, an extract of the frass was prepared. To do this, 10 g of frass sample was weighed into a 50-mL Falcon tube and homogenized in 100 mL of distilled water. The diluted samples were shaken for 1 h (at 35 % speed) using a rotary shaker (Stålprodukter, Uppsala, Sweden) and afterwards centrifuged for 15 min using a swing bucket centrifuge (Heraeus-Labofuge-400, Thermo Fisher Scientific, USA). The resulting supernatant was filtered through a paper filter (5-13 µm, VWR 413, Leuven, Belgium). The filtrates were used to test seed germination.

For the seed germination assay, 10 seeds were placed on filter paper in a 90 mm Petri dish (Sarstedt, Nümbrecht, Germany). Then, the filter paper was moistened with 10 mL of each filtrate sample or deionized water (as a control). Tests were conducted in triplicate for each filtrate sample. Afterwards, Petri dishes were randomized and incubated in the dark at 20 °C in a well-ventilated chamber (Binder FP 115, Tuttlingen, Germany). Germination was assessed after 5 days of seed incubation by counting the number of emerged seeds and measuring their radicle length using a digital calliper (VWR, Leuven Belgium). These values were used to calculate the germination index (GI).

3.4.2 Microbial analyses

Isolation and enumeration of Pseudomonas *spp.*

For the isolation of bacteria, 5 g of sample was weighed into a 100-mL sterile Erlenmeyer flask. Then, 45 mL of peptone buffer was added. Flasks were covered with sterile aluminium foil and homogenized by shaking at 120 r.p.m. for 30 min (Heidolph Rotamax 120, Schwabach, Germany). This suspension was considered the initial dilution from which 10-fold dilution series were prepared in test tubes using peptone buffer. For each dilution, the contents of the test tube were homogenized using a vortex (Vortex-Genie 2; Scientific Industries, New York,

USA). By repeating these steps, dilutions from 10^{-1} to 10^{-5} were prepared. Using a manual pipette, 0.1 mL of the desired dilution was dispensed onto PSA agar plates. The liquid was spread evenly on the agar surface across the plate using an inoculation spreader (VWR). Five replicate plates from selected dilutions were prepared for each sample. Plates were incubated at 28 °C for 48 h (VWR INCU-Line). Afterwards, the bacterial colonies on each plate were enumerated. Based on this, the colony-forming units (CFU) per milliliter and their logarithm were calculated.

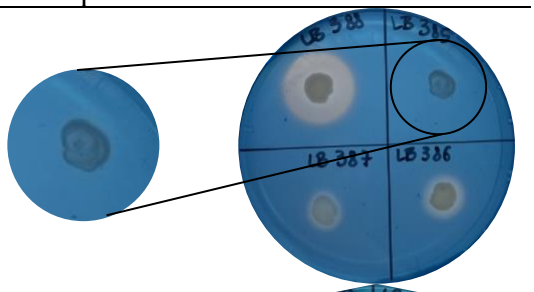
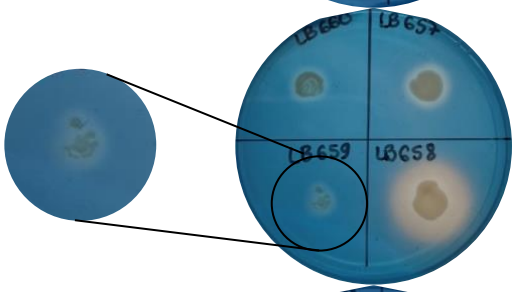
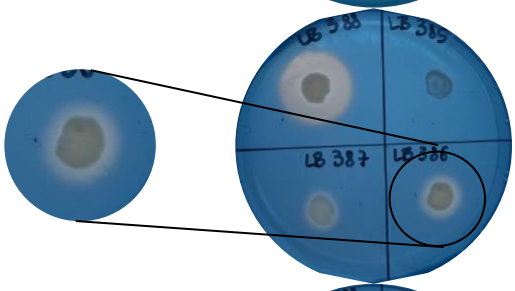
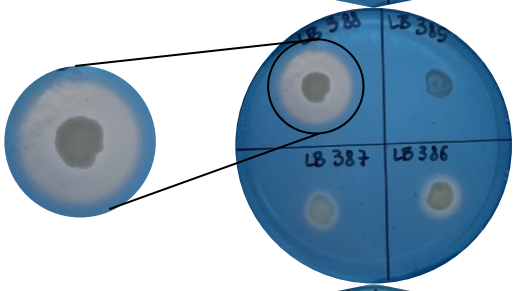
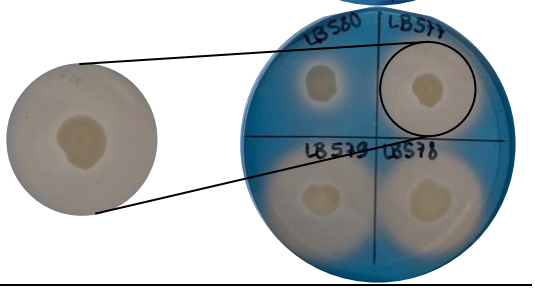
Screening bacterial isolates for siderophore production

Screening for siderophore production was performed on *Pseudomonas* spp. isolates obtained from fresh frass and rhizosphere soil.

For this purpose, *Pseudomonas* spp. colonies that had developed on the *Pseudomonas* selective agar plates after 48 h of incubation at 28 °C were used. Material was collected from the edge of a colony using an inoculation loop (Sarstedt, Nümbrecht, Germany). This material was then transferred to the surface of the CAS agar plate in the form of a circle with a diameter of about 3 mm. The CAS agar plates were visually divided into quarters, and one circle was transferred to the middle of each quarter. Each selected and transferred colony was assigned an ID for subsequent screening. The CAS agar plates were then incubated at 28 °C for 48 h (VWR Incu-Line).

After incubation, the isolates were screened for siderophore production. In the CAS assay, halos form around the isolates if they are capable of producing siderophores. Since the size of the halos is associated, among other things, with the amount and type of siderophores produced, the different sizes observed were recorded. To do this, the distance between the outer edge of the colony and the outer edge of the halo was measured in milimeters. This distance was measured at three points for each isolate with a halo to account for the fact that the colonies and halos are not always perfectly circular, and the average was then calculated. Categories ranging from 0 to 4 were defined based on different halo sizes to evaluate the results. The screened halos were assigned to these categories based on their size (see Table 3).

Table 3. Categorization of halos obtained from the CAS assay of Pseudomonas *spp. isolates, based on the average diameter size of halos in mm. Halos were scored after incubating CAS agar medium plates for 48 h at 28 °C. "*x*" represents the size of the halo to be classified.*

Category	Halo size (mm)	Example
0	0	
1	<1	
2	1 ≤ x < 2	
3	2 ≤ x < 4	
4	x ≥ 4	

3.4.3 Plant growth parameters

For the parameter “number of leaves”, the number of fully developed leaves, excluding the cotyledons, was counted. Plant height was measured from the soil surface to the node of the lowest leaf. To determine the above-ground biomass, the above-ground section of the plant was harvested and dried at 65 °C for 48 h in the oven. The dried above-ground part was then weighed. Leaves from three of the randomly selected plants in each treatment were detached and photographed from a fixed height using a phone camera attached to a stand. Leaf images were processed using ImageJ software (NIH, Bethesda, USA).

3.5 Calculations

Bioconversion efficiency
The bioconversion efficiency was calculated on the basis of total solids as:

$$BCE = \frac{Larvae_{Tot.TS}}{Pig\ manure_{Tot.TS}} \times 100$$

where the TS of larvae and pig manure is given in g and BCE is expressed as %.

Material reduction
The material reduction was calculated on the basis of total solids as:

$$Material\ reduction\ _{TS.} = 1 - \frac{Frass_{Tot.TS}}{Pig\ manure_{Tot.TS}} \times 100$$

where the TS of frass and pig manure is given in g and material reduction is expressed as %.

Seed Germination Index
The seed germination index (GI) was calculated as:

$$GI = \frac{number\ of\ germ.seeds\ in\ extract}{number\ of\ germ.\ \ seeds\ in\ control} \times \frac{root\ length\ in\ extract}{root\ length\ in\ control} \times 100\%$$

where root length is given in mm, and GI is expressed as %.

3.6 Statistics

The datasets were first tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test) with a significance level set at $\alpha = 0.05$. Subsequently, a one-way analysis of variance (ANOVA) was conducted to test the significance of the differences between the results. In cases where significant differences were revealed by the ANOVA, Tukey's post-hoc test was performed for pairwise comparisons to identify significant differences in the tested results. A significance level of $\alpha = 0.05$ was applied for the Shapiro-Wilk test, Levene's test, the one-way ANOVA, and Tukey's post-hoc test. Statistical analysis was performed using R Core Team (2020).

4. Results

4.1 Frass parameters

After bioconversion, the produced frass had an average TS and VS of about 50 % and of 83 %, respectively (Table 4). The bioconversion efficiency was about 11 %, while the material reduction was about 25 % on a TS basis (Table 4). The pH of the fresh frass was 7.2.

A seed germination test was used as a proxy for evaluating the presence of substances causing phytotoxicity in the obtained frass, revealing an average GI of 120 %. Since the GI is calculated relative to the control, values above 100 % may occur if the sample has better germination rates than the control.

*Table 4. Process parameters and frass characteristics after bioconversion of pig manure using black soldier fly larvae (BSFL). Total solids (TS) and volatile solids in percent of total solids (VS). Bioconversion efficiency (BCE) and material reduction in percent based on TS, and pH of the fresh frass. Seed germination index was evaluated based on kale (*Brassica oleracea*) seeds (GI). Values presented are mean ± standard deviation (SD).*

Parameter	Value
TS (%)	49.8 ± 2.7
VS (% of TS)	82.9 ± 0.2
BCE_{TS} (%)	10.8 ± 0.4
Material $reduction_{TS}$ (%)	25.1 ± 2.0
pH	7.2
Germination index (%)	120.4 ± 30.5

4.2 Seed germination and seedling emergence

Germination and emergence of kale seeds were assessed 10 days after planting and followed two and three weeks after planting (Figure 2). During the first eight days, emergence rates seemed to have increased more rapidly in treatments with sterile soil amended with sterile frass (X) and fresh frass (Y) (47 % and 53 % of seeds had emerged by then) compared to those for unamended sterile soil (C) and fresh soil with fresh frass (Z) (20 % and 17 % of emerged seeds at that time) (Figure 2a).

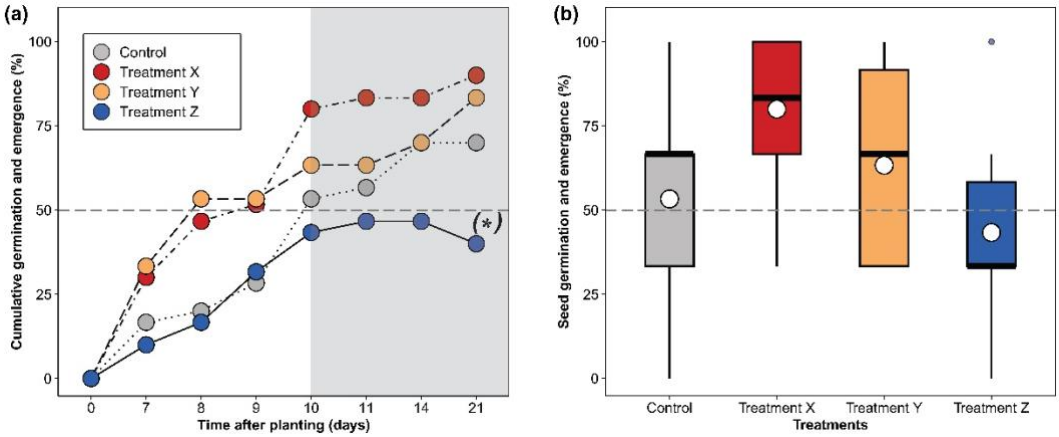


*Figure 2. Impact of black soldier fly frass on germination and emergence of kale (*Brassica oleracea*) seedlings in the greenhouse pot experiment: a) line graph showing the trends in cumulative germination and seedling emergence of kale for a period of 21 days after planting. Seed germination was considered complete and recorded on day 10 but was followed until day 21 (shaded region). The asterisk (*) denotes dead seedlings observed; b) Boxplots summarizing germination and seedling emergence of kale recorded at 10 days after planting. Control (sterile soil), Treatment X (sterile soil amended with sterile frass), Treatment Y (sterile soil amended with fresh frass) and Treatment Z (fresh soil amended with fresh frass). Different letters indicate statistically significant differences. Boxes display interquartile range (IQR), the bold line inside each box represents the median, the white circle represents the mean, and the range of data is represented by whiskers (vertical lines). Data points farther than 1.5 × IQR from boxes denote outliers.*

At day 10, germination and seedling emergence did not differ significantly among treatments ($p = 0.051$; Figure 2b). However, germination and seedling emergence was highest in Treatment Y (60 %) and Treatment X (80 %), compared to about 50 % in the control and Treatment Z (Figure 2b).

Until day 10, the number of germinated seeds for X and Y increased more prominently than those for C and Z. By day 10, which is the germination period specified by the manufacturer, cumulative emergence ranked X 80 %, Y 63 %, C 53 % and Z 45 %. After day 11, emergence for Treatment Z plateaued at 47% and later decreased to 40 % by week 3 due to the observed mortality of two seedlings. Between day 14 and 21 after planting, seed emergence in Treatment X reached the highest final value of 90 %, followed by Y with 83 % and the control, which remained with only 70 % of seeds emerged. During the experiment, 72 % of the planted seeds across all treatments emerged.

4.3 Plant growth parameters

Plant growth parameters were measured at termination of the experiment. The plant growth parameters assessed were above-ground biomass, leaf area, plant height, and number of leaves (Figure 3). Above-ground biomass differed significantly between treatments ($F = 25.4$, $p < 0.001$). Above-ground biomass was highest in Treatment Y (sterile soil with fresh frass) with 1.08 g, followed by Treatment X (sterile soil with sterile frass) with 0,69 g (Figure 3a). The control (sterile soil) and Treatment Z (fresh soil with fresh frass) had lower above-ground biomass (0.37 g and 0.32 g), which did not differ significantly.

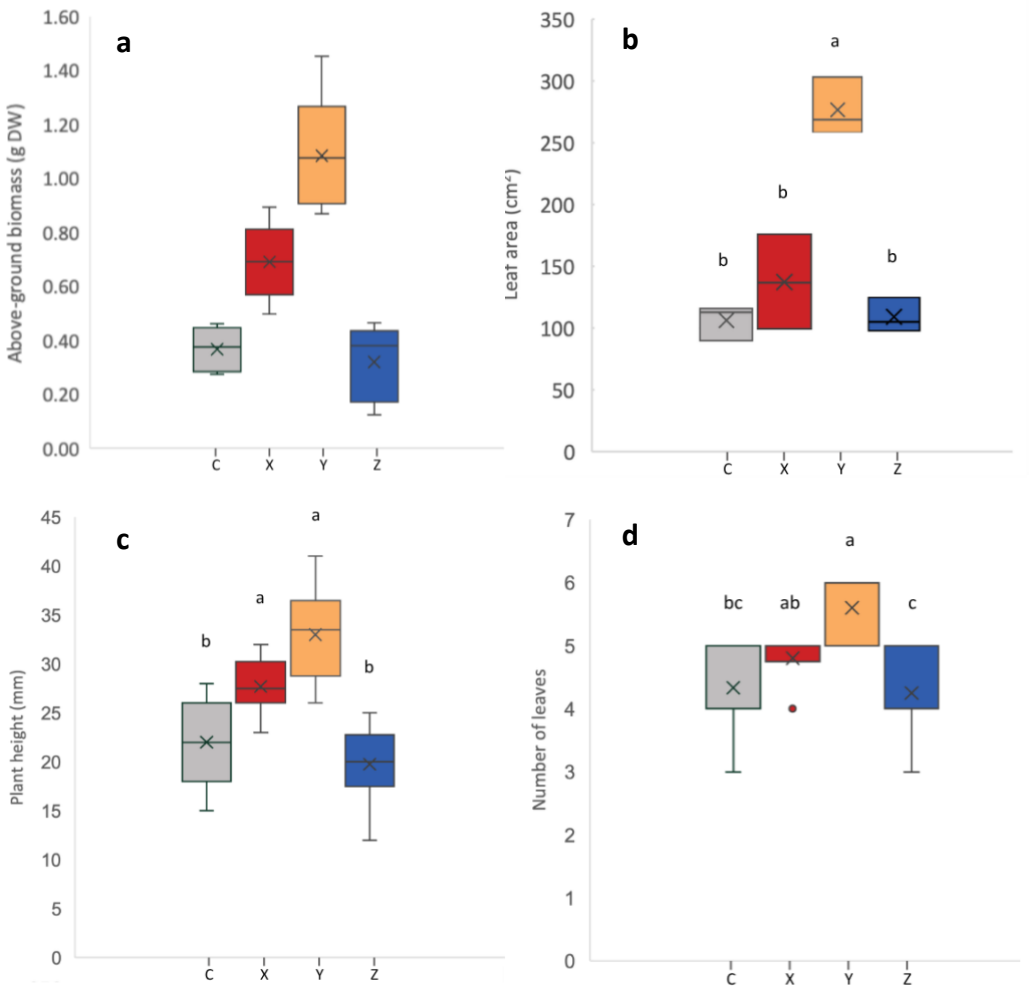


Figure 3. Plant growth parameters of kale plants from the greenhouse experiment with four different treatments, obtained 38 days after planting. a) above-ground biomass (g DW) b) leaf area (cm²) c) plant height (mm) d) number of leaves. Treatments: ***C*** *sterile soil,* ***X*** *sterile soil with sterile frass,* ***Y*** *sterile soil with fresh frass,* ***Z*** *fresh soil with fresh frass. Different letters indicate statistical differences (p = 0.05). Boxes display interquartile range (IQR); the bold line inside each box represents the median, the "×" represents the mean, and the range of data is represented by whiskers (vertical lines). Data points farther than 1.5 × IQR from boxes denote outliers.*

Leaf area differed significantly among treatments ($F = 30.1$, $p < 0.001$) (Figure 3b). The highest value for leaf area was observed in Treatment Y (276.4 ± 25.0 cm^2), whereas the other treatments did not differ significantly. Plant height was significantly different between treatments ($F = 11.7$, $p < 0.001$) (Figure 3c). Treatment Y, with an average of 33.0 ± 4.74 mm, and Treatment X with 28.0 ± 3.02 mm, had significantly ($p < 0.05$) higher values than the control (C) and Treatment Z. The number of leaves was significantly different among treatments ($F = 10.9$, $p < 0.001$) (Figure 3d). Treatment Y displayed the highest value, with an average of 5.6 ± 0.49 leaves per plant, whereas the other treatments did not differ significantly. Across all parameters, the plants in the treatment with sterile soil and fresh frass (Y) showed the highest values.

Additionally, visual differences in root architecture were observable between the treatments at the end of the experiment, 38 days after planting (Figure 6). Plants in treatments with sterile soil combined with sterile (X) and fresh frass (Y) seemed to have denser and more branched root systems than plants in the control (C) and the treatment with fresh soil and fresh frass (Z).

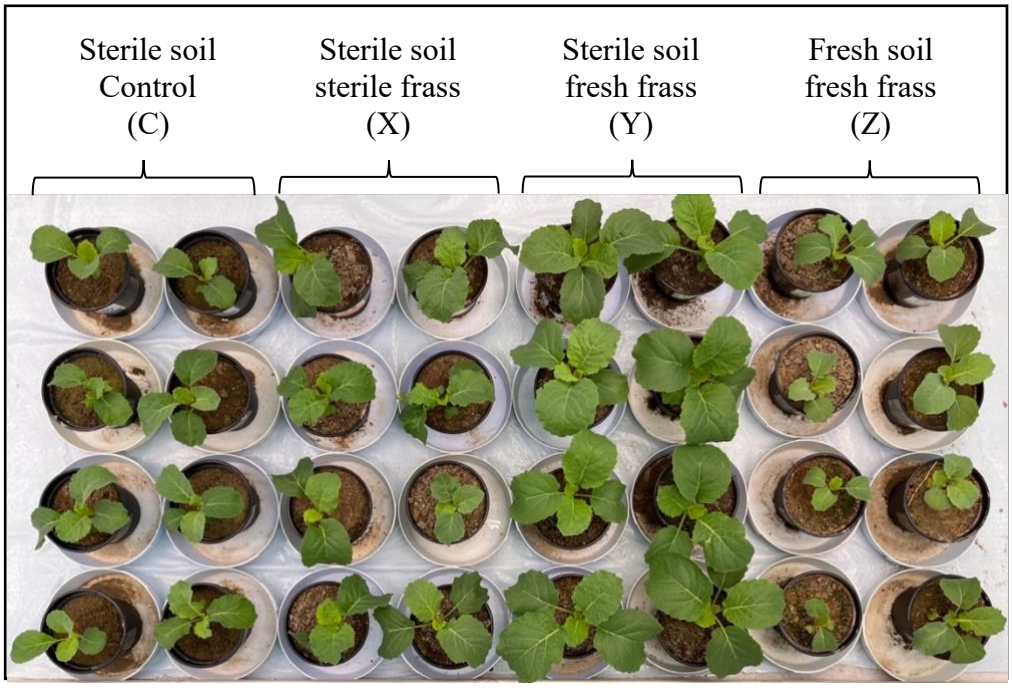


Figure 4. Comparative top view of the different treatments at the end of the greenhouse experiment 38 days after planting. Pots were rearranged according to treatments for photography.

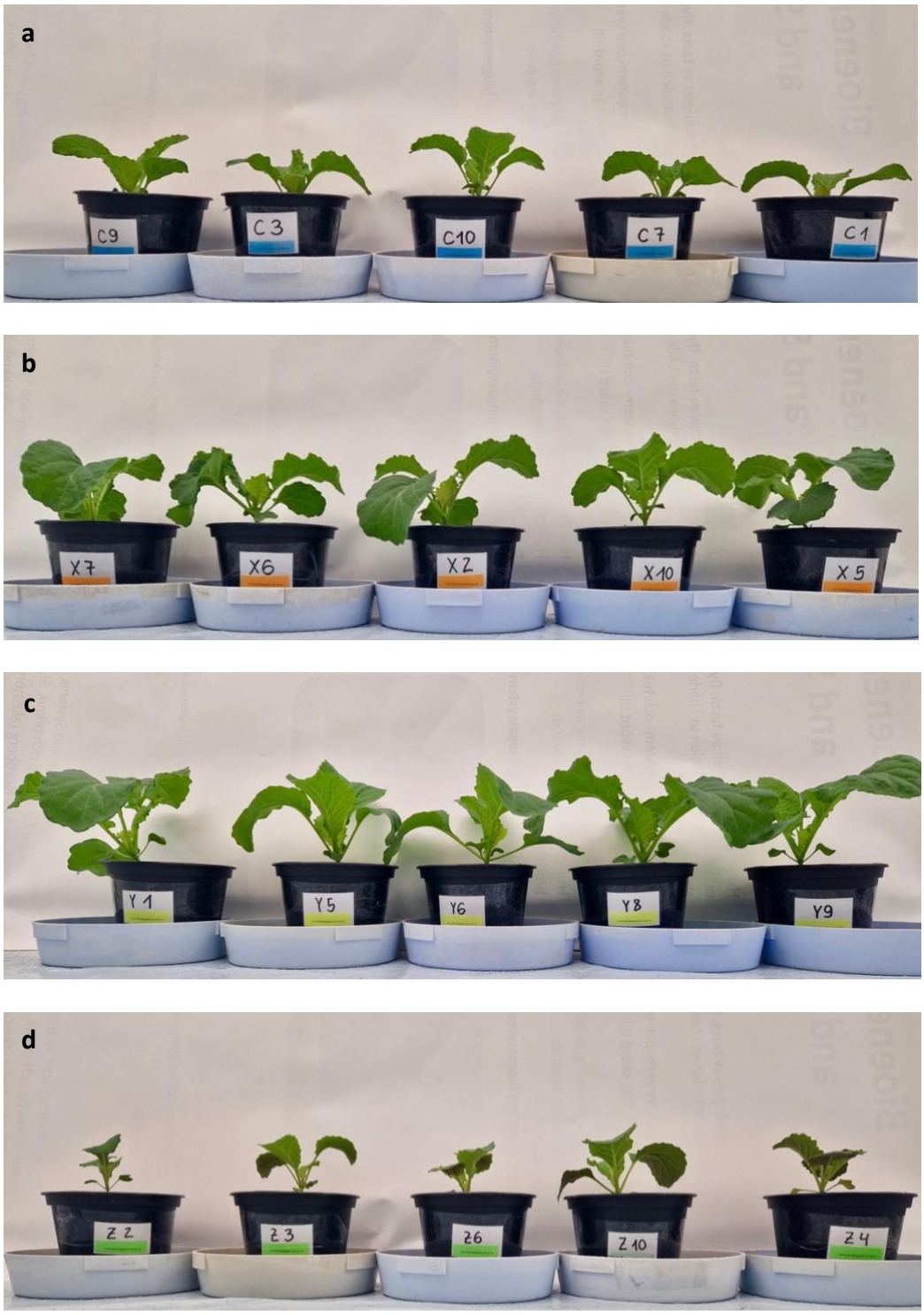


Figure 5. Vertical view of above-ground section of five randomly selected kale plants from each treatment at the end of the greenhouse experiment (38 days after planting): a) sterile soil (C), b) sterile soil with sterile frass (X), c) sterile soil with fresh frass (Y), d) fresh soil with fresh frass (Z).

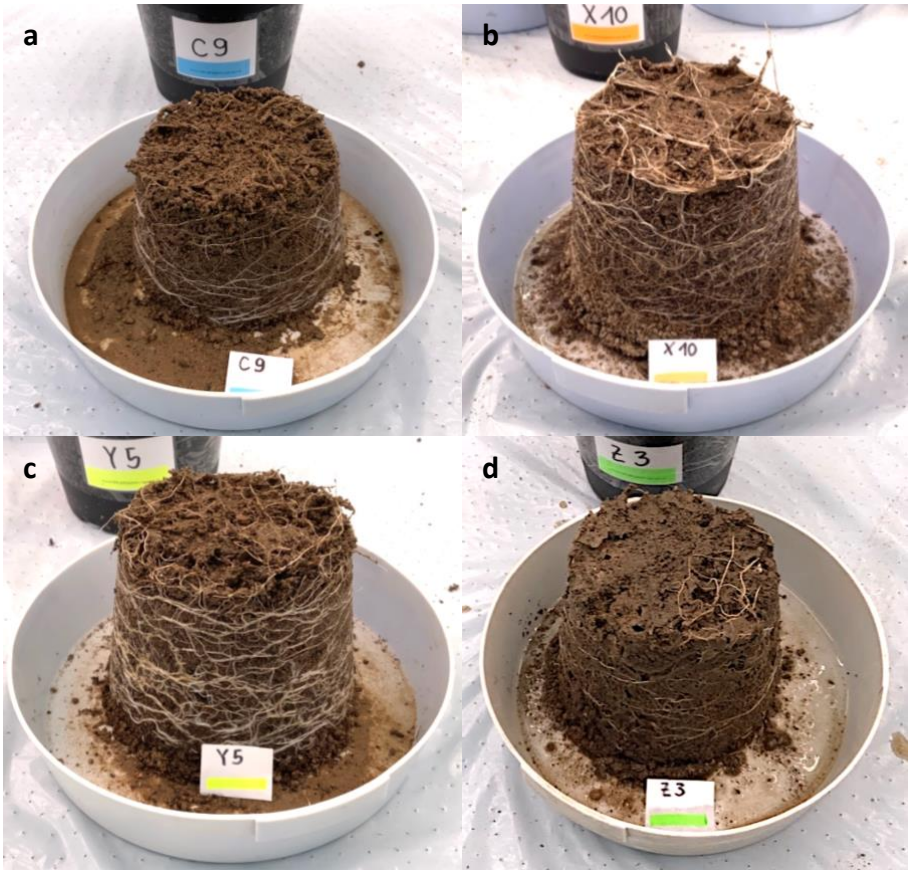


Figure 6. Representative root mass of kale plants from the different treatments at the end of the experiment (38 days after planting): a) control (C), b) sterile soil with sterile frass (X), c) sterile soil with fresh frass (Y), d) fresh soil with fresh frass (Z).

4.4 Siderophore-producing *Pseudomonas* spp.

4.4.1 Frass analysis

The isolation and enumeration of *Pseudomonas* spp. in the produced fresh frass from three replicate boxes showed an average value of 7.06 $\log_{10}$ CFU g^{-1}.

On average, 41.3 ± 1.2 % of the isolates assayed for siderophore production did not form halos on the CAS agar medium and were therefore classified as Category 0 (*Pseudomonas* spp. that do not produce any type of siderophore) (Figure 7). The largest proportion of *Pseudomonas* spp. isolates (42.9 ± 7.8 %) formed halos smaller than an average diameter of 1 mm and were therefore assigned to Category 1. On average, 11.2 ± 6.9 % of the colonies formed Category 2 halos, which were between 1 and 2 mm in diameter size. The smallest proportion, on average 4.5 ± 3.4 %, consisted of isolates that produced the largest halos, ranging from 2 to 4 mm in average diameter size.

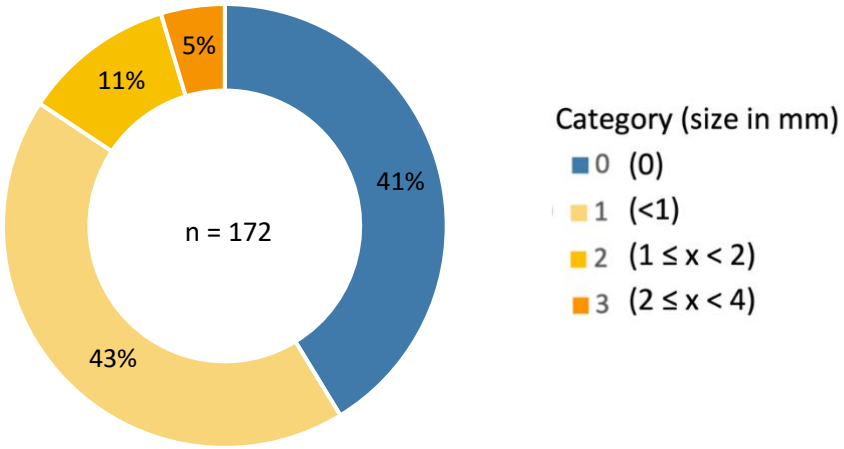


Figure 7. Categorization of CAS (chrome azurol S) assay results from Pseudomonas *spp. isolates (n = 172) after 48 h at 28 °C incubation. Categories were assigned based on the size of the halos formed. See Table 3 for an overview of the categories. Category 0: no visible halo formed, 1: < 1 mm, 2: 1 ≤ x < 2 mm, 3: 2 ≤ x < 4 mm. Results are presented as average percentages of three replicate boxes. n = number of screened isolates.*

4.4.2 Rhizosphere soil analysis

Enumeration of Pseudomonas *spp.*

The number of *Pseudomonas* spp. colonies was significantly different in the rhizosphere soil across treatments ($p < 0.05$) (Table 5). The highest number of *Pseudomonas* spp. colonies was obtained in the rhizosphere soil of plants from Treatment X (sterile soil and sterile frass), with 7.44 $\log_{10}$ CFU g^{-1}. The control (sterile soil) had the lowest number of *Pseudomonas* spp. colonies, with 5.99 $\log_{10}$ CFU g^{-1}. No differences in *Pseudomonas* spp. colonies were found in the rhizosphere of Treatment Y and Treatment Z.

Table 5. Occurrence of Pseudomonas *spp. in rhizosphere soil of kale plants grown in frass-amended and non-amended soils in the greenhouse. Treatments:* ***C*** *sterile soil,* ***X*** *sterile soil with sterile frass,* ***Y*** *sterile soil with fresh frass,* ***Z*** *fresh soil with fresh frass. Results are presented as mean ± standard deviation (SD). Different letters indicate statistical differences (p = 0.05).*

Treatment	*Pseudomonas* spp. ($\log_{10}$ CFU g^{-1})
Sterile soil (control, C)	5.99 ± 0.19^{c}
Sterile soil with sterile frass (X)	7.44 ± 0.15^{a}
Sterile soil with fresh frass (Y)	6.91 ± 0.22^{b}
Fresh soil with fresh frass (Z)	7.09 ± 0.39^{b}

CAS assay categorization of Pseudomonas *spp. isolates*

The distribution of halo size categories obtained from the CAS assay differed among the *Pseudomonas* spp. isolated from the rhizosphere soil in the greenhouse experiment (Figure 8). The largest number (34.2 %) of isolates that did not produce a halo was observed in the control (sterile soil). A similar proportion (35.8 %) of isolates showed halos with a diameter of less than 1 mm (Category 1). Only a small proportion of isolates in the control had halos with larger diameters (Categories 2 - 4). *Pseudomonas* spp. isolates from Treatment X (sterile soil with sterile frass) showed a more even distribution across the categories, with the highest values observed in Category 1 (25.0 %) and Category 3 (27.5 %). In contrast, Treatment Y (sterile soil with fresh frass) had the overall highest abundance of Category 1 halos (78.3 %), the overall smallest of Category 0 (6.7 %) and no halos of Category 3. Treatment Z (fresh soil with fresh frass) also showed a large proportion of Category 1 halos (46.6 %), whereas Categories 2 - 4 halos were only produced by a small proportion of isolates. Overall, Treatment X (sterile soil with sterile frass) showed the highest abundance of larger halo categories (Categories 3 - 4), whereas Category 1 halos were the most frequent across all treatments.

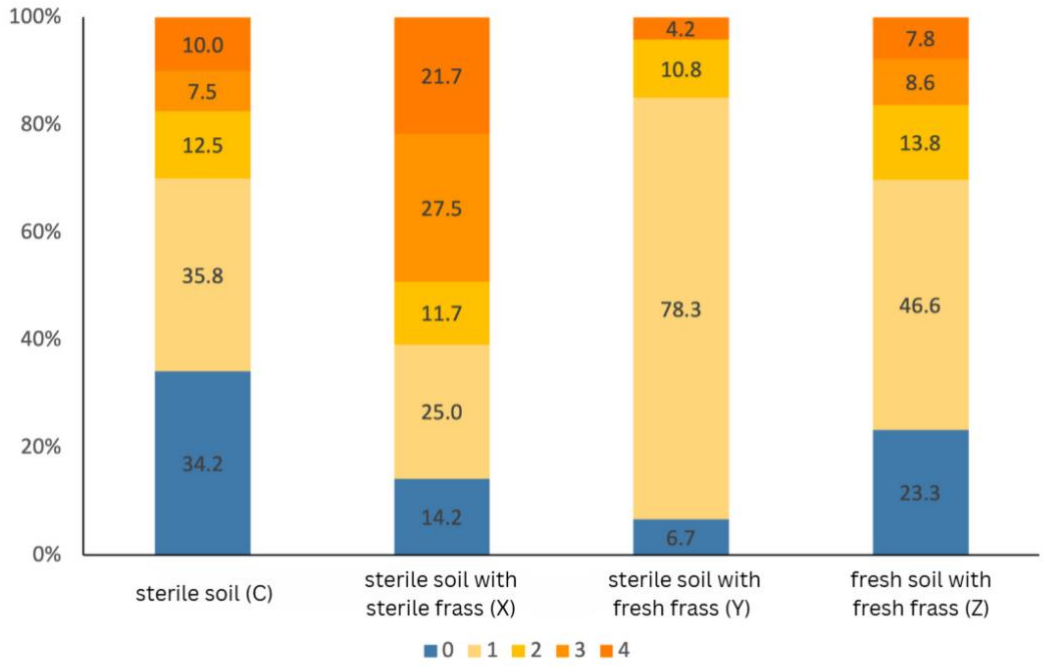


Figure 8. Percentage distribution of chrome azurol S (CAS) assay halo size categories obtained from Pseudomonas *spp. isolates (n = 476) of rhizosphere soils from four different greenhouse treatments. Size of halos scored after 48 h incubation of isolates on CAS agar medium at 28 °C. Treatments:* ***C*** *sterile soil,* ***X*** *sterile soil with sterile frass,* ***Y*** *sterile soil with fresh frass,* ***Z*** *fresh soil with fresh frass. See Table 3 for an overview of the categories. Categories* ***0****: no visible halo formed,* ***1****: < 1 mm,* ***2****: 1 ≤ x < 2 mm,* ***3****: 2 ≤ x < 4 mm,* ***4****: x ≥ 4 mm.*

5. Discussion

5.1 BSFL frass and abundance of siderophore-producing *Pseudomonas* spp.

5.1.1 BSFL frass parameters

The frass produced through the bioconversion of pig manure substrate by black soldier fly larvae contained approximately 50 % TS and 83 % VS (Table 4). The total solids content of the frass was slightly lower than the values reported in previous analyses of frass from bioconversion of pig manure, at 55.9 % (Naser El Deen et al. 2023) and 62 % (Veldkamp et al. 2021). This variation might partly result from differences in the process parameters (Gärttling & Schulz 2022). The pH of the analysed fresh frass was 7.2, which is in accordance with previously reported values of frass pH summarized by Gärttling & Schulz (2022) and Lopes et al. (2022), with average pH values of 6.8 and 7.5, respectively. The bioconversion efficiency of 10.8 % on a TS basis (Table 4) is consistent with previous studies, which reported values of 12.5 % for the bioconversion of pig manure (Parodi et al. 2021) and 11.8 % for mixed biowaste (Lalander et al. 2015). The material reduction on a solids basis was about 25 % (Table 4). This value is lower than the results reported in other studies using various feed substrates, which were summarized by Lopes et al. (2022), ranging from 30.7 % - 79.9 %. Lalander et al. (2015) reported a 55.1 % material reduction through the bioconversion of mixed biowaste containing pig manure. They suspected that the substrate was less degradable because of the straw content in pig manure, which larvae cannot consume. Since considerable amounts of straw were also found in the pig manure in this study, this correlation could be one possible explanation for the relatively low material reduction.

The seed germination test of frass revealed a germination index (GI) of 120 % (Table 4), which means that the frass is not phytotoxic but rather promotes seed germination, as the seedlings developed more vigorously in the water-extracted frass than in the control group treated with pure water. This finding corroborates the findings of Liu et al. (2019), in which frass produced from BSFL-converted pig manure also exhibited a GI above 100 %.

5.1.2 *Pseudomonas* spp. in BSFL frass

Microbiological analysis by isolation on selective agar revealed *Pseudomonas* spp. in fresh frass, at an average concentration of 7 $\log_{10}$ CFU g^{-1}. The presence of *Pseudomonas* spp. in BSFL frass has previously been reported, primarily based on culture-independent approaches. For example, using 16S rDNA gene sequencing, Shumo et al. (2021) detected *Pseudomonas* spp. in frass derived from BSFL-

processed kitchen waste and chicken manure. Similarly, using the same metagenomic approach, Fuhrmann et al. (2022) identified *Pseudomonas* spp. in frass derived from BSFL bioconversion of spent malted barley grains, while Praeg & Klammsteiner (2023) observed this microorganism in fresh BSFL frass derived from a commercial insect farm.

The isolated *Pseudomonas* spp. from the analyzed frass may originate from either the pig manure substrate or the BSFL themselves. Previous studies detected bacteria of the genus *Pseudomonas* spp. in fresh pig manure using distinct methodologies, such as PCR-DGGE (Zhang et al. 2011) and rRNA metabarcoding (Wang et al. 2017). Several studies identified *Pseudomonas* spp. in the guts of BSFL, despite differences in experimental setup, and therefore hypothesized that *Pseudomonas* spp. might be a member of a possible core gut microbiota of BSFL (Wynants et al. 2019; Klammsteiner et al. 2020; Shumo et al. 2021; Tanga et al. 2021). Therefore, *Pseudomonas* spp. may be present in frass as a result of larval excretion (Gold et al. 2020) or from the pig manure.

5.1.3 Siderophore-producing *Pseudomonas* spp. in BSFL frass

Analysis of selected *Pseudomonas* spp. isolates using chrome azurol S (CAS) assay revealed that 59 % formed halos, which indicates siderophore production (Figure 7). The halos were qualitatively categorized based on the diameter. Specifically, Category 1 (< 1 mm halo) isolates were dominant, but Category 2 (1 - 2 mm halo) and Category 3 (2 - 4 mm halo) isolates were also detected.

Siderophore-producing *Pseudomonas* spp. have already been identified in the frass of other industrially relevant insect species, such as the yellow mealworm (*Tenebrio molitor*) (Poveda et al. 2019). In the CAS assay, the competition for iron (Fe III) between siderophores and the dye complex is influenced by various factors that determine the size of the halos. These include the varying structures and binding constants of different siderophore types (Schwyn & Neilands 1987) and the quantities of excreted siderophores (Shin et al. 2001). Additionally, with the same amount of siderophores produced per cell, faster-growing colonies form larger halos due to their higher cell count. Therefore, the growth rate of bacterial colonies influences the size of the halo, causing slow-growing isolates to produce smaller halos (Schwyn & Neilands 1987).

The dominance of Category 1 isolates suggests that BSFL frass contains *Pseudomonas* spp. that produce low quantities of siderophores or siderophore types with a low binding constant, or that the growth rates of the isolates are low. Various studies classify the CAS agar assay as a purely qualitative method to determine whether bacterial isolates generally produce siderophores or not (Milagres et al. 1999; Shin et al. 2001; Arora & Verma 2017).
The presence of siderophore-producing *Pseudomonas* spp. in frass provides the microbiological basis for analyzing possible plant growth-promoting effects.

5.2 Effect of BSFL frass on plant growth

5.2.1 Effect on seed germination and seedling emergence of kale (*Brassica oleracea*)

Germination and seedling emergence increased over time (Figure 2a). However, according to the germination period specified by the manufacturer, seeds were considered fully germinated and recorded on day 10 after planting. Germination and seedling emergence on day 10 did not differ significantly among treatments, but were higher in sterile soils amended with frass than in controls or fresh soils amended with fresh frass (Figure 2). These findings demonstrate that soil amendment with BSFL frass can promote germination and seedling emergence in kale, but these effects depend on soil and frass processing. Importantly, these findings contrast the results obtained in the seed germination index for frass (Table 4). This suggests that the reduced germination in the greenhouse study is not linked to frass phytotoxicity, but rather to other, as yet unknown factors in the soil environment.

Seed germination is a key developmental transition in plants and the foundation of most global food production (Gómez-Álvarez et al. 2023). Seed germination is a complex process that begins with the initial uptake of water (i.e. imbibition) by a dry seed and concludes with the emergence of the embryonic root. The nutrition in the initial germination phase is independent of external soil nutrients (Bewley et al. 2012). As soon as the seedling's leaves are sufficiently developed to perform photosynthesis, the roots begin to absorb nutrients from the surrounding soil or substrate (Ha et al. 2017b).

Several studies have shown that incorporating other types of compost into the soil improves the soil's physical, chemical and biological properties (Aggelides & Londra 2000; Evanylo et al. 2016; Dolit et al. 2022). Aggelides & Londra (2000) showed that the incorporation of compost derived from town waste and sewage sludge improved various physical properties of the treated soil, such as an increase in soil porosity and a reduction in bulk density and penetration resistance. Penetration resistance has already been identified as a possible influencing factor for the success of seed germination. Stibbe & Terpstra (1982) found that as penetration resistance increased, the percentage of germinated corn (*Zea mays L.)* seeds decreased linearly. Taken together, soil amendment with frass could have modified soil physical properties, such as bulk density and porosity, and thereby improved the conditions, such as water availability, which support seed germination.

In the control soil, the absence of frass could have resulted in soil compaction, thereby causing reduced water percolation, aeration and light penetration, which is known to induce secondary dormancy (Karssen 1980). In contrast, in Treatment Z, where fresh (non-sterile) soil was amended with fresh frass, the seedlings may have encountered unfavourable soil conditions. In particular, the fresh soil may harbor soil-borne pathogens and pests that cause pre-emergence seed decay or post-emergence collapse and thereby reduce germination and seedling emergence. Consistent with this, dead seedlings were observed in some pots of Treatment Z (Figure 2a). Soil-borne fungi and oomycetes such as species of *Rhizoctonia*,

Fusarium, *Phytophthora* and *Pythium* are ubiquitous in soil and are known to cause seed decay, root infection, as well as pre- and post-emergence damping-off. Similarly, plant-parasitic nematodes, e.g. *Meloidogyne* spp., *Pratylenchus* spp., and *Hoplolaimus* spp., are omnipresent and cause root injury, stunting and increased susceptibility to other pathogens (Mennan & Handoo 2006).

Regarding chemical properties, frass is rich in macronutrients and micronutrients relevant to plant nutrition (Gärttling & Schulz 2022), which could support plant growth. While seed germination largely depends on nutrients stored in the seed, exogenous supply of nutrients is required for seedling growth and emergence, particularly as the seedling transitions to the autotrophic phase (Ha et al. 2017b). For example, Zhang et al. (2017) showed that macronutrients such as N, P, and K in the substrate have a significant influence on the growth of *B. oleracea* seedlings. Therefore, the addition of frass may have increased the nutrient content in the soil and thereby improved the seedling development of kale.

Regarding biological soil properties, frass could have introduced microorganisms that are beneficial for seed germination and growth of seedlings. In Treatment Y, the native soil microbiome was eliminated by autoclaving. According to the principle of the priority effect, the timing and order of colonization of a vacant habitat determine the composition of the microbial community (Fukami 2015). Therefore, it can be assumed that the seeds in Treatment Y were primarily surrounded by the frass-associated microbiome, as the competing native soil microorganisms were absent. The microorganisms present in frass have been analyzed in several previous studies (Wynants et al. 2019; Gold et al. 2020), and microorganisms belonging to groups known for their plant-growth-promoting properties have been identified in frass (Lopes et al. 2022). Plant growth- promoting bacteria colonize plant roots and exhibit their beneficial effects through different possible mechanisms, such as the production of phytohormones, nutrient solubilization or suppression of pathogens (Qessaoui et al. 2019).

The plant growth-promoting rhizobacteria also include *Pseudomonas* spp., whose plant growth-promoting traits have already been shown to have beneficial effects on seed germination (Noumavo et al. 2013; Qessaoui et al. 2019). For example, Noumavo et al. (2013) reported that maize seeds inoculated with *P. fluorescens* and *P. putida* exhibited a significantly higher seed germination percentage (phytohormones). Qessaoui et al. (2019) inoculated tomato seeds (*Solanum lycopersicum*) with *Pseudomonas* spp. isolates and observed an increase in seed germination and improved seedling height. The *Pseudomonas* isolates studied were able to colonize the roots and produce siderophores, phytohormones, and solubilize phosphate. Species of the genus *Pseudomonas* have already been found in BSFL frass in previous studies (Fuhrmann et al. 2022; Praeg & Klammsteiner 2023). Research on other plant growth-promoting rhizobacteria from BSFL frass on seed germination suggested that certain bacterial species, such as *Bacillus* spp. and *Enterococcus* spp., present in the frass exert plant growth-promoting effects on seedling development (Green 2023; Lomonaco et al. 2026). Poveda et al. (2019) identified various bacterial isolates with plant-growth promoting properties, including members of the genus *Pseudomonas* that produced siderophores in mealworm frass.

The microbiome, which was likely introduced into the sterile soil by fresh frass, may be one reason why seed germination and seed emergence showed better results in Treatment Y compared to the control (sterile soil). This would also be consistent with the results discussed in Section 5.1, which showed that BSFL frass contains a significant abundance of siderophore-producing *Pseudomonas* spp. bacteria.

By contrast, with 43 % of total germinated seeds, germination and seedling emergence in Treatment Z (fresh soil amended with fresh frass) were lower than the control (sterile soil only), Treatment X (sterile soil with sterile frass), and Treatment Y (sterile soil with fresh frass) (Figure 2). In addition, two seedlings from Treatment Z did not continue to develop after emergence and eventually withered. The only difference between Treatments Z and Y was that, in Treatment Z, the fresh frass was added to fresh soil, rather than to autoclaved soil. It can therefore be assumed that the soil's native microbiome was present in Treatment Z. Treatment Z contained both nutrients and the microbiome from the fresh frass, which were already discussed in the previous section and suspected to be possible reasons for the improved germination of Treatment X and Treatment Y compared to the control. Therefore, the lower germination results of the seeds from Treatment Z, despite the potential benefits of the fresh frass, suggest that the reasons relate to the microbiome of the fresh soil.

According to the principle of priority effects, the microbial community that first colonizes an ecological niche modifies it through various mechanisms, such as niche pre-emption and niche modification, and thereby either inhibits or facilitates the establishment of subsequently arriving microbial communities in that niche (Fukami 2015). Because the native fresh soil likely already had an established microbiome and was present in greater quantities, there is a possibility that it exerted an inhibitory priority effect, which caused microorganisms introduced through frass addition to be unable to establish themselves. This could have inhibited the potential positive effects of the frass microbiome on seed germination and emergence.

5.2.2 Effect on plant growth parameters of kale

Plant growth was assessed using growth parameters such as plant height, above-ground biomass, the number of leaves and leaf area. Overall, plant growth followed the treatment order Y > X > Z ≈ Control. These findings suggest that soil amendment with BSFL frass can promote plant growth in kale, but these effects depend on soil and frass processing (i.e. the effect of fresh versus autoclaved soil and frass). Plant height is an important growth trait that can influence vegetative biomass production in kale (Liu et al. 2014). Consistent with that, the number of leaves, leaf area and above-ground biomass were highest in the treatments with the tallest plants (Figure 3). These findings are in line with previous field and pot studies where soil amendment with frass has been shown to promote plant growth in various crops including leafy vegetables (Chiam et al. 2021; Chirere et al. 2021), cereals (Beesigamukama et al. 2020; Wang et al. 2024) and fodder crops (Klammsteiner et al. 2020). In a pot study, Gurung et al. (2025) investigated the impact of BSFL frass on the growth of chilli (*Capsicum annuum*). Soils were amended with 0.2 - 1.2 % w/w frass derived from BSFL bioconversion of animal manure and carcasses alongside non-amended controls and synthetic

fertiliser. The 0.6 % frass rate increased shoot and root dry weight by 12 % and 17 %, respectively, relative to the negative control, while shoot nitrogen content rose with higher application rates. Moreover, frass amendment enhanced nutrient bioavailability and uptake (Gurung et al. 2025). In a study using a multi-storey "Wonder Garden," Abiya et al. (2022) showed that amendment with BSFL frass produced the tallest kale plants and highest leaf numbers, with maximum heights under daily irrigation. Frass amendment also produced the largest yield and dry matter increase over synthetic fertiliser (Abiya et al. 2022).

Physical factors

The results show that the plants in Treatment X (sterile soil with sterile frass) achieved significantly higher plant height and above-ground biomass, and slightly higher number of leaves and canopy area, compared to the control (sterile soil only) (Figure 3). Since Treatment X differed from the control only in its addition of sterile frass, the factors that influenced the results of this treatment likely relate to the chemical and physical aspects.

As already described in the section on physical factors affecting seed germination, it is assumed that the addition of frass affects the physical structure of the soil, although no studies have been conducted on this topic to date (Lopes et al. 2022). Soil physical properties play a major role in plant growth, as they are directly related to the supply of water and air to plant roots and, indirectly, to nutrient availability (Letey 1985). Previous studies analyzed the effect of different compost amendments on soil physical properties, and all found that adding and incorporating compost reduces the soil's bulk density and increases its porosity (Aggelides & Londra 2000; Celik et al. 2004; Dolit et al. 2022; Noor et al. 2023).

These results suggest that adding sterile frass to the sterile soil in Treatment X may have improved its physical properties, thereby providing the kale plants with better growth conditions and resulting in higher values of the plant growth parameters compared to the control.

Chemical factors

By adding sterile frass in Treatment X compared to the control (sterile soil only), not only physical but also chemical factors were modified. As previously described in the section on chemical factors affecting seed germination, the results of the soil analyses from the different treatments showed higher levels of macro- and micronutrients in all treatments with frass addition compared to the control without frass (see Appendix 3).

The macro- and micronutrients present in the BSFL frass have been analyzed in various studies (Gärttling & Schulz 2022; Salomon et al. 2025) and have shown that frass often contains higher levels of macronutrients than other composts (Gärttling & Schulz 2022; Salomon et al. 2025). The nutrients present in the frass and their possible impact on plant growth resulting from frass amendments have been analyzed in several previous studies (Beesigamukama et al. 2020; Anyega et al. 2021; Salomon et al. 2025). Anyega et al. (2021) analyzed the effect of BSFL frass and other fertilizers on the growth of kale (*Brassica oleracea*), tomatoes (*Solanum lycopersicum*), and French beans (*Phaseolus vulgaris*), with regard to parameters such as plant growth, yield, and nutritional quality. BSFL frass addition showed similar or better performance than tested mineral or organic

fertilizers. The authors suggested that the observed increased nutritional quality may be attributed to the uptake of nitrogen and other nutrients from the frass. Salomon et al. (2025) found that with increasing N application rates of BSFL frass on greenhouse-grown tomato plants, significant positive effects on plant biomass production were obtained. Beesigamukama et al. (2020) analyzed the impact of BSFL frass addition on maize growth and observed greater plant height, nutrient uptake (N and P), and increased chlorophyll concentration and assumed that these effects could be attributed to the increased nutrient availability resulting from the frass. Depending on the nutrient ratio, the addition of the macronutrients N, P, and K resulted in increased chlorophyll content and root weight in *Brassica oleracea* seedlings (Zhang et al. 2017).

The addition of sterile frass in Treatment X (sterile soil with sterile frass) presumably increased the content of plant-relevant nutrients compared to the control (sterile soil only). This additional nutrient input may explain the improved plant-growth parameters observed in Treatment X as compared to the control.

Biological factors

The results of Treatment Y (sterile soil with fresh frass) showed the significantly highest values for above-ground biomass and canopy area, with 195 % and 160 % in comparison to the control, respectively (Figure 3). In contrast to the frass in Treatment X, the frass in Treatment Y was not autoclaved and therefore still contained its microbiome when it was added to the sterile soil.

As previously discussed in the section on biological factors in seed germination, the microbiome of frass has already been analyzed in various studies (Wynants et al. 2019; Gold et al. 2020), and microorganisms that belong to the plant-growth promoting bacteria group have been identified (Lopes et al. 2022; Kreft et al. 2026).

Bacterial species identified in the frass include, among others, *Enterococcus* (Green 2023; Kreft et al. 2026), *Klebsiella* (Kreft et al. 2026), *Morganella* and *Providencia* (Lopes et al. 2026), as well as members of the families *Lactobacillaceae* and *Bacillaceae* (Lomonaco et al. 2026; Lopes et al. 2026). Depending on the species, these microorganisms were associated with plant-growth-promoting traits such as phytohormone production and nutrient solubilization (Kreft et al. 2026). As discussed in Section 5.2.1, previous studies have identified *Pseudomonas* spp. in the frass microbiome that are known to produce siderophores and thereby promote plant growth.

Soil analysis of Treatment Y (sterile soil with fresh frass) revealed a pH of 8.0. At this pH, iron is mostly present in ferric forms (Fe III), which are less available for plants due to their low solubility (Section 2). Microbial analysis (Section 5.1) showed that siderophore-producing *Pseudomonas* spp. were present in the fresh frass and in the rhizosphere soil samples, which will be further discussed in Section 5.3. Siderophore-producing *Pseudomonas* strains have been shown to improve iron availability for plants, even under iron-limited conditions, such as high soil pH (Gao et al. 2022). Since iron is essential for many processes in plant metabolism, improved iron availability can promote plant growth (Marschner 2011).

These results suggest that siderophore-producing *Pseudomonas* spp. were introduced into Treatment Y by the addition of fresh frass, which may have

improved the plants' iron availability. This could possibly explain the higher values of plant growth parameters observed in Treatment Y in comparison to Treatment X and the control. However, since a similar number of *Pseudomonas* spp. were detected in the rhizosphere soil of Treatment X (with sterile frass) as in Treatment Y (with fresh frass), it is possible that the increased growth of Treatment Y is not attributable to the effect of the Pseudomonas added by the frass microbiome. The ability of *Pseudomonas* spp. to protect plants against soilborne fungi (as presented in Section 2) presumably did not play a role in Treatment Y, since the soil microbes were eliminated by repeated autoclaving (Carter et al. 2007).

By contrast, Treatment Z (fresh soil with fresh frass) showed significantly lower results in plant growth than Treatment Y (sterile soil with fresh frass). Both treatments were amended with fresh frass but differed in soil condition: the soil in Treatment Z was fresh, while the soil in Treatment Y was autoclaved, which eliminated the present soil microbiome. This suggests that the microbiome still present in the soil of Treatment Z may have directly or indirectly influenced the plant growth. Since the greenhouse experiment did not include a control treatment consisting solely of fresh soil, possible explanations for the significantly weaker growth can only be hypothesized.

Based on the principle of the priority effect (discussed in Section 5.2) the microbiome already established in the fresh soil may have prevented the added frass microbiome from colonizing, through mechanisms such as niche preemption or modification. As a result, microorganisms with plant growth-promoting properties from the frass would not have been able to exert their beneficial effects, such as improved iron bioavailability and biocontrol activity against soilborne fungi by siderophore-producing *Pseudomonas* spp. (Fukami 2015; Chen et al. 2025). This could offer one possible explanation for the significantly poorer growth in Treatment Z, even though it contained the same frass microbiome as Treatment Y.

Treatment Z (fresh soil with fresh frass) received the same amount of nutrients through frass amendment as treatments X and Y, as confirmed by the soil analysis results. However, the results for the plant growth parameters of Treatment Z were similar to those of the control (sterile soil only), which did not receive any additional nutrients. One possible explanation for this could be that already established microorganisms in the fresh soil temporarily immobilized the nutrients added through frass addition, making them less available for plant growth. For example, Beesigamukama et al. (2021) amended soil with fresh frass and found that the macronutrient nitrogen (N) was immobilized over a period of 30 - 60 days, before being mineralized and released again. Since plant growth in this experiment was assessed after 38 days, such temporary immobilization of plant essential macronutrients could be one of the reasons why Treatment Z showed poorer results, despite frass addition.

The fact that two seedlings already showed mortality during the seed emergence phase could be interpreted as a symptom of damping-off disease and thus as an indicator of the presence of soilborne pathogens that may have inhibited plant development.

The results from Treatment Z suggest that the observed positive effects on plant growth resulting from the addition of fresh frass in treatments X and Y may

have been hindered by the use of fresh soil. Given the potential involvement of multiple interacting physicochemical and biological mechanisms, the available data do not allow for a more specific explanation.

5.3 Abundance of siderophore-producing *Pseudomonas* spp. in rhizosphere of BSFL frass-amended soil

5.3.1 Abundance of *Pseudomonas* spp. in rhizosphere of BSFL frass- amended soil

The significantly lowest amount of *Pseudomonas* spp. isolates was detected in the control with about 6 $\log_{10}$ CFU g^{-1}. The treatments with fresh frass addition to sterile soil (Y) and fresh soil (Z) showed significantly higher values (6.9 and 7.1 $\log_{10}$ CFU g^{-1}) but did not differ from one another. The significantly highest abundance of *Pseudomonas* spp. was found in Treatment X (sterile soil with sterile frass), with approximately 7.4 $\log_{10}$ CFU g^{-1}.

The rhizosphere of the control (sterile soil only) contained the lowest abundance of *Pseudomonas* spp. Because the soil had been sterilized by autoclaving, no microorganisms were present in the soil at the start of the greenhouse experiment. However, the *Brassica oleracea* seeds used were not autoclaved before planting and thus retained their seed microbiome. Species of the genus *Pseudomonas* have already been identified in the seed microbiome of *Brassica oleracea* (Tyc et al. 2020) and in a wide range of other plant species (Johnston-Monje & Raizada, 2011; Simonin et al., 2022). It has been shown that bacteria of the seed microbiome are able to colonize the rhizosphere and thereby alter the rhizosphere microbiome (Shao et al. 2021). In the absence of the soil microbiome, the *Pseudomonas* spp. may have colonized and proliferated in the rhizosphere. These findings suggest that the *Pseudomonas* spp. isolated from the rhizosphere of the control treatment may have originated from the seed microbiome.

The rhizosphere of Treatment X (sterile soil with sterile frass) contained the highest abundance of *Pseudomonas* spp. Since both the soil and the frass were sterilized in this treatment, the substrate likely did not contain any microorganisms. Therefore, as suggested for the control, it could be assumed that the *Pseudomonas* spp. were introduced into the treatment via the seed microbiome, which was the first microbial community to establish in the treatment. The addition of sterile frass in Treatment X resulted in the supply of additional macro- and micronutrients compared to the control, as confirmed by the soil analysis. Watson et al. (2021) found that a 5 % BSFL frass addition stimulated the growth of soil microbial biomass and attributed this to the nutrient content of the frass. Therefore, it could be assumed that *Pseudomonas* spp. from the seed microbiome colonized the rhizosphere and were able to proliferate more effectively due to the increased nutrient availability provided by the sterile frass.

In the rhizosphere of Treatment Y (sterile soil with fresh frass), the number of isolated *Pseudomonas* spp. was significantly lower than in Treatment X (sterile soil with sterile frass). The addition of fresh frass in Treatment Y likely introduced an additional frass microbiome to the potential seed microbiome in comparison to the control and Treatment X, while the concentration of nutrients remained constant. The result can be interpreted in the context of the community coalescence framework, which describes the interactions that arise from the convergence of different entire microbial communities (Rillig et al. 2015).

5.3.2 Siderophore-producing *Pseudomonas* spp. in rhizosphere of BSFL amended soil

Analysis of selected *Pseudomonas* spp. isolates derived from the rhizosphere of the different treatments using the CAS assay revealed differences in the proportions and types of the observed categories (Figure 8).

In the control (sterile soil), 65.8 % of the *Pseudomonas* spp. isolates tested formed halos, which represents the smallest proportion compared to the other treatments. Among the positive isolates, those of Category 1 (< 1 mm halo) were dominant, suggesting that the rhizosphere of the control contains *Pseudomonas* spp. that produce low quantities of siderophores or siderophore types with a low binding constant, or that the growth rates of the isolates were low (Schwyn and Neilands, 1987). Since the control consisted only of sterile soil with non-sterile kale seeds, it is likely that *Pseudomonas* spp. capable of siderophore production entered the control via the seed microbiome. In the sterile control soil, these *Pseudomonas* may have found an unoccupied ecological niche and, in accordance with the concept of priority effects, may have been the first to establish themselves there.

Treatment X (sterile soil with sterile frass) showed that 85.8 % of *Pseudomonas* spp. isolates formed halos. Of these, nearly 50 % belonged to Categories 3 (2 - 4 mm halo) and 4 (≥ 4 mm halo). The categories with larger halos suggest that Treatment X contained *Pseudomonas* spp. isolates that either produced a large number of siderophores, or siderophore types with a high binding constant, or that the colonies were fast-growing. Since both the soil and the frass were sterile, the seed microbiome of the kale seeds may have found an unoccupied ecological niche that it could colonize first, in accordance with the priority effect (Fukami 2015). Additionally, the amendment with frass provided extra nutrients. The reasons for the increased proportion of *Pseudomonas* spp. isolates that formed halos in the higher categories are complex and can only be speculated upon due to insufficient data. One possible reason could be that siderophores, as public goods (i.e. compounds that benefit other cells in the producer's environment), may influence complex interactions between siderophore-producing and “cheating” bacteria. Thereby influencing the competition within microbial communities in the soil (Butaitė et al. 2017). The higher proportion of siderophore-producing *Pseudomonas* spp. isolates compared to the control, as well as a potentially greater capacity for iron binding due to the higher proportion of isolates belonging to higher categories, may be a reason for

the improved plant growth compared to the control. However, it is also possible that this effect is due solely to the nutrient addition via frass amendment discussed in Section 5.2.

Treatment Y had the highest overall proportion of *Pseudomonas* spp. isolates that tested positive for siderophore production, at 93.3 %. Plant growth in Treatment Y in the greenhouse experiment showed the highest values, suggesting that the addition of fresh frass promoted plant growth. This finding is consistent with the studies by Lomonaco et al. (2026) and Beesigamukama et al. (2020). It was expected that a particularly high number of siderophore-producing isolates would be found in the rhizosphere of Treatment Y, which was also confirmed. However, the isolates primarily belonged to Category 1 (< 1 mm halo), indicating that they produce low quantities of siderophores or siderophore types with a low binding constant, or that the growth rates of the isolates were low. This could suggest that the siderophore-producing isolates of Category 1 do not contribute as significantly to the plants' iron availability as those of higher categories. Thus, the promotion of plant growth in Treatment Y could be partially attributed to the siderophore-producing *Pseudomonas*, but is likely attributed in large part to other factors. These could include the factors already discussed in Section 5.2, such as improved nutrient availability and soil structure resulting from frass addition, as well as the influence of other beneficial microorganisms or bioactive compounds from the frass.

In Treatment Z (fresh soil with fresh frass), 76.7 % of the *Pseudomonas* spp. isolates tested formed halos. More than half of these were classified as Category 1 (< 1 mm halo), suggesting that the rhizosphere mostly contained *Pseudomonas* spp. that produce low quantities of siderophores or siderophore types with a low binding constant, or that the growth rates of the isolates were low (Schwyn and Neilands, 1987). In Treatment Z, the soil microbiome encountered the seed and frass microbiomes. This fusion of pre-existing individual microbial communities, known as community coalescence, leads to interactions and changes in the resulting community (Rillig et al. 2015). The outcome is likely influenced by factors such as mixing ratios or interaction interfaces (Rillig et al. 2015). Although *Pseudomonas* spp. isolates capable of siderophore production were detected in the rhizosphere of this treatment, the plants in the greenhouse exhibited plant growth similar to that of the control.

Various studies classify the CAS assay as a purely qualitative method for assessing the isolate's general ability to produce siderophores (Milagres et al. 1999; Shin et al. 2001; Arora & Verma 2017). If the CAS assay results were interpreted using only the qualitative assessment regarding siderophore production (yes/no), the pattern would align with plant growth: Treatments C and Z, which had the lowest percentage of positive CAS results, also showed the weakest plant growth in the greenhouse experiment. Treatments X and Y, which had higher percentages of positive CAS assay results, also showed significantly better plant growth. This could suggest that the higher number of siderophore-producing *Pseudomonas* isolates in treatments X and Y may have promoted plant growth through enhanced iron availability. However, the distribution of the different categories of halos presents a much more complex picture. To evaluate the actual role of siderophore-producing *Pseudomonas* spp. in promoting plant

growth and iron nutrition, more comprehensive analyses and further experiments would be necessary.

5.4 Limitations and recommendations

Limitations

The results of the greenhouse study were likely influenced by several factors which could not be analysed separately, as this was beyond the scope of the present study.

Autoclaving the soil and frass may have altered their physical and chemical properties, potentially resulting in additional differences between the treatments.

The kale seeds were not surface-sterilised and probably still contained their natural seed microbiome and likely influenced the greenhouse treatments.

The microbiome of the fresh soil probably played a role in the growth of Treatment Z. However, the effect of the fresh soil alone was not tested in a separate greenhouse experiment. These seed and soil microbiomes probably also influenced the subsequent results of the microbial analysis of the rhizosphere soil samples. The contribution of nutrients supplied via the frass can only be determined indirectly from the soil analyses of the respective greenhouse treatments, since no separate analysis of the fresh frass itself was available.

The limitations of microbial analysis relate to the fact that not all *Pseudomonas* spp. are capable of growing on PSA, which may lead to an underestimation of the abundance of *Pseudomonas* spp. in the sample.

Further research recommendations

Based on the findings of the present study, the following recommendations are made for future research on this topic.

In order to assess the potential role of siderophore-producing *Pseudomonas* spp. from BSFL frass in promoting plant growth and enhancing plant iron nutrition in more detail, future studies should identify both the *Pseudomonas* spp. species present in the frass and the types and quantities of siderophores they produce. Furthermore, quantifying the iron content in plant tissue would allow for a more direct assessment of whether siderophore-mediated iron uptake leads to an improved iron supply to the plants, rather than relying solely on growth parameters. To capture more realistic plant responses, longer growth periods in a greenhouse or field studies would be advisable. Seed inoculation with siderophore-producing *Pseudomonas* strains isolated from frass-amended rhizosphere could provide further insight into their actual effect on plant growth.

6. Conclusion

The aim of this study was to investigate whether siderophore-producing *Pseudomonas* spp. contribute to the documented biostimulant properties of BSFL frass. The results show that frass contained *Pseudomonas* spp., of which 59 % of screened isolates were positive for siderophore production. Soil amendment with BSFL frass derived from the bioconversion of pig manure promoted the growth of kale by up to a 195 % increase in above-ground biomass compared to the negative control, although these effects depend on whether the frass and soil were sterile or fresh. Additionally, frass-amended rhizosphere soil of the plants contained up to 93.3 % siderophore-producing *Pseudomonas* spp. of the isolates screened.

These findings support the hypothesis that plant-growth promotion by BSFL frass is, in part, mediated by frass-associated microorganisms. However, whether siderophore-producing *Pseudomonas* spp. specifically contribute to this effect by enhancing plant iron nutrition, remains unresolved based on current data.

To the best of the authors' knowledge, no studies have yet been conducted on the possible role of siderophore-producing *Pseudomonas* spp. from BSFL frass in promoting plant growth. Should this mechanism be confirmed by future studies, BSFL frass may have extra relevance for agricultural practice as a biofertiliser for iron-limited soils. Limitations that must be taken into account in this study include the potential influence of the seed microbiome and the fresh soil microbiome, which could not be examined separately.

Future studies should identify both the *Pseudomonas* spp. present in the frass and the types and quantities of siderophores they produce in order to assess the potential role in promoting plant growth. To capture more realistic plant responses to BSFL frass amendments, longer growth periods in a greenhouse or field studies would be advisable. Seed inoculation with siderophore-producing *Pseudomonas* strains isolated from frass-amended rhizosphere could provide further insight into their actual effect on plant growth.

This study highlights the potential of BSFL frass as a biofertiliser with possible relevance for iron-limited agricultural soils, while underscoring the need for further mechanistic research into the role of frass-associated microorganisms.

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Popular science summary

Insect Poop as Plant Food: how bacteria in black soldier fly frass might promote plant growth of kale

Black soldier fly larvae can break down large amounts of organic waste, such as pig manure. Next to the valuable insect protein, this process also produces a by-product called "frass": a mixture of larval excrement and undigested substrate residues. Frass is increasingly being discussed as a sustainable, high-value and low-cost fertilizer that can promote plant growth. But why frass has this positive effect on plants is still poorly understood.

One possible explanation lies in bacteria that naturally occur in frass. This study specifically investigated a group of bacteria called *Pseudomonas* spp., some species which are known to produce special molecules called siderophores. These siderophores act like tiny "iron magnets": they bind iron in the soil that would otherwise be difficult for plants to access and make it available to plant roots instead. Since iron is essential for many processes in plants, this mechanism could help explain part of frass's growth-promoting effect.

The study was carried out in two phases. First, pig manure was consumed by fly larvae, and the resulting frass was analysed, including testing whether it contained *Pseudomonas* spp. bacteria and whether these bacteria could produce siderophores. Next, a greenhouse experiment tested how kale plants grew under different conditions: with and without frass, and using either sterilized or fresh soil and frass. The root zone was then tested to determine whether it contained *Pseudomonas* spp. bacteria and whether these bacteria could produce siderophores.

The results showed that frass did contain *Pseudomonas* spp. bacteria, more than half of which were able to produce siderophores. In the greenhouse, kale plants that were fertilized with frass showed better growth, however, this depended on the condition of the soil and the frass (fresh or sterilized). The root zone of the fertilized plants also contained numerous siderophore-producing *Pseudomonas* spp. bacteria.

These findings support the idea that bacteria in frass contribute to its plant-growth promoting effect. Whether iron supply through siderophores specifically plays a key role, however, could not be fully confirmed by this study. Further research is needed to clarify this. If this mechanism is confirmed in future studies, insect-based fertilizer could become particularly valuable for soils where plants naturally struggle with iron deficiency, which is a common agricultural challenge worldwide.

Appendix 1 – Bioconversion process parameters

Table 1. Pig manure substrate and larvae parameters after bioconversion.

	TS (%)	VS (% of TS)	VS total	Ash (%)
Pig manure substrate	23.03 ± 0.21	86.07 ± 0.18	19.82 ± 0.18	13.93 ± 0.18
Larvae after bioconversion	28.55 ± 0.61	78.43 ± 0.15	22.39 ± 0.52	21.57 ± 0.15

Table 2. Fixed parameters of the bioconversion process.

Fixed bioconversion parameters	Value
Total VS / larva	0.2
Larvae / cm^2	5
Feeding events	3
Box area (cm^2)	2400
Box height (cm)	5-8
Replicats	3

Table 3. Calculations of larvae and substrate quantities.

Calculated parameter	Quantity
Larvae / box	12000
Weight / larva (g)	0.00179
Total larvae mass / box (g)	21.47
Substrate VS / box	2400
Substrate / box (g)	12109.54
Total amount substrate (g)	36328.61
Substrate per feeding (g)	4036.51

Table 4. Average values of larval parameters following bioconversion of 3 replicate boxes.

	Total larva mass (g)	Average weight / larva (g)	Amount larvae / box	Larvae survival (%)
Mean of 3 replicate boxes	1042.90	0.117	9009.2	75.08

Appendix 2 – Overview randomized pot arrangement

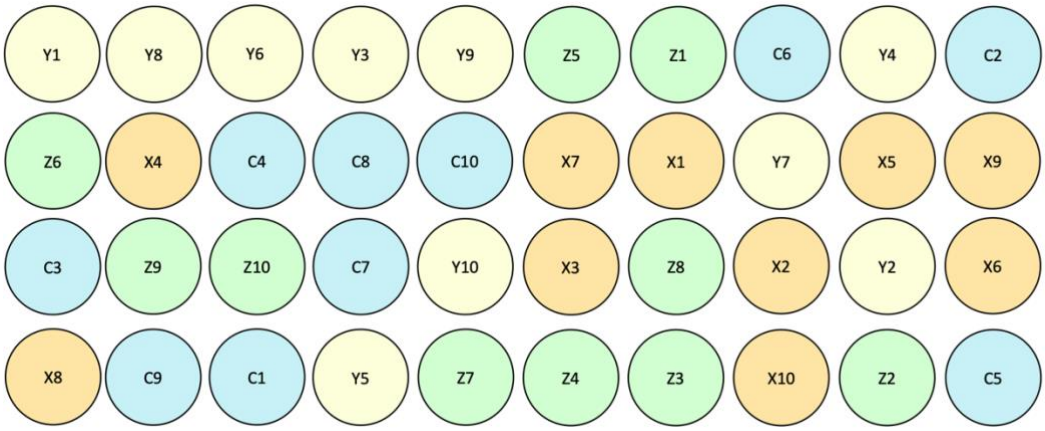

Figure 1. Schematic overview of randomized arrangement of pots in the greenhouse experiment. The codes in each circle represent: letters for the treatment (C, X, Y and Z) and numbers for the replicate (1–10).

Legend:

- C – sterile soil
- X – sterile soil with sterile frass
- Y – sterile soil with fresh frass
- Z – fresh soil with fresh frass

Appendix 3 – Soil analysis results

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		pH	P-AL mg/100 g lufttorr prov	P-AL klass*	K-AL mg/100 g lufttorr prov	K-AL klass*	Mg-AL mg/100 g lufttorr prov	K/Mg-kvot*	Ca-AL mg/100 g lufttorr prov	B-CAT mg/kg lufttorr prov	Mn-CAT mg/kg lufttorr prov	Fe-CAT* mg/kg lufttorr prov	Cu-CAT mg/kg lufttorr prov	Zn-CAT mg/kg lufttorr prov
06230043	Fresh soil	8.6	5.9	III	9.9	III	17	0.6	1400	< 0,10	10	17	1.0	1.0
06230044	C	8.5	5.3	III	7.4	II	12	0.6	1200	< 0,10	17	10	0.6	0.5
06230045	X	8.2	110	V	53	V	65	0.8	1100	0.36	40	22	2.3	20.2
06230046	Y	8.0	96	V	53	V	60	0.9	1100	0.28	34	18	1.9	16.3
06230047	Z	8.6	86	V	64	V	62	1.0	1200	0.18	16	17	3.1	10.8

Analys	Metod	Mätosäkerhet	Lab
B-CAT	VDLUFA Methodenbuch Band I, 3. Teillieferung, Kapi	± 43.2%	EUDEJE2
Ca-AL	SS 028310:1995-12	± 12%	EUDEJE2
Cu-CAT	VDLUFA Methodenbuch Band I, 3. Teillieferung, Kapi	± 32.21%	EUDEJE2
Fe-CAT*	VDLUFA Methodenbuch Band I, 3. Teillieferung, Kapi	± 26.87%	EUDEJE2
K-AL	SS 028310:1995-12	± 16%	EUDEJE2
Mg-AL	SS 028310:1995-12	± 23%	EUDEJE2
Mn-CAT	VDLUFA Methodenbuch Band I, 3. Teillieferung, Kapi	± 20.4%	EUDEJE2
P-AL	SS 028310:1995-12	± 11%	EUDEJE2
pH	DIN ISO 10390: 2005-12	± 4.14%	EUDEJE2
Zn-CAT	VDLUFA Methodenbuch Band I, 3. Teillieferung, Kapi	± 23.54%	EUDEJE2

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