

ASSESSING AND IMPROVING SUSTAINABILITY OF *CAMELINA SATIVA*
THROUGH RHIZOBACTERIAL INOCULANTS
AND SOIL ENZYMATIC ACTIVITY

by

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ABSTRACT

Camelina sativa is an oilseed crop with potential to be used in biofuel production as an alternative to contemporary fossil fuels. To ensure biofuels are a more sustainable alternative, considerations and improvements must be made regarding the inputs and land-use needs of producing biofuel feedstocks. This research assessed the beneficial effects of inoculating *C. sativa* plant growth-promoting rhizobacteria candidates in greenhouse trials. Additionally, we explored agronomic responses of *C. sativa* and bulk soil enzymes in field trials across Montana to nitrogen and sulfur fertilizer treatments applied as pelleted urea and gypsum respectively. Co-inoculations of *Pseudomonas putida* ATCC 12633 and *Bacillus thuringiensis* ATCC 33679 were associated with seed yield increases of 60% relative to the uninoculated control. Co-inoculations of *Pseudomonas brassicacearum* 36D4 and *B. thuringiensis* ATCC 33679 were associated with significantly shortened root lengths of early seedlings but did not reduce total biomass. Field trials found a strong seed yield response to nitrogen treatments, with yields increasing with each treatment up to 168 kg N/ha. No significant yield response to sulfur treatments was observed. Additionally, fertilizer treatments did not have any significant effects on the activities of arylsulfatase, β -glucosaminidase, β -glucosidase, or urease in soils sampled at crop flowering. Rhizobacterial inoculants have potential to improve crop yields without additional inputs and should be tested on *C. sativa* in field settings. Urea applications can be used to improve *C. sativa* yields without any short term effects on soil enzymatic activity, but longer-term studies are needed to accurately determine the effects of the crop and its inputs on soil properties.

CHAPTER ONE

LITERATURE REVIEW OF *CAMELINA SATIVA*, BIOFUELS, PLANT GROWTH PROMOTING
RHIZOBACTERIA, AND SOIL ENZYMES*Camelina sativa*

Camelina sativa (L.) Crantz is an oilseed crop in the family *Brassicaceae*. Human cultivation of *Camelina* species dates as far back as 500 BC, likely grown for human consumption of its oil and as fodder for livestock (Bouby, 1998). It was produced intermittently throughout Europe and North America until the mid-1800s (Murphy, 2016). Currently it has gained popularity as a potential feedstock for biofuel production, particularly biodiesel and jet-fuels (Moser, 2010). *C. sativa* has multiple characteristics that make it a suitable candidate for sustainable biofuel production, including low nutrient needs, favorable seed oil compositions, and a robust range of suitable habitats.

C. sativa is considered a low input crop, with a low to moderate nitrogen (N) demand (Zubr, 1997). Crop response to nitrogen application rates vary greatly between studies. Maximum yields have been found at N rates of 120 kg/ha or greater (Jiang et al., 2013), while other studies found no significant yield increases at N rates greater than 80 kg/ha (Urbaniak et al., 2008). Others have found maximum yields at N rates as low as 54 kg/ha (Obeng et al., 2021). The need for sulfur (S) application is debated, as higher yields have been reported with applications of 25 kg S/ha at N rates higher than 120 kg/ha (Jiang et al., 2013). Other studies have found no effect from applications of sulfur (Mohammed et al., 2017; Obeng et al., 2021). Additionally, *C. sativa* is a frost tolerant crop, with low optimal temperatures relative to other

oilseeds, and seedling emergence is possible at 0 °C (Allen et al., 2014; Hosseini Sanehkoori et al., 2021).

Oil and meal derived from *C. sativa* has multiple potential industrial and pharmaceutical applications. Crude *C. sativa* oil is generally low in free fatty acids relative to other crops which allows it to be used directly for biodiesel production via transesterification without the need for pretreating the oil (Moser, 2010). Additionally, the seed meal which remains after pressing seeds for oil can be used as animal feed for poultry and ruminants, increasing potential economic returns for producers (Murphy, 2016). *C. sativa* oil may also be a potential source of wax esters, which are used in a variety of industries as industrial lubricants and cosmetic surfactants (Domergue & Miklaszewska, 2022; Iven et al., 2016). More research is needed to determine the ecological and economical effects of producing *C. sativa* at an industrial scale, but attributes of the crop suggest production would be suitable in the Northern Great Plains of North America.

Biofuels

Energy production derived from non-fossil biomass is referred to as bioenergy and energy-rich solid, gaseous, or liquid products derived from biomass are often classified as biofuels (Taymaz et al., 2021). Continual growth of greenhouse gas emissions and anthropogenic climate change has increased the pressure for global policy makers and energy sectors to find alternative energy sources such as biofuels (Gaurav et al., 2017). There are many forms of biomass that can be used in biofuel production, including but not limited to: food and feed crops, oilseeds, lignocellulosic biomass, farmed and foraged residues, and algal biomass (Ho et al., 2014).

Two liquid biofuels, biodiesel and bio-jet fuels can both be produced using plant derived oils. Biodiesels are produced through transesterification of animal or plant derived triglycerides, in which a catalyst is used to separate alkyl esters from their glycerol backbone (Knothe, 2016). Bio-jet fuels, similarly use triglycerides, which are hydrogenated, cracked, and isomerized to produce hydrogenated esters and fatty acids (Gollakota et al., 2021). Plant oils have been considered a potential fuel for diesel engines since at least the early 1900s, when a diesel engine was successfully powered by peanut oil at the Paris World's Fair at the behest of the French government. The potential use of peanut oil in diesel engines was later supported by Rudolf Diesel himself (Knothe, 2010). Biomass derived jet fuels are a relatively new concept and are one of the few known potential replacements for fossil-fuels in the aviation sectors that is readily available today (Gollakota et al., 2021).

The sustainability of biofuels from agriculturally sourced biomass has been debated, as large-scale agriculture production requires the use of fossil-fuel derived agrochemical inputs, heavy machinery, and intense land usage, all of which may cause ecosystem disturbances (Brentrup et al., 2004; Lark et al., 2022; Spawn-Lee et al., 2021). Additionally, some biofuels are derived from edible feedstocks, which may cause some to question the ethical use of food sources when many are unable to meet their nutritional needs. This has encouraged the use of non-edible feedstocks such as some oilseeds or lignocellulosic biomass (Ho et al., 2014). While there is variation between crops used, lifecycle analyses suggests that biofuel production decreases overall greenhouse gas emissions relative to fossil-fuels (Sieverding et al., 2016). An ideal biofuel crop is one that can be produced with minimal inputs of agrochemicals (low nutrient needs and pest/weed resistant) and would cause minimal ecological disturbance when produced at scale. Additionally, application of bacterial inoculants could enhance microbial

processes such as biological nitrogen fixation and nutrient solubilization, and help meet plant nutrient needs while reducing agrochemical inputs.

Plant Growth Promoting Rhizobacteria

The rhizosphere is the direct interface between plants and soils. It is defined as the zone including and surrounding a root in which the plant, soil, and biota can interact through physical and chemical means (Lynch & de Leij, 2012). Plant growth promoting rhizobacteria (PGPR) are soilborne bacteria that form associations directly with plant roots, subsisting off root exudates and providing some benefit that increases plant health or hardiness (Gouda et al., 2018). Microbe-mediated benefits such as nutrient solubilization (Ku et al., 2018) and phytohormone production (Damam et al., 2016) are direct PGPR effects. Indirect effects include the neutralization or prevention of an external stressor on the plant, such as limiting nutrient availability to potentially pathogenic soil microbes (Kloepper et al., 1980). PGPR effects vary widely from crop and strain type but offer a unique potential to augment fertilizer use or suppress common crop diseases.

Many free-living bacteria exist in bulk soils, subsisting off rhizo-deposits and unassociated soil organic matter (Parke, 1991) and can be “recruited” into a plants rhizosphere. As plant roots develop throughout the soil, they release exudates such as non-structural carbohydrates, mucilage, or enzymes. These exudates can promote motility from nearby microbes through chemotaxis (Feng et al., 2019; Xiong et al., 2020), this stimulus can drive bulk soil bacteria towards the rhizosphere. Many biotic and abiotic factors can help or hinder rhizosphere colonization. Root exudate composition, extracellular compounds produced by the microbe, and membrane permeability of plant and microbe all affect the colonization of the

rhizosphere (Lugtenberg et al., 2001). The rhizosphere is composed of many microbial species and some studies suggest that plants can influence the composition of the microbial community (Grayston et al., 1998). Once established within the rhizosphere, bacterial metabolites can aid in plant growth through various means, such as producing compounds that include indole-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate deaminase (ACCd), and siderophores (Baharlouei et al., 2011; Damam et al., 2016; Kloepper et al., 1980).

Ideal PGPR candidates for oilseed crops would increase the yield, oil content, and nutrient use efficiency of the crop. Given the diversity of bacteria within the soil, it can be difficult to select strains to test. Selections can be narrowed by looking to previous experiments to determine which bacterial taxa show the most potential in providing benefits to oilseed crops. The plant-microbe relationships of selected inoculants must first be established in smaller scale experiments and incrementally increased to field-scale for results applicable to agricultural production.

Studies have shown a variety of plant species are responsive to PGPR. Sunflowers inoculated with *Planomicrobium* and *Bacillus* species display decreased drought stress, increased root dry mass, and increased root and shoot lengths when compared to the drought stressed control (Khan et al., 2018). The authors suggest the stress alleviation could be due to production of IAA by the inoculant strains, which stimulates cell division in plant roots. Many bacterial strains of genus *Pseudomonas* also promote plant growth. *P. fluorescens* LBUM677 improves oilseed yields of *Brassica napus* (Jiménez et al., 2020), and field trials of *P. brassicacearum* strain CDVBN10 also improves *B. napus* yields (Jiménez-Gómez et al., 2020). Additionally, root lengths of canola increase when inoculated by *Pseudomonas putida* GR12-2 which produces ACCd (Hall et al., 1996).

Naturally occurring rhizosphere communities are generally comprised of many bacterial species, thus co-inoculating with multiple species may be more beneficial to plants than individual inoculants. Multiple strains of PGPR, referred to as consortia, can produce metabolites that benefit one another, creating a positive feedback that encourages the proliferation of beneficial rhizobacteria (Pandey et al., 2012). In soybean trials, co-inoculation of *Bacillus thuringiensis*-KR1 and *Bradyrhizobium japonicum*-SB1 were more effective than respective individual inoculants (Mishra et al., 2009), and a consortia of three *Aeromonas spp.* improved the salt-tolerance of wheat (Rajput et al., 2018).

PGPR have potential to provide benefits for future food and fuel crops, but there is often a disparity between experimental results in greenhouse/laboratory trials and field conditions. Most studies regarding PGPR are greenhouse- or laboratory-based experiments. Transitioning from such controlled environments to field-trials introduces a myriad of new challenges, such as field microclimates, variability in soils, preexisting microbial communities, and reliance on weather regimes, all of which may impact the function of the PGPR.

Soil Enzymes

Almost all nutrient cycles interface with soils, and some argue that soils may be examined as a biological entity of their own (Tabatabai, 1994). It is crucial that the health and nutrient cycling capacities of soils be examined when considering changes to agricultural practices, such as incorporating oil seed crops. Adequately assessing the health of a soil can be difficult, and assessments differ from focusing on the belowground biodiversity from an ecological perspective while others consider potential productivity in the context of agriculture (Guerra et al., 2020; Maikhuri & Rao, 2012). The logistics of soil health assessments must also

be considered since producers may be limited by funds and time. Soil enzyme activity is a potential metric for assessing soil health that is relatively low cost and increasingly easier to implement as protocols have standardized (Dick, 1994; Sainju et al., 2022). Soil enzyme assays are currently recommended as part of soil health assessments by numerous public and private interests including the USDA NRCS and Soil Health Institute (Acosta-Martinez et al., 2018).

Soil enzymes can be sourced from plants, animals, and microbes, however it is generally expected that microbial exudates and lysed cells are the primary source (Dick, 1994). Enzymes catalyze chemical reactions, playing a key role in nutrient cycles and transforming molecules into plant-available forms (Klose et al., 2011; Tabatabai, 1994). Soil enzyme activity may also serve as an early warning of soil degradation (Sainju et al., 2022). Measuring soil enzymatic activity is done through *in-vitro* assays typically carried out in conditions optimized for each enzyme (pH, temperature, etc.) (Bandick & Dick, 1999; Rao et al., 2014). Due to the nature of such assays, they do not represent the *in-situ* enzymatic activities of sampled soils, rather an optimized potential of activity. Despite this disparity, assay results can still be indicative of soil health, as enzymatic rates can be strongly correlated to soil organic matter (Deng & Tabatabai, 1996), and nitrogen mineralization rates (Ekenler & Tabatabai, 2007). These results may also act as indices of potential productivity, as soil enzyme activity can be sensitive to changes in biogeochemical cycling (Acosta-Martínez et al., 2011; Acosta-Martínez et al., 2019).

Individual enzyme activities can be examined as one step in a nutrient cycle, such as urease activity representing the hydrolysis of urea (Tabatabai & Bremner, 1972). The activity of multiple enzymes can be viewed as an overall trend of nutrient cycling in soils and are a component of soil health (Sainju et al., 2022). A common metric is the geometric mean of all measured enzymatic activity. This central tendency has been linked to the overall productivity of

the soil and is sensitive to management practices (García-Ruiz et al., 2008). The trends of enzymatic activity may be used in crop production to assess potential effects of the crop or management practices on the soils.

Research Objectives

The purpose of this research was to evaluate sustainable methods of *C. sativa* production through use of inoculants, optimization of fertilizer application rates, and evaluating the belowground response to fertilizer application. Potential PGPR benefits were examined through greenhouse trials that evaluated four strains of PGPR candidates and co-inoculation with two PGPR strains for efficacy in enhancing *C. sativa* agronomic performance. Field trials were performed to evaluate *C. sativa* oil and seed yield in response to different rates of nitrogen and sulfur fertilizer application at multiple Montana State University Agricultural Research Centers. Belowground ecology responses were assessed through assays examining the activity of soil enzymes under *C. sativa* cultivation. Target enzymes were involved in carbon, nitrogen, and sulfur cycling.

CHAPTER TWO

INVESTIGATING GROWTH PROMOTION AND YIELD ENHANCEMENT OF *CAMELINA SATIVA* BY APPLYING PLANT GROWTH-PROMOTING RHIZOBACTERIAIntroduction

Camelina sativa (L.) Crantz is a promising oilseed crop with increasing popularity as a biofuel feedstock for providing a sustainable means of biodiesel and jet fuel production (Moser, 2010). *C. sativa* is a member of the family *Brassicaceae*. While it is a less popular crop compared to counterparts such as canola, it can grow comparably and even outperform other *Brassica* oilseeds when grown in adverse conditions such as drought (Gugel & Falk, 2006). Yields of current *C. sativa* cultivars can vary widely depending on growth conditions, likely contributing to producers preferentially adopting other lower-risk *Brassica* oilseed crops (Murphy, 2016). The pressure to counter growing greenhouse gas emissions has driven energy-sector interests towards alternative energy sources such as biofuels (Gaurav et al., 2017). There is some debate regarding the sustainability of agriculturally sourced biofuels, as machinery and chemical inputs needed to produce these crops at scale are often reliant on fossil fuels and can cause various forms of ecosystem disturbances (Brentrup et al., 2004). The extent of life cycle greenhouse gas usage varies greatly depending on the biofuel feedstock and refining methods (Jeswani et al., 2020).

The sustainability of biofuel production must be assessed through agricultural, ecological, and economical perspectives. Dedicating agricultural resources to oilseed cultivation can decrease land availability for food and fiber crops and expanding new lands for agricultural

production may compete with urban expansion or natural land restoration. Adjusting common crop rotations and cover crop practices to incorporate biofuel feedstocks may allow for production with minimal land-use changes (Taheripour et al., 2022). However, replacing summer fallow with *C. sativa* in a wheat-fallow system is associated with decreases in subsequent wheat yields (Obeng, 2018). The profitability of *C. sativa* as a fallow replacement may outweigh the decreased wheat yield compared to fallow rotations. Adding *C. sativa* into a wheat-fallow rotation can disrupt the microbial composition in the soil, significantly decreasing bacterial and fungal diversity (Hansen et al., 2020). The incorporation of oilseeds in common cropping systems needs to be further refined and tested to provide best results for producers and the environment. Additionally, oilseed crops are often associated with low nitrogen use efficiency, which can be improved to some extent through nutrient management such as timed fertilizer regimes (Rathke et al., 2006). Nutrient demands of oilseeds may be improved further through plant growth promoting rhizobacteria (PGPR) associations. The desired outcome of advances in *Camelina*-associated PGPR is to improve oilseed yields without increases in chemical inputs, thus increasing the sustainability of agricultural and transit sectors through biodiesel production.

There are many agronomic and industrial practices being developed and tested to curb the environmental impacts of conventional agriculture and improve sustainability, from farm system management to microbial soil inoculations. Large-scale implementations such as conservation and precision agriculture adjust management at the systems-level (Gebbers & Adamchuk, 2010; Kulagowski et al., 2021), and strategies are being developed to better utilize and preserve soil biota (Cappelli et al., 2022). Soil microbiota such as PGPR can form close associations with plants through the rhizosphere, the zone surrounding roots in which the plant can chemically and physically interact with the soil and soil biota (Lynch & de Leij, 2012). From an agronomic

perspective, PGPR have potential to increase yields of economically important crops with minimal increases in agrochemical inputs (Gouda et al., 2018).

The purpose of this work was to examine the effects of several PGPR candidate strains on the agronomic performance of *Camelina sativa*. PGPR candidate strains were selected based on previously demonstrated microbial traits that benefit target crops, specifically production of secondary metabolites, and yield improvement. *Bacillus thuringiensis* ATCC 33679 can produce iron-binding siderophores, which promote plant growth by depriving competing microbiota of soilborne iron (Kloepper et al., 1980; Wilson et al., 2006). Additionally, the efficacy of some PGPR may increase in the presence of certain *B. thuringiensis* strains (Mishra et al., 2009). Co-inoculations including other strains of *B. thuringiensis* improve disease resistance in tomatoes, possibly through biocontrol traits and synergistic associations with other PGPR (Yanti & Hamid, 2021; Yanti et al., 2018). *Bacillus cereus* ATCC 14579 can act as a biocontrol agent protecting tomato from infection of *Ralstonia solanacearum*, a pathogenic bacterium that infects a broad variety of plants (Yanti et al., 2018). *Pseudomonas putida* ATCC 12633 can solubilize inorganic phosphate, increase its bioavailability in soils (De Bolle et al., 2013), and enhance the biomass and protein content of *Arabidopsis thaliana* (Liffourrena & Lucchesi, 2018). *Pseudomonas brassicacearum* strain 36D4 can produce indole acetic acid and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, two compounds associated with plant growth promotion (Gislason & de Kievit, 2020). The goal of this research was to evaluate the effect of these PGPR inoculants on the agronomic performance of camelina.

We hypothesized that the application of selected PGPR candidates or a combination thereof would be associated with an increased aboveground biomass of young and mature *C. sativa*. PGPR applications would be associated with an increased mean seed biomass in matured

C. sativa. And co-inoculation of *B. thuringiensis* with other strains would be more effective than individual inoculations. We tested these effects through two greenhouse trials examining effects on plants to maturity, and one growth chamber experiment to study the effects on early seedling development.

Methods

PGPR Candidate Culturing and Preparation

Four bacterial strains were used as plant inoculants, three were acquired through ATCC (Manassas, VA) (*B. thuringiensis* ATCC 33679, *P. putida* ATCC 12633, and *B. cereus* ATCC 14579), and one was isolated from agricultural field soil in Moccasin, MT (47.062422, -109.947653). Sanger sequencing was performed on this isolate (Eurofins, Louisville, KY, USA) and 16S amplicon BLAST results showed 93.6% pairwise identity with *Pseudomonas brassicacearum* strain 36D4. All strains were individually cultured in King's Medium B liquid media (King et al., 1954) and incubated for 48 hours in a 28° C oscillating water bath at 80 rpm. Cultures were decanted into 50 ml falcon centrifuge tubes and centrifuged at 10,000 RCF for 5 minutes, the supernatant was removed, and remaining pellets were resuspended to 50 ml volume with sterile water, this process was repeated two additional times. One milliliter samples were taken from each suspension and the optical density was measured at a wavelength of 600nm (OD₆₀₀) and cultures were diluted to a uniform density. The resulting suspensions were then used for rhizosphere inoculations.

C. sativa Agronomic Response to PGPR

Two greenhouse experiments were conducted to evaluate *C. sativa* response PGPR inoculation. In the first experiment, *C. sativa* seeds were surface sterilized by soaking for 5 minutes in a 5% sodium hypochlorite solution and thoroughly rinsed with sterile water. 6-inch diameter plastic pots were filled with either untreated or autoclaved soil (MSU Plant Growth Center (PGC) 50:50 soil mix, by volume half MSU Mix (Equal parts (by volume) of loam soil, washed concrete sand, Canadian Sphagnum peat moss and one pound per cubic yard AquaGro 2000 G wetting agent.) and half Sunshine Mix #1 (Canadian Sphagnum Peat Moss, perlite, vermiculite, starter nutrient charge, wetting agent, and Dolomitic lime.)). Soils were autoclaved for 1 hour rested for 24 hours before autoclaving again. Four *C. sativa* seeds were sown into each pot and watered daily. Seedlings were grown in the MSU PGC West-Wing greenhouse, set temperatures were 72° F days and 65° F nights, with a maintained 16-hour photoperiod utilizing SON AGRO 430-WATT High Pressure Sodium bulbs. At 14 days each pot was thinned so only one plant remained per pot.

C. sativa seedlings were inoculated after 14 days of growth by pipetting the bacterial suspensions directly to the base of the seedling stem, as previously described (Grobela et al., 2015). Inoculation treatments were divided into ten groups (Table 1) and were treated with 2ml of their respective inoculant. Control plants were treated with sterile water. Each treatment was replicated in 5 pots for a total of 50 plants, and inoculated pots were arrayed in a randomized block design on a greenhouse bench with positions shuffled every 14 days to avoid spatial pattern in environmental conditions influencing results.

Table 1. Rhizobacterial and soil sterilization treatments for *C. sativa* in initial greenhouse experiment.

Rhizobacterial Treatment	Soil Treatment	
	Sterilized	Non-Sterile
Control (No Inoculant)	C-S	C-NS
<i>Bacillus thuringiensis</i>	BT-S	BT-NS
<i>Bacillus cereus</i>	BC-S	BC-NS
<i>Pseudomonas putida</i>	PP-S	PP-NS
<i>Pseudomonas brassicacearum</i>	PB-S	PB-NS

After inoculation, plants were watered to soil capacity every 48 hours until seed pods began to form, at which point watering was decreased to once every 72 hours until all seed pods had set, and watering was ceased. Plant heights were measured two weeks after inoculation and at flowering, days from sowing to flowering was noted, and number of functional (green/not senesced) leaves were counted. Once plants were fully matured, all aboveground biomass was harvested and dried for 48 hours at 40 °C before individual plant biomass and seed yields were measured.

Inoculation With Multiple PGPR Strains

A second experiment was performed to evaluate multi-strain co-inoculations and further explore single strain effects. All methods were the same as the prior experiment unless otherwise noted. Plants were sown in 1.5-inch plastic cone-tainer pots instead of 6-inch pots and replicate numbers were increased. The same soils were used but were steam pasteurized at 70 °C for 1 hour prior to use in lieu of autoclaving. This was due to the decrease in biomass and seed weights of the sterile control treatments relative to the non-sterile treatments observed in the first experiment. BC-S and -NS were removed from the treatments as the initial experiment suggested

BC had either no effect or detrimental effects to the plants relative to the control (Figures 1 and 2.).

C. sativa seedlings were inoculated after 14 days of growth by pipetting the bacterial suspensions directly to the base of the seedling stem. The inoculation protocol was adapted from a previous report (Grobela et al., 2015). Inoculation treatments were divided into five groups: 2ml of *B. thuringiensis* suspension (BT), 2ml of *P. putida* suspension (PP), 2ml of *P. brassicacearum* suspension (PB), 1ml each of *P. putida* and *B. thuringiensis* suspensions (PPBT), and 1ml each of *P. brassicacearum* and *B. thuringiensis* suspensions (PBBT). The control treatment received 2ml of sterile water and no bacterial suspensions. Each treatment was replicated in 49 pots for a total of 294 plants. Once inoculated, all pots were randomly placed within pot racks and the racks were randomly placed on greenhouse bench space with positions being rotated every 14 days.

After inoculation plants were watered to soil capacity every 48 hours until seed pods began to form, at which point watering was decreased to once every 72 hours until all seed pods had set, and watering was ceased. Plant heights were measured two weeks after inoculation and at flowering, days from sowing to flowering was noted. Flowering was noted as stage 605 on the Biologische Bundesantalt, Bundessortenamt and Chemische Industrie scale, 50% or more buds fully opened (Martinelli & Galasso, 2011). Once plants were fully matured, all aboveground biomass was harvested and dried for 48 hours at 40 °C before individual plant biomass and seed yields were measured.

Effects of PGPR on Early Seedling Development

An additional experiment was performed to examine the effects of the rhizobacterial inoculants on early seedling development from imbibition to 14 days post-germination. BT, PB, and PP inoculants were prepared as described in the previous experiments. Once washed, inoculants were resuspended in 10ml of sterile water, and 10ml co-inoculants (PPBT and PBBT) were prepared as equal parts of their respective inoculants. *C. sativa* seeds were surface sterilized by soaking in 5% sodium hypochlorite solution for 10 minutes, and thoroughly rinsed with sterile water. To inoculate, seeds were fully immersed in suspensions of respective inoculants for 30 minutes, control seeds were soaked in sterile water in lieu of inoculants. Eight seeds were evenly placed on plates of Hoagland's agar prepared with 50% nitrogen availability to better emulate the lower N availability of the MSU PGC 50:50 mix. Three plates were prepared for each treatment, with six treatments (C, BT, PP, PB, PPBT, PBBT) for a total of 144 seedlings and 24 seedlings per treatment.

Plates were stored vertically in a CONVIRON growth chamber with a day/night cycle of 18 and 6 hours, at 22 and 17 °C respectively, for 14 days. Root and shoot lengths were measured using an Epson Perfection® V850 Pro and winRHIZO™ Pro software. All seedlings (roots and shoots intact) were removed from the agar and dried at 40 °C for 48 hours. Total dry biomass of all seedlings was recorded to the nearest tenth of a milligram.

Statistical analysis

All recorded data were compiled using Microsoft Excel and exported to RStudio 4.1.1 (R Core, 2021). Prior to any analysis of results, residuals were used to determine if datasets met all assumptions for ANOVA: normal distribution, homogeneity of variance, and observation independence. Outliers were determined using interquartile range and removed if influential.

ANOVA was used to determine if there were differences in treatment means for each normally distributed quantitative parameter (Chambers et al., 2017), those with significant differences were then examined further using Tukey's Honest Significant Differences from the "multcomp" package as a post-hoc analysis (Hothorn et al., 2008). Plant heights at flowering, days to from sowing to flowering, functional leaves at flowering, and total dry biomass were all normally distributed. Because seed biomass was left skewed in the second experiment, and remained non-normal throughout multiple transformations (log, natural log, and exponential), Kruskal-Wallis and pairwise Wilcoxon post-hoc tests from the package "stats" were used to assess relationships of seed biomass to the rhizobacterial treatments (Hollander et al., 2013). Shoot lengths showed heavily bimodal distributions so Kruskal-Wallis and pairwise Wilcoxon tests were used. Correlations between quantitative variables were assessed using Spearman's ranked correlation tests from the "stats" package (Best & Roberts, 1975). For both parametric and non-parametric tests a p-value threshold of 0.05 was used to determine significance.

Results

Biomass

The mean dry biomass of mature *C. sativa* was consistent with no significant differences between rhizobacterial treatments across both greenhouse experiments. The mean dry biomass of *C. sativa* grown in the first experiment was significantly different between sterilized and unsterilized soils (Two-way ANOVA, $F_{1,39} = 5.92$, $p = 0.02$). Post-hoc analysis estimated the difference in mean dry biomass between *C. sativa* in the sterile and non-sterile treatments was -0.30g [95% CI: -0.57, -0.03] (Figure 1A, Table 2.). Plants grown in the cone-tainers had a mean dry biomass of 0.14 ± 0.03 g (One-way ANOVA, $F_{5,152} = 1.79$, $p = 0.12$) (Figure 1B, Table 3).

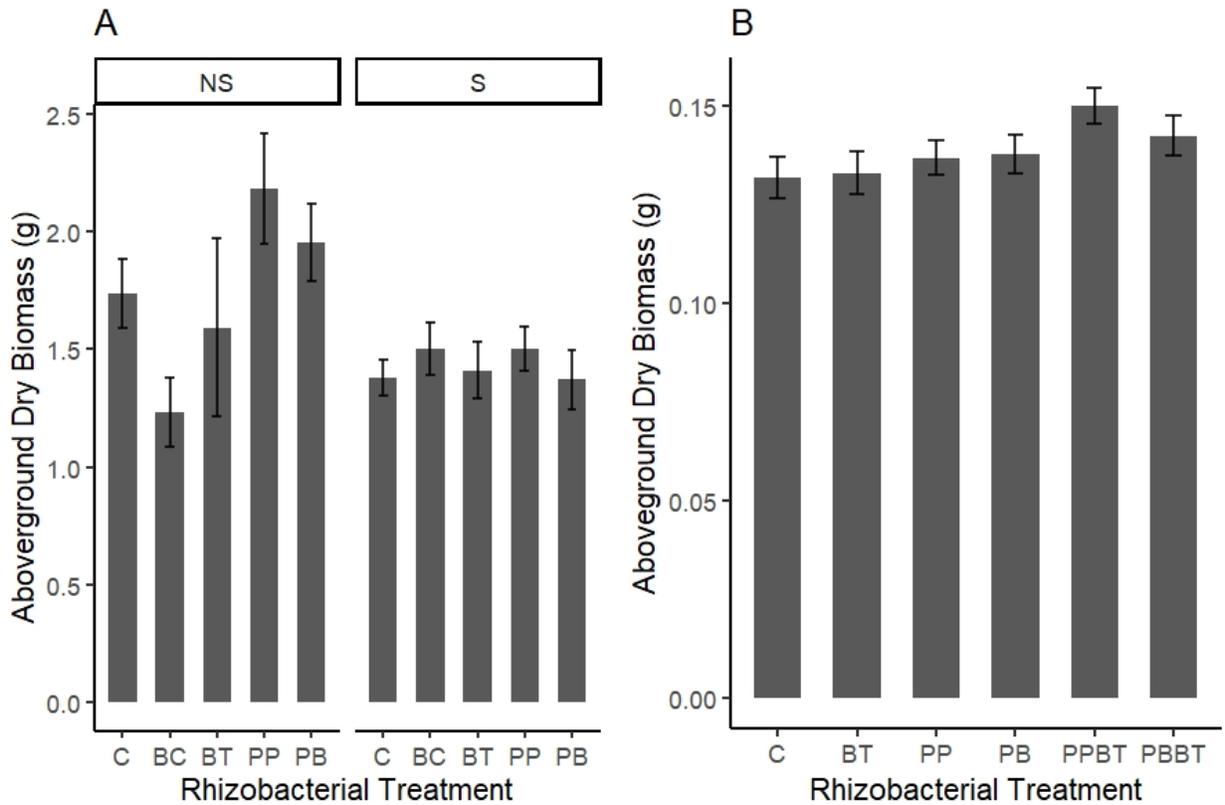


Figure 1. Mean dry biomass of individual *C. sativa* plants by rhizobacterial inoculants, error bars represent standard error of individual means. A) Mean dry aboveground biomass of fully matured *C. sativa* plants grown in non-sterile (NS) and sterilized soils (S), both groups were grown in 6-inch pots, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=45). B) Mean dry aboveground biomass of fully matured *C. sativa* plants grown in steam pasteurized soil in 1.5in cone-tainers, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=158).

Shoot Length

The mean shoot length at flowering of *C. sativa* grown with a single PGPR inoculant was 46.5 ± 6.5 cm, no significant differences were found in means between rhizobacterial inoculants, sterilization treatments, or interactions (Figure 2A, Table 2). The mean shoot length at flowering of plants grown with multiple PGPR inoculants was significantly different between rhizobacterial inoculants (One-way ANOVA, $F_{5,156} = 3.96$, $p = 0.002$). Estimated mean shoot length increased in PPBT inoculated plants relative to the control, with an estimated difference

of 3.6cm [95% CI: 0.6, 6.7], plants inoculated with PB had an increased shoot length relative control, with an estimate mean difference of 3.4cm [95% CI: 0.4, 6.3] (Figure 2B) (Table 3).

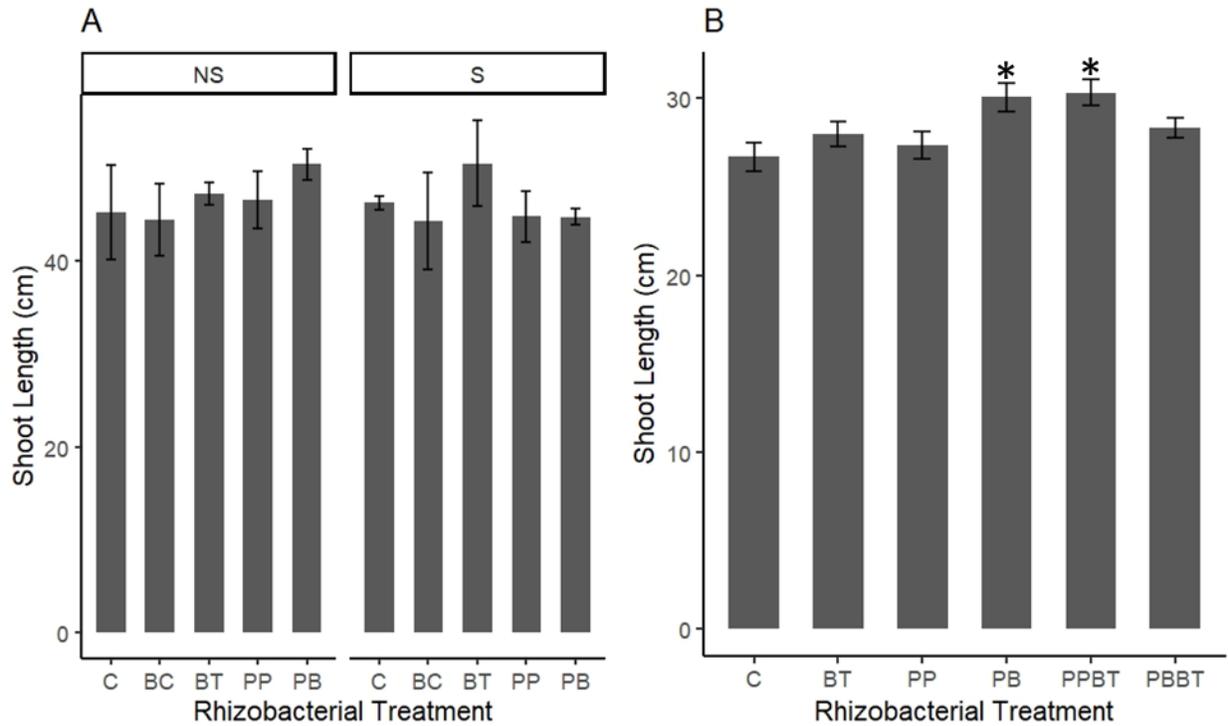


Figure 2. Mean shoot length in centimeters of individual *C. sativa* plants by rhizobacterial inoculants, error bars represent standard error of individual means. An asterisk (*) above the bar denotes a significant difference relative to the control. A) Mean shoot length of fully matured *C. sativa* plants grown in non-sterile (NS) and sterilized soils (S), both groups were grown in 6-inch pots, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=45). B) Mean shoot length of fully matured *C. sativa* plants grown in steam pasteurized soil in 1.5in cone-tainers, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=158).

Differences in Days from Sowing to Flowering

The mean time from sowing to flowering of *C. sativa* grown with single PGPR inoculants was 46 ± 3 days, there were no differences in time to flowering between rhizobacterial inoculants (Two-way ANOVA, $F_{4,35} = 0.46$, p-value = 0.77), sterilization treatments (Two-way ANOVA,

$F_{1,35} = 3.34$, p -value = 0.08), or interactions of sterilization and inoculants (Two-way ANOVA, $F_{4,35} = 1.11$, p -value = 0.37) (Figure 3A, Table 2).

Control plants had a mean time to from sowing to flowering of 49 ± 4 days and there was a significant difference in mean days to flowering between rhizobacterial inoculants (One-way ANOVA, $F_{5,154} = 2.66$, $p = 0.025$). Post-hoc analysis indicated time to flowering was shortened in plants inoculated with PPBT relative to the control, with an estimated mean difference of -2 days [95% CI: -5, 0]. Plants inoculated with PPBT flowered sooner than those inoculated with only BT, with an estimated mean difference of -3 days [95% CI: -5, 0]. No differences were found in remaining pairwise comparisons (Figure 3B, Table 3).

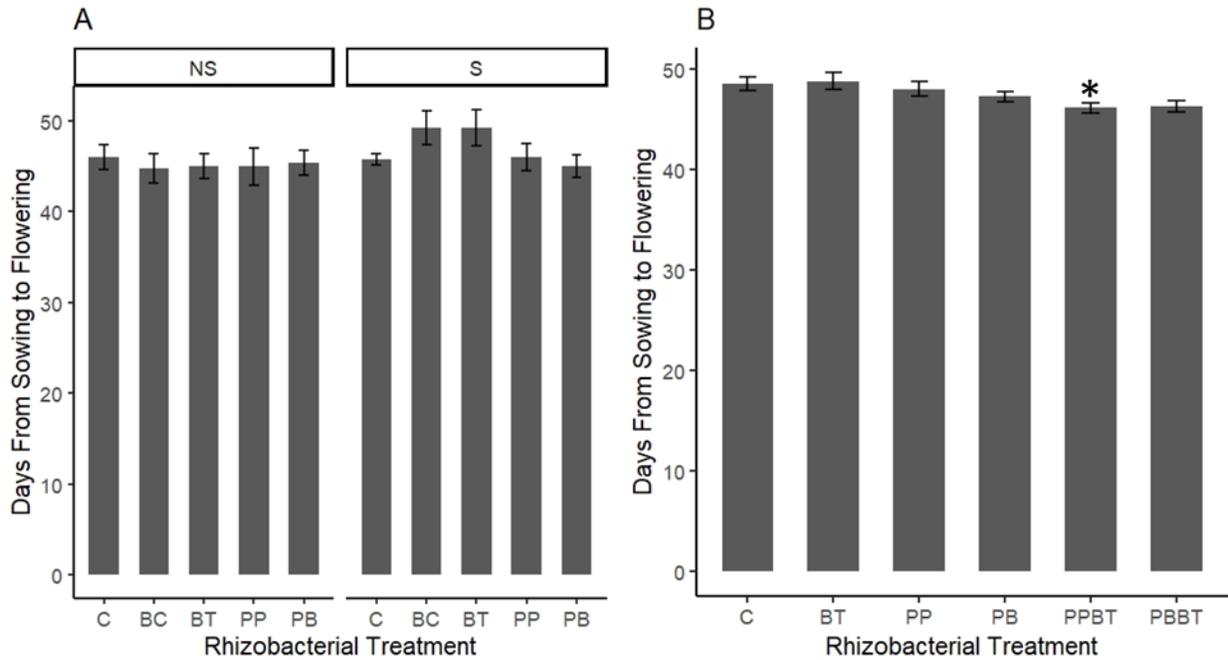


Figure 3. Mean time in days from sowing to flowering of *C. sativa* plants by rhizobacterial inoculants, error bars represent standard error of individual means. An asterisk (*) above the bar denotes a significant difference relative to the control. A) Mean time to from sowing to flowering of *C. sativa* plants grown in non-sterile (NS) and sterilized soils (S), both groups were grown in 6-inch pots, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=45). B) Mean time from sowing to flowering of *C. sativa* plants grown in steam pasteurized soil in 1.5in cone-tainers, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=158).

Differences in Seed Yields

The mean seed yield of *C. sativa* grown with single PGPR inoculants was 0.26 ± 0.14 g per plant, yields were consistent with no differences between rhizobacterial treatments (Two-way ANOVA, $F_{4,35} = 0.74$, p-value = 0.6), sterilization treatment (Two-way ANOVA, $F_{1,35} = 0.09$, p = 0.8), or from interactions thereof (Two-way ANOVA, $F_{4,35} = 0.98$, p = 0.4) (Figure 4A, Table 2).

In the second experiment the mean seed biomass of the *C. sativa* uninoculated control was 11.4 ± 9.3 mg. There were significant differences of mean seed biomass between rhizobacterial inoculants (One-way ANOVA, $F_{5,157} = 2.78$, p = 0.02). Post-hoc analysis estimated

that the mean seed biomass of *C. sativa* inoculated with PPBT was higher than those of the control, with an estimated difference of 7.2mg [95% CI: 0.3, 14]. Additionally, the mean seed biomass of *C. sativa* inoculated with PPBT was higher than that of BT with an estimated mean difference of 7.6mg [95% CI: 0.6, 14.6] (Figure 4B, Table 3).

A significant correlation of -0.62 was estimated between seed biomass and days from sowing to flowering ($p < 0.0001$, Figure 5A). Additionally, a correlation of 0.16 was estimated between seed biomass and shoot length at flowering ($p = 0.039$, Figure 5B). Both correlations determined using data from the only the second experiment.

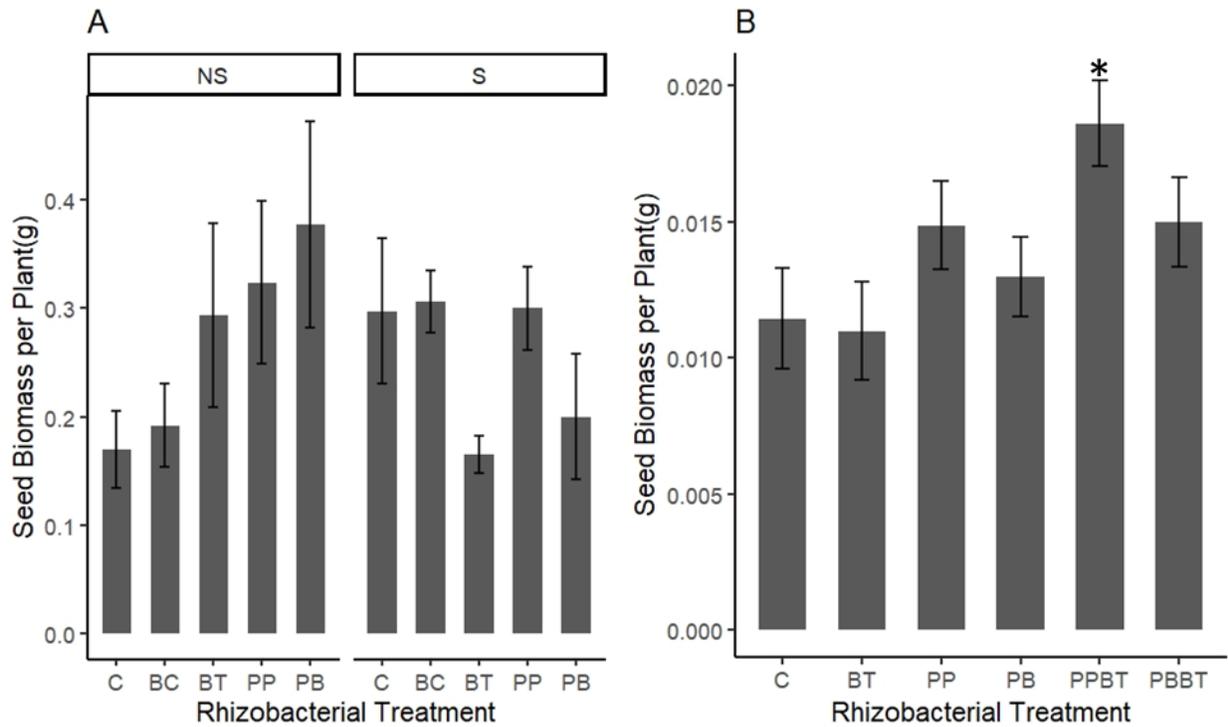


Figure 4. Mean seed biomass in grams of *C. sativa* plants by rhizobacterial inoculants, error bars represent standard error of individual means. An asterisk (*) above the bar denotes a significant difference relative to the control. A) Mean seed biomass of fully matured *C. sativa* plants grown in non-sterile (NS) and sterilized soils (S), both groups were grown in 6-inch pots, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=45). B) Mean seed biomass of fully matured *C. sativa* plants grown in steam pasteurized soil in 1.5in cone-tainers, plants were inoculated 14 days after sowing and harvested 90 days after sowing (n=158).

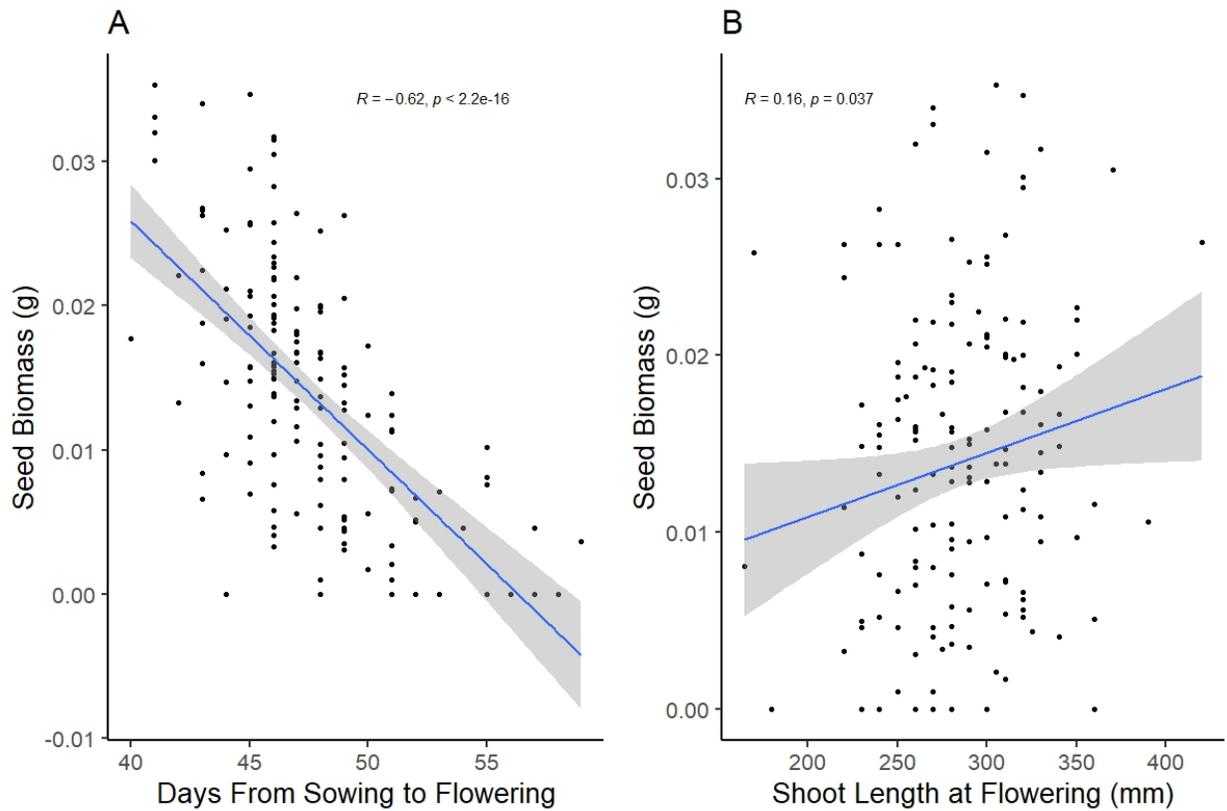


Figure 5. Total seed biomass per plant of *Camelina sativa* as a function of agronomic parameters, a trendline prediction interval of 95% confidence is represented in dark grey along the blue trendline. R = Pearson's correlation coefficient. A) Seed biomass per plant as a function of days from sowing to flowering. B) Seed biomass per plant as a function of shoot length at flowering.

Table 2. Two – Way ANOVA results of agronomic measurements of *Camelina sativa* by rhizobacterial inoculant and soil sterilization, boldface p-values indicate statistical significance.

Aboveground Biomass	DF	F-value	P-value
Inoculant	4	1.91	0.13
Sterilization	1	1.10	0.02
Inoculant*Sterilization	4	0.35	0.13
Residuals	35	-	-
Shoot Length			
Inoculant	4	0.60	0.66
Sterilization	1	0.12	0.73
Inoculant*Sterilization	4	0.53	0.71
Residuals	35	-	-
Days to Flower			
Inoculant	4	0.46	0.77
Sterilization	1	3.34	0.08
Inoculant*Sterilization	4	1.11	0.37
Residuals	35	-	-
Seed Biomass			
Inoculant	4	0.74	0.57
Sterilization	1	0.09	0.77
Inoculant*Sterilization	4	0.98	0.43
Residuals	35	-	-

Table 3. One-Way ANOVA results of agronomic measurements of *Camelina sativa* by rhizobacterial inoculant, boldface p-values indicate statistical significance.

Aboveground Biomass	DF	F-value	P-value
Inoculant	5	1.79	0.12
Residuals	152	-	-
Shoot Length			
Inoculant	5	3.49	0.01
Residuals	156	-	-
Days to Flower			
Inoculant	5	2.66	0.03
Residuals	158	-	-
Seed Biomass			
Inoculant	5	2.78	0.02
Residuals	157	-	-

Early Seedling Development

The mean shoot height of *C. sativa* seedlings at 14 days was 2.69 ± 0.78 cm, no significant differences in mean shoot height was found between rhizobacterial inoculants (One-way ANOVA, $F_{5,113} = 0.20$, $p = 0.96$, Figure 6A.). Root lengths of *C. sativa* seedlings at 14 days differed significantly (Kruskal-Wallis test, $H_{df=5} = 46.4$, $p = <0.0001$). Post-hoc pairwise Wilcoxon tests estimated significant differences in mean root length in multiple comparisons. The mean root length of control plants was significantly shorter compared to those inoculated with PP ($p = 0.02$) and longer than those with PBBT ($p = 0.0002$). The mean root length of plants inoculated with BT was significantly longer than those inoculated with PPBT ($p < 0.0001$). The mean root length of plants inoculated with PP was significantly longer than those of PPBT ($p = 0.007$), and the mean root length of plants inoculated with PB was significantly

longer than those of PBBT ($p = 0.002$, Figure 6B.). The mean dry biomass of *C. sativa* seedlings harvested at 14 days was $1.9 \pm 1\text{mg}$ and no significant differences in mean dry biomass were found between rhizobacterial inoculants or the control (One-way ANOVA, $F_{5,113} = 0.75$, $p = 0.6$, Figures 6C and 7).

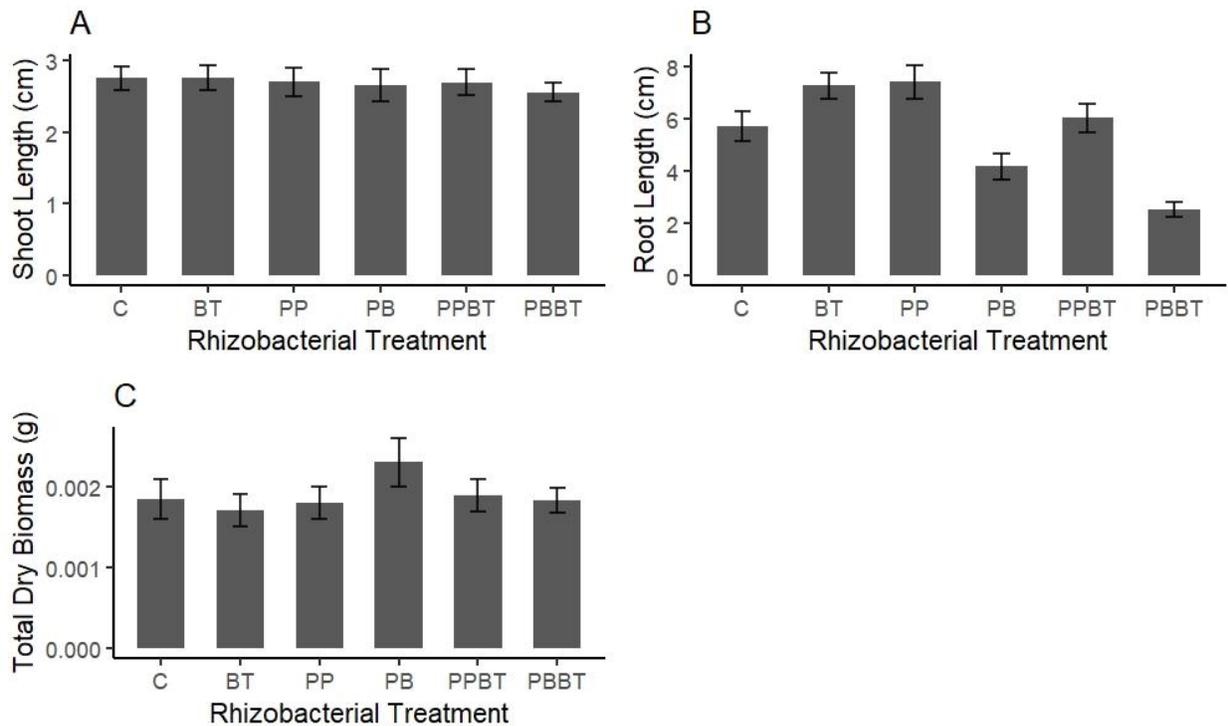


Figure 6. Mean root and shoot lengths in centimeters, and dry biomass in grams of *Camelina sativa* at 14 days by rhizobacterial inoculant, all seedlings were grown on 0.5N Hoagland's agar (n total = 119). Error bars represent standard error of individual means. A) Mean shoot lengths by rhizobacterial inoculant. B) Mean root lengths by rhizobacterial inoculant. C) Mean total dry biomass.

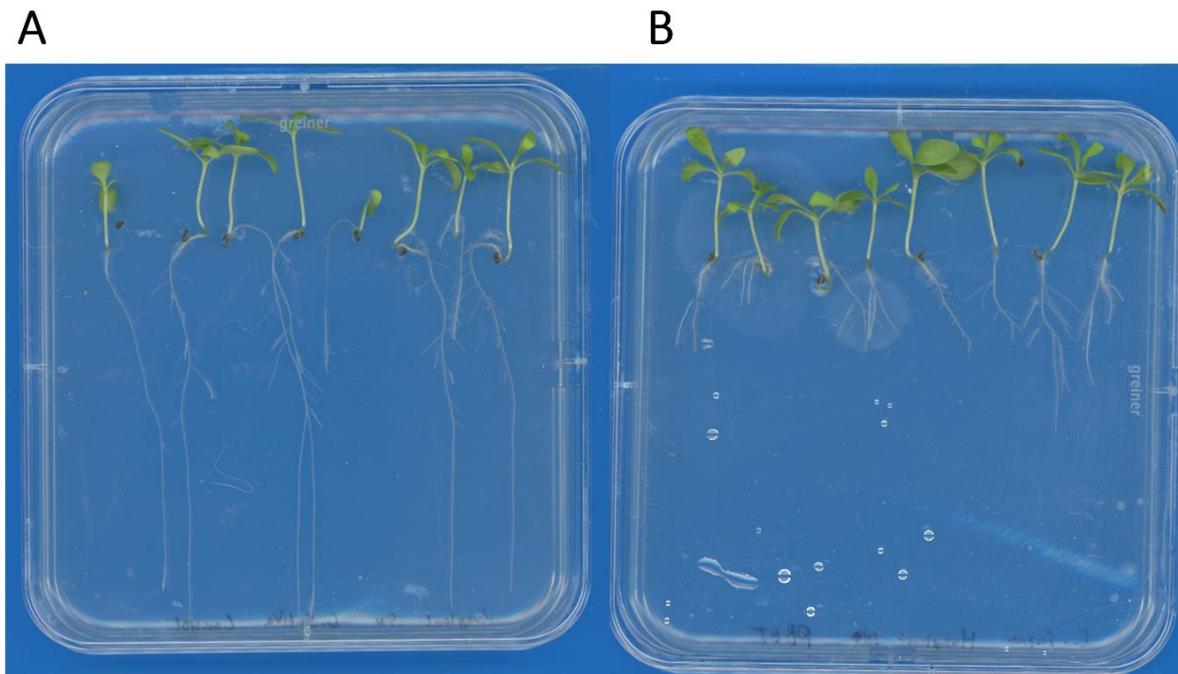


Figure 7. *Camelina sativa* treated with rhizobacterial inoculants grown on 0.5N Hoagland's agar at 14 days post-sowing. A) Control rhizobacterial treatment, sterile water bath. B) Combined *P. brassicacearum* and *B. thuringiensis* rhizobacterial treatment.

Discussion

Improving seed yields without increasing fertilizer inputs would increase the viability of *C. sativa* as a sustainable biofuel crop. Higher yields with fewer inputs would allow for higher agricultural production with lower lifetime carbon emissions (Brentrup et al., 2004; Gaurav et al., 2017). In this study we found that individual and co-inoculations of PGPR candidate strains can result in small but significant changes in several agronomic parameters of *C. sativa*.

Time to Flowering

Both co-inoculations PPBT and PBBT reduced time to flowering relative to the control (Figure 3B). This faster development in flowering time may be caused by enhanced nutrient availability by the inoculations in the case of PPBT. This strain of *P. putida* is capable of

solubilizing inorganic phosphorous and increasing plant-available P (De Bolle et al., 2013). Increased P availability can reduce time to flower initiation in *Brassica oleracea* (Elahi et al., 2015). Another possible contributing factor could be metabolite production by *P. brassicacearum* (Gislason & de Kievit, 2020), which produces ACC deaminase, an enzyme associated with more vigorous plant growth and ethylene regulation (Saleem et al., 2007).

The switch from vegetative to reproductive phases varies widely due to individual plant genetics and environments in which it is grown (Méndez-Vigo et al., 2011). Gomez and Miralles (2011) suggested that *Brassica napus* seed yields can be increased by lengthening the duration of the late reproductive phase at the expense of the vegetative phase. Additionally a negative correlation between time to flowering and final seed yield has been found in *Brassica juncea* (Saroj et al., 2021). The strong negative correlation between seed yield and days to flowering in this study suggests a similar trend in *C. sativa*. In dryland field settings in Montana, earlier flowering may increase yields, as spring precipitation can overlap with reproductive phase growth. Longer flowering stages of canola are associated with increased seed yields, and conversely early drought conditions that affect flowering stages can decrease canola silique counts and final yields (Secchi et al., 2023; Wright et al., 1995), *C. sativa* may be influenced similarly by drought. Phenological development of *C. sativa*, and its relationship with PGPR inoculants should be explored further.

Dry Biomass, Shoot Length, and Seed Yields

Dry biomass was not affected by the inoculation treatments in fully matured plants. However, there was an increase in mean plant height in both PPBT and PB inoculations relative to the control with a mean increase of 3.4 and 3.6cm respectively. In this study, there was a slight

positive correlation between plant height and plant seed yield, which is inverse to the relationship of plant height and seed yield of *Brassica juncea* reported by Saroj et al. (2021). The mean seed yield of plants inoculated with PPBT was approximately 60% higher than that of the control. This increase includes plants that failed to produce seeds but exclusion of the zero yield plants retained the significant effect. This increase is in line with similar research, in which co-inoculations including strains of *P. putida* increased seed yields of *Brassica campestris* (Dutta et al., 2017). As with the decreased time to flowering, the P-solubilizing of the PP inoculant may have influenced the final seed yield (de Freitas et al., 1997). The mean seed yields of plants with the combined PPBT inoculants were also roughly 60% higher than those of the BT inoculants, but no differences were found between PPBT and PP mean seed yields. The mean seed yield of plants in the PP treatment was numerically higher than those of the control, but the difference was non-significant. Co-inoculations may have more robust metabolite production that bolster the rhizobacteria and the plants they are associated with (Pandey et al., 2012), however, these experiments did not identify mechanisms that explain why the PPBT to significantly outperform the control while the PP do not.

Early Seedling Effects

While the PPBT inoculum affected multiple traits in mature *C. sativa* it had little influence on the plants during early development. The only significant differences noted between rhizobacterial treatments was root length, with a longer mean root length in the PP treatment relative to the control, and shorter in the PBBT treatment. *P. brassicacearum* 36D4 is closely related to other *Pseudomonas* species that are plant pathogens, and has characteristics of both

PGPR and phytopathogens, such as genes that encode Hrp1 T3SS, a protein associated with both pathogenicity and commensal relationships (Gislason & de Kievit, 2020).

It would be expected that ACC deaminase activity and IAA produced by *P. brassicacearum* would induce root elongation, since low concentration of IAA stimulates root growth in canola (Patten & Glick, 2002). While root lengths were suppressed, shoot lengths and total biomass did not differ from the control, suggesting that the reduced seedling root growth did not reduce overall growth of plants treated with PBBT. Root-shoot ratios should of *C. sativa* at multiple developmental stages should be assessed to see if this trend persists throughout the lifecycle of the crop.

Future Direction

While PGPR studies must begin *in vitro* as proof of concept, field trials are critical to accurately translate findings to agricultural settings. Field trials would ideally entail multiple inoculants over a range of nutrient application rates to test the response under variable field conditions. Additionally, this study was conducted entirely with the Suneson variety of *C. sativa*, testing inoculants on multiple lines of *C. sativa* and similar crops such as *B. napus* would improve the future prospects of these strains in PGPR applications.

This study examined the effects of multiple inoculants on *C. sativa*, but not the beneficial mechanisms associated with them. Biochemical properties of the ATCC strains are well documented, but further studies to quantify growth and metabolite production of the combined strains are needed. Understanding the dynamics of interactions between PGPR strains would provide insight into how these inoculants provided growth promotion.

Conclusions

Biofuels may provide a sustainable means of liquid fuel production, but improvements must be made regarding the agrochemical needs of growing the feedstocks. PGPR may provide an avenue for such changes. The strains used in this study, particularly the combined application of *P. putida* ATCC 12633 and *Bacillus thuringiensis* ATCC 33679, can induce earlier flowering and improve the seed yields of *Camelina sativa* without any addition of fertilizers. This study did not consider nutrient availability and future research should explore if yield improvement is consistent at differing levels of fertilizer, and how much fertilizer can be offset by these PGPR. Additionally, how are these nutrient dynamics altered by the presence of PGPR, as suggested by the altered root morphologies in the early seedling trials. Ideally, if these effects can be replicated in field applications, overall production of *C. sativa* for biofuels can be increased with limited need to increase agrochemical inputs.

CHAPTER THREE

NITROGEN FERTILIZATION AFFECTS *CAMELINA SATIVA* SEED YIELDS WITHOUT
ALTERING BULK SOIL ENZYMATIC ACTIVITY IN DRYLAND CROPPINGIntroduction

The oilseed crop *Camelina sativa* (L.) Crantz is a potential feedstock for biodiesel and jet fuel production (Moser & Vaughn, 2010). It can have inconsistent yields relative to similar crops in the family *Brassicaceae* in standard conditions but can outperform crops such as canola in drought conditions (Gugel & Falk, 2006; Murphy, 2016). These traits make *C. sativa* a suitable crop for dryland cropping systems in the Northern Great Plains of the United States and Canada, but further agronomic research and market recognition is needed for producers to consider adoption of the crop (Obour et al., 2015). A driving pressure to increase oilseed production for biofuels comes from the need to counter greenhouse gas emissions in transit and agriculture sectors (Gaurav et al., 2017). The sustainability of biofuel production from agricultural feedstocks has been debated due to the fossil fuel usage of agrochemical inputs and heavy machinery needed in large-scale agriculture, and land usage may also cause ecosystem disturbances (Brentrup et al., 2004). Lifecycle assessments of biofuel production suggest overall decreases in emissions relative to fossil fuels (Sieverding et al., 2016).

Due to low adoption of the crop, there is limited research regarding nutrient recommendations for *C. sativa*. Studies have shown it responds positively to increasing nitrogen fertilization in grain and oil yields, but will decrease in seed oil content (Jackson, 2008; Sintim et al., 2015). Field studies in Montana that tested grain response to N, phosphorous (P), potassium

(K), and sulfur (S) found the highest yields with maximum N-P-K-S treatments of 134-22-22-28 kg/ha, compared to a non-significant decrease in yield with treatments of low N only (Mohammed et al., 2017). The effects of sulfur applications on *C. sativa* are unresolved; in the previous study, sulfur applications increased seed yield relative to the control, but were not significantly different from the yields with 50-0-0-0 (N-P-K-S). Field trials in eastern Canada observed significant increases to *C. sativa* yields when applying sulfur (25 kg S/ha) at higher levels of nitrogen fertilization (≥ 80 kg N/ha)(Jiang et al., 2013). Pot experiments testing combined effects of N and S found no yield effects from increased S applications (Lošák et al., 2011). Other *Brassica* oilseeds can respond positively to increased S applications, with increases in seed oil content (Ahmad et al., 2000). Determining optimum nutrient application rates for maximum profitable seed/oil yields may improve adoption and sustainability of the crop.

Understanding the nutrient needs and belowground effects of a crop and management practices is essential in assessing the sustainability of its production. Plant species can alter soil microbial communities, *C. sativa* temporarily decreased soil microbial abundance when added into a wheat-fallow rotation (Hansen et al., 2020) and Brassicas generally do not form mycorrhizal associations (Lambers & Teste, 2013). Both effects may alter soil communities when introduced into a cropping system and these alterations may have broader effects on soil biogeochemical processes.

In addition to plant impacts on soil microorganisms, agronomic practices that include intensive fertilization have reduced plant dependence on microbial processes mediating nutrient cycling. This has effectively uncoupled plant productivity from microbial processes (Murphy et al., 2017). Field trials by Bolton et al. (1985) found that soil organic matter and enzymatic activity was generally lower in fields treated with mineral fertilizers than those treated with

organic inputs such as green manures. Additionally, a meta-analysis by Bebber and Richards (2022) found that mineral fertilizers were associated with an increase in microbial functional diversity relative to unfertilized controls but was lower relative to organic fertilizer treatments and did not change the bacterial taxonomic diversity. Understanding potential effects of needed inputs is essential in assessing the feasibility of a biofuel crop.

Soil enzymes catalyze the biogeochemical reactions which drive nutrient cycling (Tabatabai, 1994). Various biota are sources of soil enzymes, but the majority are sourced from microbial exudates and biomass (Dick, 1994). Soil microbes, enzymatic activity, and capacity for nutrient cycling are tightly linked (DeAngelis et al., 2008; Tabatabai et al., 2010). As a result, soil enzymatic activity can be used as a biological indicator to detect changes in biogeochemical activity and soil health in response to agricultural management practices (Acosta-Martínez et al., 2019). Due to the nature of enzyme assays, the results represent potential activity at the time of sampling and do not reflect the actual activity occurring *in situ* (Dick, 1994). Assays are carried out in conditions optimized for each enzyme, therefore their results should be interpreted as a maximum potential activity. As there are many different enzymes, indices can be used as a synthesized representation of the total potential enzymatic activity. When performing assays for multiple enzymes, the geometric mean of all enzymatic activities from the same sample can be used as an index for overall enzymatic activity that is sensitive to soil management practices (Paz-Ferreiro et al., 2012).

Enzymatic activities often represent individual steps of various nutrient cycles. Urease activity can partially describe soil biological capacity for hydrolysis of urea to CO₂ and NH₃, a plant-available form of nitrogen (Tabatabai, 1994). N-acetyl-β-D-glucosamine hydrolysis as measured through N-acetyl-β-D-glucosaminidase (NAGase) assays can provide an index for N-

mineralization and plays a large role in C and N cycling in soils (Ekenler & Tabatabai, 2002; Ekenler & Tabatabai, 2007). Arylsulfatase catalyzes the hydrolysis of ester sulfates and is integral to providing plant available S (Klose et al., 2011). β -glucosidase is a ubiquitous enzyme in carbon cycling and is a potential limiting factor in soil cellulose degradation, and its activity releases energy sources for soil microbes (Bandick & Dick, 1999; Turner et al., 2002).

Soil arylsulfatase activity decreases in response to urea applications (Davies et al., 2022), decrease from low pH due to N acidification (Wang et al., 2016) and is generally sensitive to low soil pH (Deng & Tabatabai, 1997). Increasing applications of $MgSO_4$ decreases arylsulfatase activity (Baligar et al., 2005) while long-term $CaSO_4$ applications increase activity (Inagaki et al., 2016). Urease activity is decreased by $MgSO_4$ applications (Baligar et al., 2005). β -glucosidase was unaffected by urea applications in *Zea mays* field trials in Minnesota (Davies et al., 2022). A meta-analysis of the effects of nitrogen applications on soil enzyme rates found that N fertilization significantly increased β -glucosidase and urease activities, but did not have a significant effect on NAGase activity (Jian et al., 2016).

Changes in soil enzyme activity in response to fertilizer application have implications for soil health as indicators that are sensitive to changes in biogeochemical cycling (Acosta-Martínez et al., 2019; Sainju et al., 2022). This has led to the adoption of enzyme activity metrics for soil health by the Soil Management Assessment Framework (SMAF) (Stott et al., 2010) and the USDA NRCS (Kim et al., 2020). However, there are questions regarding the efficacy of enzyme activity as an indicator of soil health and more work is needed to relate activity to soil properties and crop performance (Sainju, et al., 2022).

There is limited research regarding responses of soil enzymatic activity to N and S fertilizer under *Camelina sativa* cultivation. Understanding the effects of a biofuel crop on soil

health is essential to evaluate its prospects as a sustainable product. The goal of this study was to evaluate the agronomic response of *C. sativa* to different rates of N and S application and determine the effects of fertilization on nutrient cycling within the soil microbial community. This study assayed four soil enzymatic activities, Arylsulfatase, Urease, NAGase, and β -glucosidase. Collectively, these activities were used to examine the rates of carbon, nitrogen, and sulfur cycling in the bulk soils of *C. sativa*.

Methods

Agronomic Fertilizer Trials

Agronomic trials in 2022 were conducted at five Montana State University Research Centers; Central (CARC), Eastern (EARC), Northern (NARC), Northwestern (NWARC), and Southern (SARC) Agricultural Research centers in Moccasin, Sidney, Havre, Kalispell, and Huntley Montana respectively. In 2023, studies were performed at the highest (EARC) and lowest (CARC) yielding sites regarding 2022 *C. sativa* seed yields.

Soil sampling was carried out prior to planting to determine residual nitrate and sulfate levels (Table 4)2. N fertilizer was applied to target soil fertility levels of 34, 67, 101, 134, and 168 kg/ha. The N source was pelleted urea (46-0-0). S fertilizer was applied as pelleted gypsum (CaSO_4) at rates of 0 and 22 kg Sulfate-S/acre. *C. sativa* seeding rate was 5.6 kg/acre planted at approximately 1.5cm depth. Seeds were supplied by Sustainable Oils, Inc (Great Falls, MT). Treatments were applied in a randomized block design and planted in 1.5 x 5m 5-row plots with 0.3m row spacing, with untreated plots located between treatment plots to minimize edge effects between treatments. Weeds were controlled by an initial application of glyphosate prior to planting and a broadcast application of Prowl at planting. Emergent weeds were removed by

hand. Seeds were harvested at plant maturity (pods dried out and golden/tan coloration) by combine in 2022, and by combine and hand in 2023 due to seeder malfunction causing uneven row spacing in several plots. 2023 agronomic results require further processing and were omitted from this report. Harvested seed was cleaned and weighed at all sites and seed oil content was measured for all sites except NWARC.

Table 4. Mean and standard deviations of soil properties at sampled locations. Only one sample collected at sites denoted with (*) preventing calculation of mean or standard deviation.

Location	pH	Organic Matter (%)	Nitrogen (lb/acre)	Potassium (ppm)	Sulfate-S (lb/acre)	Phosphate (ppm)
CARC	7.3 ± 0.7	4.9 ± 0.2	21 ± 11	329 ± 90	13.8 ± 2.0	47 ± 35
EARC	8.1 ± 0.1	3.2 ± 0.1	24 ± 12	358 ± 31	46 ± 11	18 ± 3
NARC*	6.2	1.4	14	361	66	39
NWARC*	7.2	2.9	8	218	14	38
SARC*	7.9	2.8	3	-	-	10

Soil Extracellular Enzyme Assays

Soil samples for enzyme activity assays were collected mid-season (early to mid-flowering) from low and high N and S treatments. Bulk soils between rows were collected from 0-6" depth and a composite sample of 6 cores per plot was used for analysis. All samples were air dried, sieved to 2mm and stored in 50ml falcon tubes at -20 °C until analysis.

Enzymatic activity was measured through colorimetric assays. All assays were adapted to fit a high throughput method in 96-well plate format as described by Cordero et al. (2019). Five technical replicates of each sample were used in this format (1 sample per column of deep-well plate, with final column acting as negative control). Enzyme incubations were performed in 96 2ml deep well polypropylene plates, and colorimetric readings were carried out in 96-well microplates with a Molecular Devices SpectraMax® M2 microplate reader.

All assays followed the same protocol unless otherwise noted. Enzyme specific substrates and buffers are shown in Table 5. Two grams of soil was weighed out and suspended in 40ml of buffer. For arylsulfatase, the weighed soil was incubated with toluene for 1 hour prior to suspension. Soils and buffer were mixed with a magnetic stir rod and the soil slurry was transferred to each well of a deep-well column. The enzyme-specific substrate was added to the top 5 wells of each column, while the remaining 3 wells were used as sample-specific negative controls. The plate was then sealed and incubated. For the β -glucosidase activity assay, toluene was added to all wells prior to incubation. Plates were incubated on a 37 °C shaker at 250RPM for 1 hour, Urease plates were incubated at 18 °C for 2 hours. Stop solutions were added to all wells after incubation to halt the reactions, arylsulfatase reactions were halted in a 0 °C ice bath, urease reactions were incubated for an additional 30 mins after substrate was added to extract ammonia from soils. Enzyme-specific substrates were added to all negative control wells. Plates were centrifuged at 2250 RCF for 6 minutes, and supernatants were transferred to respective wells in a clear 96-well microplate. Additional reagents were added to the microplates after centrifugation for arylsulfatase and urease assays to adjust pH prior to recording absorbance (Table 5). The absorbance of each well was measured at a wavelength of 405nm, with the exception of Urease plates, which were measured at 650nm. Soil enzymatic activity was measured by P-nitrophenol released given a standard calibration curve of 0-10 μ g/ml of P-nitrophenol (Urease activity as ammonium released given a standard curve of 0-3.5 μ g/ml ammonia). Arylsulfatase was only assayed for CARC and EARC.

Statistical Analysis

All recorded data were compiled using Microsoft Excel and exported to RStudio 4.1.1 (R Core, 2021). Oil yields were calculated by multiplying the yield of each plot by the measured oil content percentage, and enzymatic geometric means were calculated by taking the product of all enzymatic activity to the fourth root (Equations 1 and 2). Site years were assessed individually unless otherwise noted. Prior to any analysis of results, residuals were examined to determine if datasets met all assumptions for ANOVA: normal distribution, homogeneity of variance, and observation independence. Outliers were determined using interquartile range and removed if influential (Rousseeuw & Hubert, 2011). Two-way ANOVA was used to determine if there were differences in means by N and S treatments for each normally distributed quantitative parameter (Chambers et al., 2017). Those with significant differences were then examined further using Tukey's Honest Significant Differences from the "multcomp" package (Hothorn et al., 2008) and Fisher Least Significant difference tests from the "agricolae" package for post-hoc analysis (Mendiburu, 2019).

Table 5. Substrates used for assays of enzymatic activities.

Enzyme	Substrate	Amount in well	Notes
Arylsulfatase: EC 3.1.6.1 (Whalen & Warman, 1996)	Toluene	400 μ l	Added to soil for 1-hour static incubation before suspension.
	0.5M Sodium Acetate Buffer (pH 5.8)	40ml / 800 μ l	2 grams of sieved soil suspended in full amount, then pipetted into wells.
	0.05M p-nitrophenol sulfate	200 μ l	Enzyme specific substrate.
	0.5 NaOH	100 μ l	Added to microplate after incubation
NAGase: EC 3.2.1.30 (Parham & Deng, 2000)	0.1M Sodium Acetate Buffer (pH 5.5)	40ml / 600 μ l	2 grams of sieved soil suspended in full amount, then pipetted into wells.
	10mM P-Nitrophenol-N-acetyl- β -D-glucosaminidase	250 μ l	Enzyme specific substrate.
	0.5 NaOH	600 μ l	Added to deep wells after incubation.
	0.5 CaCl ₂	150 μ l	Added to wells after incubation.
β -glucosidase: EC 3.2.1.21 (Eivazi & Tabatabai, 1988)	Modified Universal Buffer (pH 6.0) (Skujins et al., 1962)	40ml / 600 μ l	2 grams of sieved soil suspended in full amount, then pipetted into wells.
	0.05M p-nitrophenol- β -D-glucopyranoside	150 μ l	Enzyme specific substrate.
	Toluene	37.5 μ l	
	0.1M Tris(hydroxymethyl) aminomethane-NaOH solution (pH 12)	600 μ l	Added to deep wells after incubation.
	0.5 CaCl ₂	150 μ l	Added to deep wells after incubation.

Enzyme	Substrate	Amount in well	Notes
Urease: EC 3.5.1.5 (Cordero et al., 2019)	50mM sodium acetate buffer (pH 5.0)	40ml / 250µl	2 grams of sieved soil suspended in full amount, then pipetted into wells.
	50mM urea solution	100µl	Enzyme specific substrate.
	2M KCl	1000µl	Added to deep wells after incubation.
	1mg/ml dichloroisocyanuric acid sodium salt dehydrate	30µl	Oxidizing solution. Prepared during incubation. Added to microplate.
	170mg/ml sodium salicylate and 1.278mg/ml sodium nitroprusside dehydrate	75µl see notes	Color solution. Prepared during incubation as a volumetric 2:1 mixture of Color solution:Oxidizing solution before added to microplate.

$$\text{Geometric mean} = \sqrt[n]{x_1 \times x_2 \times \dots \times x_n}$$

Equation 1. n = the number of values and x = values multiplied.

$$\text{Geometric Mean of Enzymatic Activity} = \sqrt[4]{(Aryl \times \beta gluc \times NAGase \times Urea)}$$

Equation 2. Contextual example of individual sample geometric mean in which *Aryl* = assayed arylsulfatase activity, *βgluc* = assayed β-glucosidase activity, *NAGase* = assayed N-acetyl-β-D-glucosaminidase activity, and *Urea* = assayed urease activity.

Results

Agronomic Response

There was a strong response of seed yield to nitrogen treatments at all sites except CARC in 2022. Seed yields generally increased with higher nitrogen treatments with the highest yield of 2398 kg/ha from EARC at 168 kg N/ha. Yield increases were non-significant between 134 and

168 kg N/ha at sites with the additional 168 kg N/ha treatment (Table 6). There were no effects to mean seed yields from sulfur treatments (Two-way ANOVA $p > 0.05$).

Table 6. Estimated mean seed yields of *Camelina sativa* as a response to nitrogen treatments. Crops were produced in 2022 at five different agricultural research centers across Montana. Differing superscript letters indicate significant differences in yields between N-fertilization at individual sites as determined by Fisher's LSD test.

N Rate (kg/ha)	Yield by Location (kg/ha)				
	CARC	EARC	NARC	NWARC	SARC
168	-	2398 ^a	2396 ^a	-	-
134	762 ^a	2261 ^{ab}	2278 ^{ab}	2486 ^a	923 ^a
101	666 ^a	2072 ^b	2173 ^b	1874 ^b	809 ^b
67	639 ^a	1791 ^c	1874 ^c	1781 ^b	744 ^b
34	605 ^a	1686 ^c	1693 ^d	1245 ^c	569 ^c

Nitrogen applications had a significant effect on seed oil content at all locations except CARC (NWARC oil was not measured). Seed oil content generally decreased with increase nitrogen applications with the highest seed oil content of 38.6% at EARC at 67 kg N/ha (Table 7). Seed oil content was unaffected by soil sulfur treatments at all sites except CARC, where results suggested an interactive effect between sulfur and nitrogen treatments (Two-way ANOVA, $F_{3,23}$, $p = 0.02$). Post-hoc analysis estimated a mean difference of 0.99% [95% CI: 0.034, 1.95] between treatments of 101 N/22 S and 101 N/0S.

Table 7. Estimated mean seed oil content of *Camelina sativa* by weight as a response to nitrogen treatments. Crops produced in 2022 at four different agricultural research centers across Montana. Differing superscript letters indicate significantly different means determined by Fisher's LSD test.

N Rate (kg/ha)	Oil Content by Location (%)			
	CARC	EARC	NARC	SARC
168	-	37.2 ^c	36.1 ^{bc}	-
134	33.8 ^a	37.9 ^b	36.0 ^c	28.7 ^c
101	34.0 ^a	38.4 ^{ab}	36.3 ^{abc}	29.5 ^c
67	33.9 ^a	38.6 ^a	36.8 ^{ab}	31.5 ^b
34	33.0 ^a	38.4 ^{ab}	36.9 ^a	33.2 ^a

Seed oil yield was significantly affected by nitrogen treatments at all sites except CARC (NWARC oil was not measured). Oil yields generally increased with nitrogen treatments with the highest oil yield of 890 kg/ha at EARC at 168 kg N/ha. EARC and NARC had nonsignificant increases to oil yields from 134 to 168 kg N/ha. Oil yields at SARC were not significantly different between 67, 101, and 134 kg N/ha (Table 8).

Table 8. Estimate mean oil yield of *Camelina sativa* as a response to nitrogen treatments. Crops produced in 2022 at four different agricultural research centers across Montana. Differing superscript letters indicate significantly different means determined by Fisher's LSD test.

N Rate (kg/ha)	Oil Yield by Location (kg/ha)			
	CARC	EARC	NARC	SARC
168	-	890 ^a	865 ^a	-
134	258 ^a	857 ^{ab}	821 ^{ab}	265 ^a
101	226 ^a	794 ^b	788 ^b	239 ^a
67	220 ^a	690 ^c	689 ^c	235 ^a
34	205 ^a	647 ^c	625 ^d	189 ^b

Enzymatic Responses

Arylsulfatase activity was not responsive to nitrogen, sulfur, and N*S interaction effects were not observed at either CARC or EARC (Two-Way ANOVA $p > 0.05$) (Figure 8). Urease activity was not responsive to nitrogen, sulfur, and N*S interaction effects were not observed at any site (Two-Way ANOVA $p > 0.05$) (Figure 9).

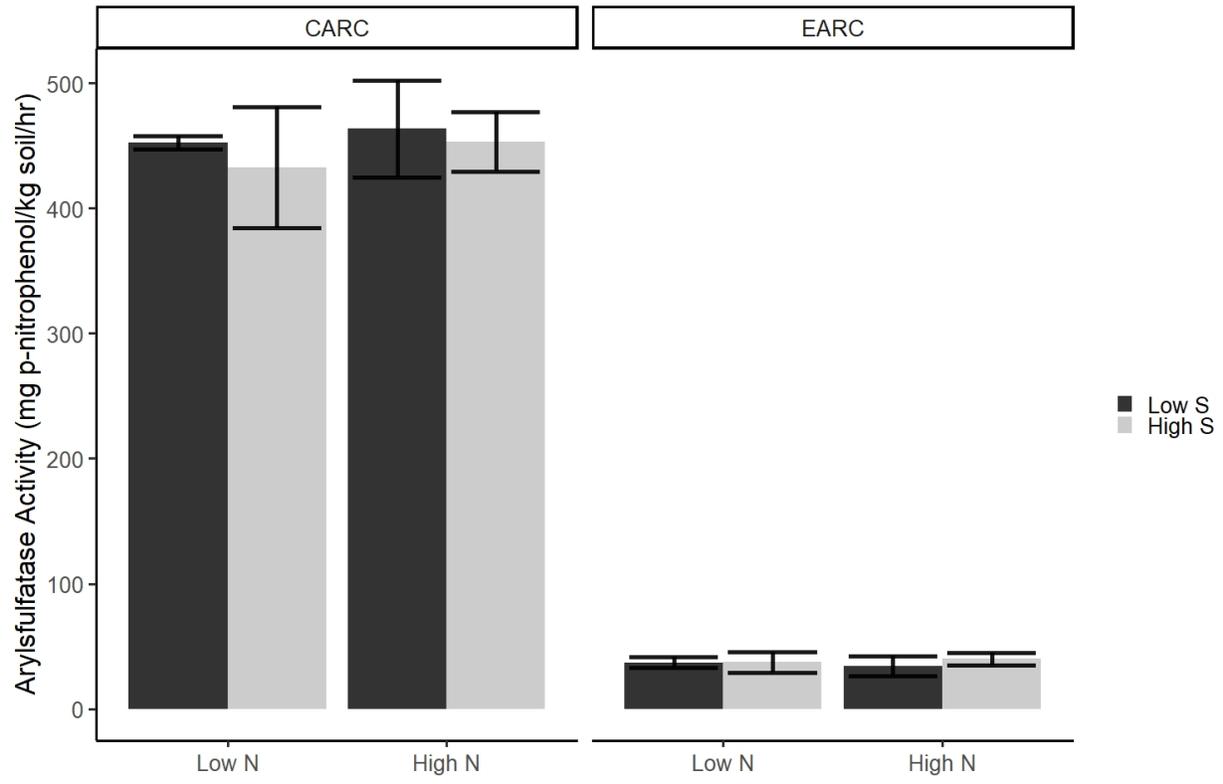


Figure 8. Arylsulfatase activity by nitrogen and sulfur treatments of soils sampled at Montana State University Central and Eastern Agricultural Research Centers in 2022 (n = 28). Error bars represent standard error of individual means. Assayed with p-nitrophenol sulfate substrate and p-nitrophenol standard concentration curve.

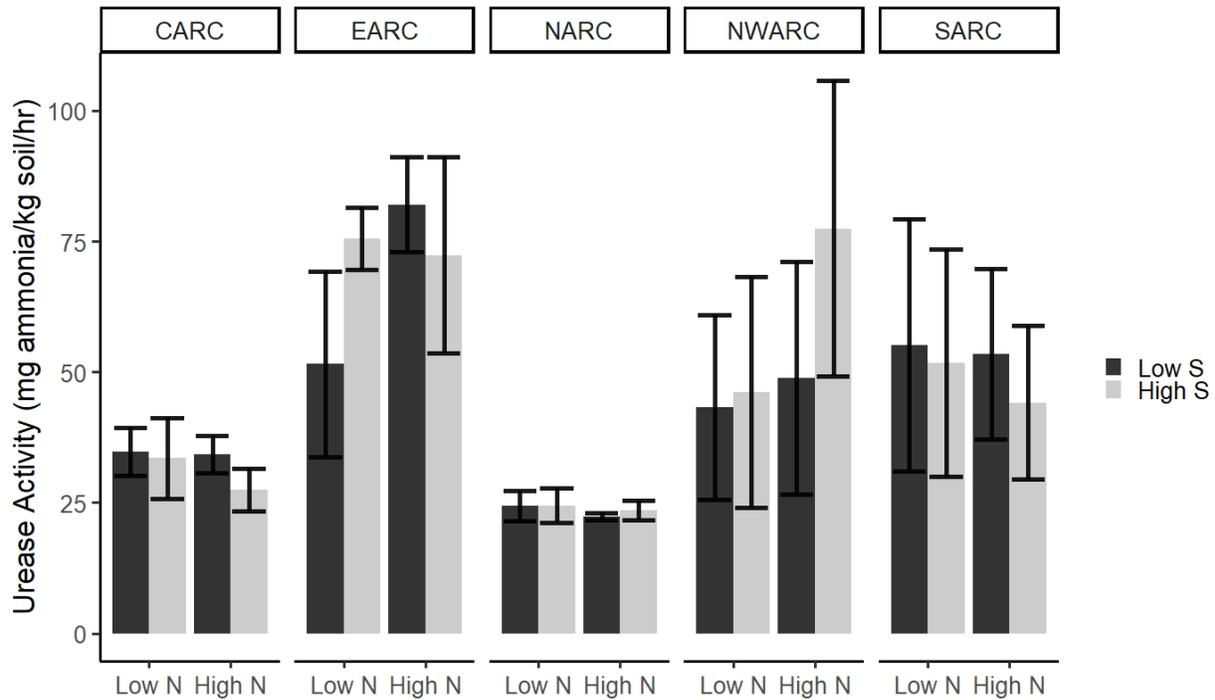


Figure 9. Mean urease activity by nitrogen and sulfur treatments of soils sampled at Montana State University Central, Eastern, Northern, Northwestern, and Southern Agricultural Research Centers in 2022 (n = 80). Error bars represent standard error of individual means. Assayed with urea substrate and ammonia standard concentration curve.

β -glucosaminidase activity was not responsive to nitrogen or sulfur treatments. There were significant N*S interaction effects found at CARC (Two-way ANOVA, $F_{1,12} = 5.9$, $p = 0.03$) and SARC (Two-way ANOVA, $F_{1,12} = 6.3$, $p = 0.03$). Post-Hoc analysis of activity at CARC found a significant difference of 102 mg p-nitrophenol/kg soil/hr [95% CI: 3, 201] between soils treat with low N/low S and high N/low S. Post-hoc analysis of activity at SARC found no significant differences in any pairwise comparisons (Figure 10).

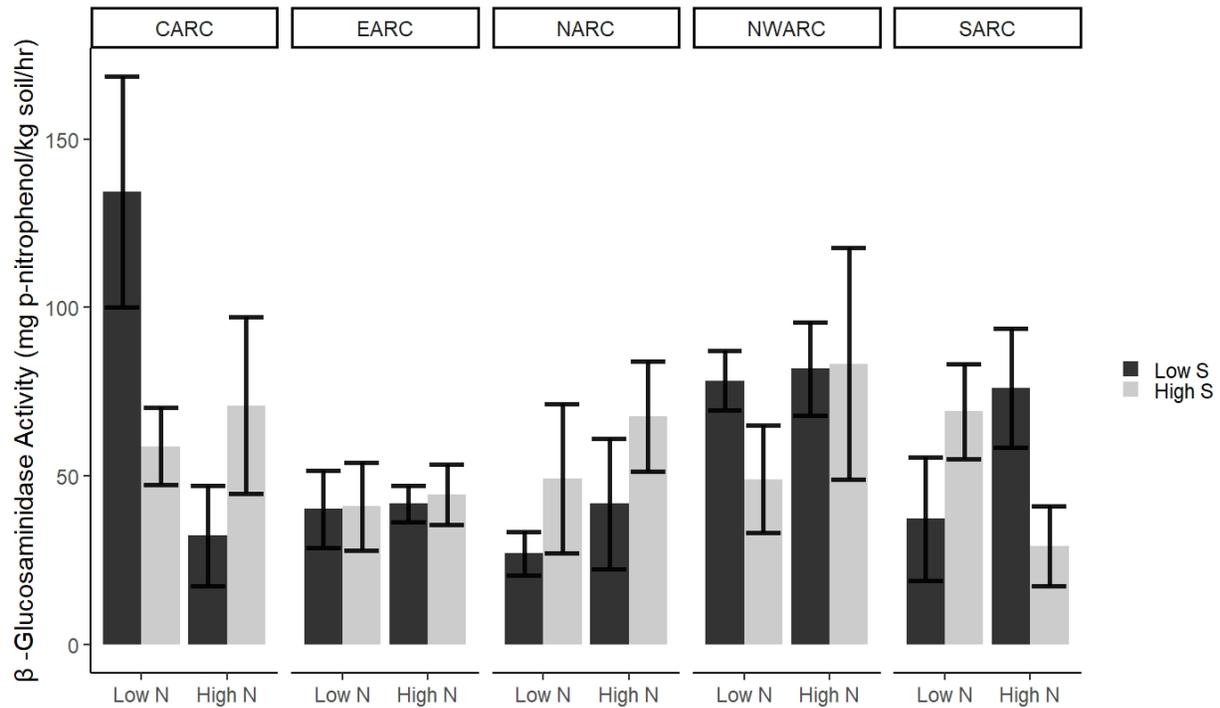


Figure 10. Mean N-acyl- β -D-glucosaminidase activity by nitrogen and sulfur treatments of soils sampled at Montana State University Central, Eastern, Northern, Northwestern, and Southern Agricultural Research Centers in 2022 (n = 80). Error bars represent standard error of individual means. Assayed with p-nitrophenol-N-acetyl- β -D-glucosaminidase substrate and p-nitrophenol standard concentration curve.

β -glucosidase activity was not responsive to nitrogen or sulfur treatments at any site (β -glucosidase not measured at NWARC). Sulfur and nitrogen treatment interactive effects were found at CARC (Two-way ANOVA, $F_{1,11}=5.9$, $p = 0.03$) and EARC (Two-way ANOVA, $F_{1,12} = 6.7$, $p = 0.02$). Post-hoc analysis found no significant differences in pairwise comparisons at either site (Figure 11).

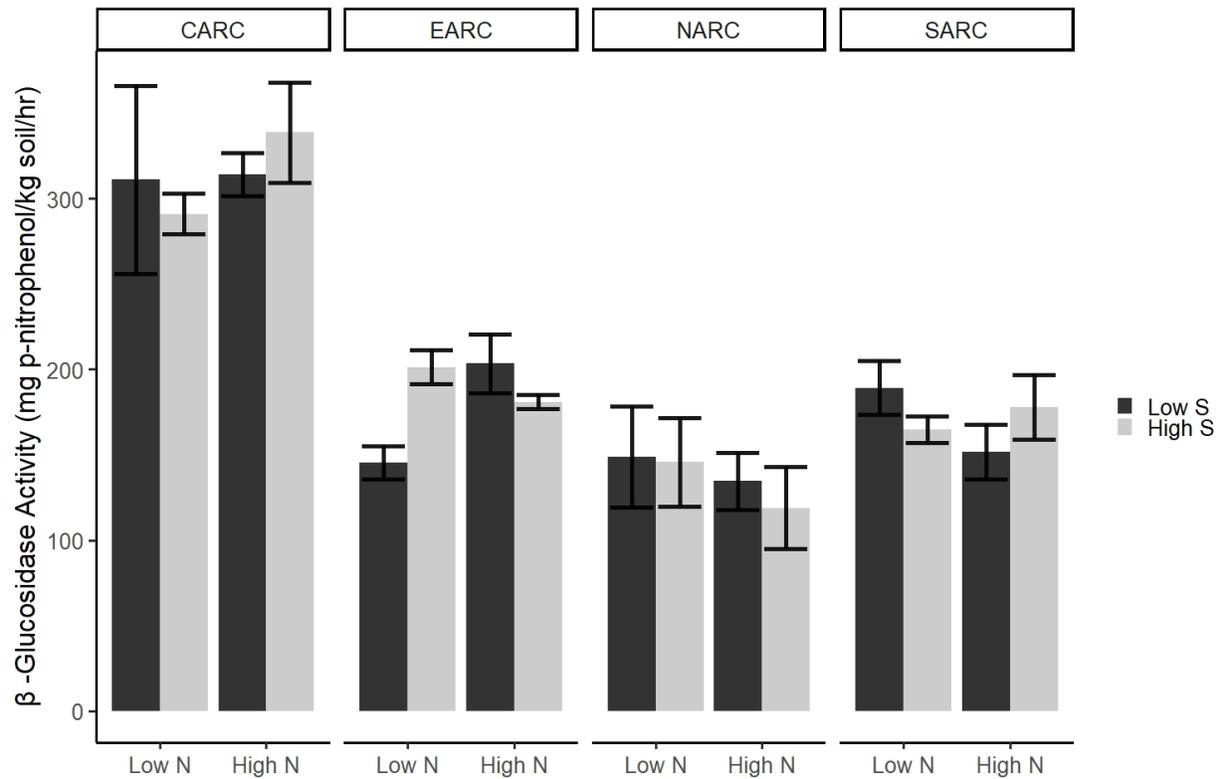


Figure 11. Mean β -glucosaminidase activity by nitrogen and sulfur treatments of soils sampled at Montana State University Central, Eastern, Northern, and Southern Agricultural Research Centers in 2022 ($n = 64$). Error bars represent standard error of individual means. Assayed with p-nitrophenol- β -D-glucopyranoside substrate and p-nitrophenol standard concentration curve.

The enzymatic geometric mean was not significantly affected by fertilizer treatments at any location in 2022 or 2023 (Table 10). A significant sulfur and nitrogen fertilization interactive effect was found at SARC in 2022 (Two-way ANOVA, $F_{1,32} = 4.6$, $p = 0.04$). However, no significant pairwise differences were found in post-hoc analysis (Figure 12).

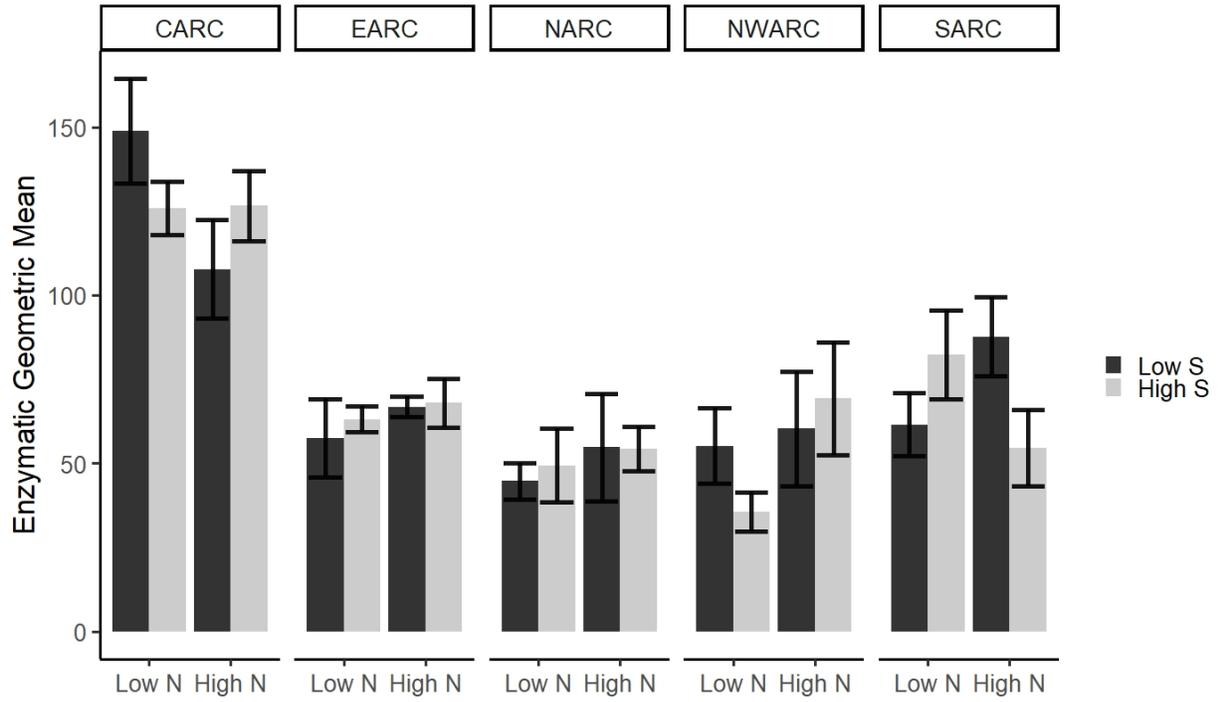


Figure 12. Mean enzymatic activity geometric means by nitrogen and sulfur treatments of soils sampled at Montana State University Central, Eastern, Northern, Northwestern and Southern Agricultural Research Centers in 2022 (n = 80). Error bars represent standard error of individual means.

Table 9. ANOVA p-values of the effects of nitrogen and sulfur fertilization on bulk soil enzymatic activity in *Camelina sativa* production, boldfaced p-values indicate a significant effect.

Effect on NAGase	ANOVA Effect p-value by Site Year						
	CARC	CARC 23	EARC	EARC 23	NARC	NWARC	SARC
Nitrogen Treatment	0.08	0.45	0.87	0.31	0.35	0.38	0.97
Sulfur Treatment	0.45	0.50	0.87	0.19	0.18	0.51	0.64
N*S Interaction	0.03	0.44	0.94	0.84	0.92	0.47	0.03
Effect on Glucosidase	CARC	CARC 23	EARC	EARC 23	NARC	NWARC	SARC
Nitrogen Treatment	0.84	0.81	0.59	0.44	0.42	-	0.44
Sulfur Treatment	0.33	0.96	0.43	0.26	0.71	-	0.96
N*S Interaction	0.03	0.32	0.02	0.36	0.80	-	0.12
Effect on Urease	CARC	CARC 23	EARC	EARC 23	NARC	NWARC	SARC
Nitrogen Treatment	0.54	0.86	0.41	0.88	0.62	0.44	0.78
Sulfur Treatment	0.46	0.07	0.69	0.53	0.84	0.51	0.77
N*S Interaction	0.61	0.39	0.19	0.98	0.83	0.59	0.89
Effect on Arylsulfatase	CARC	CARC 23	EARC	EARC 23	NARC	NWARC	SARC
Nitrogen Treatment	0.65	0.07	0.90	0.73	-	-	-
Sulfur Treatment	0.66	0.90	0.73	0.32	-	-	-
N*S Interaction	0.89	0.37	0.77	0.05	-	-	-
Effect on EGM	CARC	CARC 23	EARC	EARC 23	NARC	NWARC	SARC
Nitrogen Treatment	0.16	0.47	0.29	0.30	0.49	0.18	0.95
Sulfur Treatment	0.97	0.41	0.58	0.58	0.85	0.70	0.60
N*S Interaction	0.12	0.90	0.70	0.16	0.81	0.31	0.04

Table 10. Estimated mean and standard deviation of enzymatic activities of bulk soil in *Camelina sativa* production by site year and enzyme assayed. Superscript letters indicate significant differences between values at different site years determined by Fisher's LSD. All values are recorded as (mg p-nitrophenol released / kg soil / hour) *Urease results recorded as (mg ammonium released / kg soil / hour) **EGM is recorded as a unitless value.

Site Year	Enzyme Assayed				
	Arylsulfatase	Urease*	NAGase	Glucosidase	EGM**
CARC	450.4 ± 50.6 ^a	32.5 ± 9.9 ^{cd}	74.2 ± 57.2 ^b	313.8 ± 60.5 ^a	130.2 ± 30.1 ^a
CARC 23	136.2 ± 85.1 ^b	17.1 ± 7.2 ^d	107.2 ± 39.3 ^a	154.9 ± 78.2 ^{cd}	74.2 ± 22.3 ^b
EARC	48.8 ± 36.8 ^c	73.9 ± 23.7 ^a	41.9 ± 20.8 ^c	191.2 ± 31.6 ^b	64.5 ± 9.6 ^{bc}
EARC 23	45.8 ± 31.9 ^c	38.0 ± 15.1 ^{bc}	45.2 ± 18.0 ^c	132.6 ± 34.8 ^d	51.9 ± 14.0 ^c
NARC	-	23.8 ± 4.6 ^{cd}	46.5 ± 34.1 ^c	136.9 ± 45.6 ^{cd}	50.8 ± 19.4 ^c
NWARC	-	53.9 ± 43.4 ^b	73.2 ± 39.6 ^b	-	55.1 ± 27.3 ^c
SARC	-	50.9 ± 34.9 ^b	53.0 ± 34.8 ^{bc}	170.8 ± 30.9 ^{bc}	71.5 ± 25.0 ^b

Discussion

Seed yields from all sites ranged from 569 to 2398 kg/ha, two of the five locations (CARC and SARC) had lower yields than the remaining three, with low yield sites ranging from 569 to 923 kg/ha, and higher sites from 1245 to 2486 kg/ha. The higher site yields are comparable to similar studies of various *C. sativa* cultivars (Jiang et al., 2013; Urbaniak et al., 2008). At four of the five sites, overall seed yields were highly responsive to nitrogen applications, with the highest yields at applications of at least 134 kg N/ha at seeding. Sites that received an additional level of fertilization (168 kg N/ha) had non-significant increases to yield. Three of the five sites had a significant yield increase from 34 to 67 kg N/ha (NARC, NWARC, and SARC), and three sites had no significant increase from 67 to 101 kg N/ha (CARC, NWARC, SARC). No yield responses to sulfur applications or interactions between nitrogen

and sulfur were observed at any site, which is contrary to the results of Jiang et al. (2013) in which sulfur applications increased the yields at higher nitrogen applications.

The lower yields at CARC and SARC were likely due to adverse weather conditions, as CARC received significantly lower than average precipitation and the SARC trial was damaged by a hailstorm in the summer of 2022. Both sites had the highest estimated EGM at 130.2 and 71.5 respectively (Table 10), however, no correlation was found between EGM and seed yields. Soil enzymatic activity at flowering stage has been positively correlated with final yields of wheat (Mandal et al., 2007), generally correlations between plant yields and enzymatic activity can be highly variable (Rao et al., 2014).

Seed oil content ranged from 28.7 to 38.6% which is comparable to previously reported overall range of 30 to 40% suggested by Obour et al. (2015). Oil content followed the same trend that was reported Jiang et al. (2013), generally decreasing as nitrogen applications increased. This is likely due to higher protein synthesis in the seeds diluting the oil concentration (Rathke et al., 2005). Estimated oil yields increased with nitrogen applications at all sites except CARC, ranging from 189 to 890 kg/ha. EARC and NARC oil yields were highest at 134 kg N/ha, with non-significant increases at 168 kg N/ha. Neither oil content or oil yield was responsive to sulfur treatments, Lošák et al. (2011) suggested sulfur responses are minimal and applications are only needed in sulfur deficient soils which was confirmed by our findings.

Enzymatic activity was generally unresponsive to fertilizer treatments at all sites (Table 10). Some interactive effects between nitrogen and sulfur fertilization on three enzymes and EGM were found at CARC, EARC, EARC in 2023, and SARC (Table 10). These results are consistent with other reports that found enzymatic responses to fertilizer treatments can be highly variable and potentially contradictory between studies. Urease has been found to be unresponsive

to fertilizer treatments in bulk soils of corn (Davies et al., 2022) as well as in pea and radishes (Piotrowska & Wilczewski, 2012). In the same study of peas and radishes, β -glucosidase increased with nitrogen treatments up to 80 kg/ha, then began to decrease, our high levels of nitrogen were 120 and 150 kg/ha, potentially missing that responsive window. Additionally, long term N-fertilization strongly affect NAGase and arylsulfatase activity in multiple crop rotations (Ekenler & Tabatabai, 2002; Wang et al., 2016). Our soils were sampled roughly two months after fertility treatments so more time may be needed to see a response.

Application of CaSO_4 did not appear to affect any enzymatic activity positively or negatively. These results are contrary to both the results of Baligar et al. (2005) in which urease activity was suppressed by MgSO_4 applications, and those of soybean field trials by Zhao et al. (2008) in which sulfur applications increase urease activity. Inorganic salts inhibit β -glucosidase activity in soils (Eivazi & Tabatabai, 1990) and enzymatic activity is generally quite sensitive to soil pH, and can be hindered by N acidification (Wang et al., 2016), it is unlikely the gypsum applications would alter soil pH in a significant way as it is relatively neutral in solution.

Conclusions

Producers seeking maximum profitable seed and oil yields should apply nitrogen to an adjusted soil N of at least 101-134 kg/ha. Current seed and fertilizer costs and logistics should be considered, as the lower costs and decreased yields of the 101 kg N/ha applications may be more profitable for the producer. Additionally, soil enzymatic activity under *Camelina sativa* cultivation is not immediately affected by applications of nitrogen or sulfur fertilizers. While numerous reports in the literature suggest enzymatic activity can act as indices for soil health, it is clear from our results that additional measures such as soil organic matter, aggregate structure,

and other types of microbial community assessment should be taken to form a more robust assessment. The impact of the crop on enzymatic activity should be explored in reference to similar uncultivated or fallow sites, and further research is needed on the long-term effects of fertilizers in *C. sativa* to accurately assess the impact of the crop on soil health.

CHAPTER FOUR

SUMMARY OF FINDINGS AND FUTURE RESEARCH DIRECTION

Production of *C. sativa* for biofuels would provide a sustainable alternative to contemporary fossil-fuels, but considerations must be made for the input and land-use needs of producing the crop at an industrial scale. Understanding the fertilizer input needs of *C. sativa* and how they affect soil properties and processes allows for more accurate assessments of the impacts of the crop. Improving the productivity of the crop without the need to increase inputs improves the sustainability prospects of the crop. This purpose of this study was to assess multiple PGPR candidate strains for their abilities to improve the growth of *C. sativa*, and to evaluate the effects of N and S fertilizer inputs on soil enzymatic activity and agronomic performance in *C. sativa* production. Effects varied when *C. sativa* was inoculated with PGPR candidates, depending on strains and co-inoculants, *C. sativa* showed improved seed yields, shortened time to flowering, and decreased root length without changes to total biomass. *C. sativa* was responsive to N fertilization but not S, and there were no short-term effects from the fertilizers on soil enzymatic activity.

The most effective PGPR strains were the combined inoculations of *P. putida* ATCC 12633 and *B. thuringiensis* ATCC 33679, which were associated with a 60% increase in seed yields relative to the non-inoculated plants, as well as a decrease in time from sowing to flowering of more than two days. Seed yield and the time to flowering were negatively correlated, suggesting the plants that flowered sooner had higher yields, potentially due to their longer reproductive phases before maturity (Secchi et al., 2023; Wright et al., 1995). Additionally, inoculation with the strain *P. brassicacearum* 36D4 individually and in co-

inoculations was associated with significantly shortened roots but no changes to total biomass of early *C. sativa* seedlings.

Seed yields of *C. sativa* were responsive to N fertilization steadily increasing up to rates of 168 kg/ha. Oil yields increased with N applications, but seed oil content decreased, likely due to higher protein content in the seed diluting the amount of oil (Rathke et al., 2005). The most profitable N application rate would be dependent on prices at the time of use. Sulfur applications had no effects on yields but these applications should be explored further, potentially in sulfur-deficient soils (Lošák et al., 2011), as *C. sativa* has been found to be responsive to S applications in other studies (Jiang et al., 2013). While the fertilizer applications were not associated with any short-term effects on soil enzymatic activity, further studies should explore additional soil attributes, such as physical aggregate structures, microbial biomass and community composition to provide a more robust assessment of the effects on soil health (Dick, 1994). *C. sativa* can alter the microbial communities of crop rotations (Hansen et al., 2020) but more research is needed to determine the effects of *C. sativa* itself on soil health and productivity relative to other crops and to undisturbed soils to provide adequate reference points. Additionally, longer term studies (5-10 years) including time-series assays of enzymatic activity in crop rotations including *C. sativa* would offer a more accurate assessment of how the crop impacts soil processes, as enzymatic activity can be sensitive to management strategies (Dick, 1994; Paz-Ferreiro et al., 2012).

Further research should explore the metabolites and nutrient solubilization abilities of the most effective inoculants and co-inoculants to determine the mechanisms used to benefit the host plants. These strains should also be tested with existing PGPR consortia to potentially improve artificial rhizobacterial communities. While no fertilizer treatments were applied in the PGPR study, similar trials with varying levels of NPK fertilizers are needed to assess what level of

fertilizers the inoculants can offset (measured as differences in yields) and if the benefits they provide are consistent in the presence of such inputs. Additionally, developing field trials to determine if these strains provide similar benefits to *C. sativa* at a larger and more environmentally diverse scale is essential to assess the feasibility of using PGPR in *C. sativa* agricultural production (Gouda et al., 2018).

This work contributes to the ongoing efforts of making biofuels, specifically those derived from *C. sativa*, more accessible and available for sustainable production. This research showed that *P. putida* ATCC 12633 and *P. brassicacearum* 36D4 have strong potential to improve the growth and yields of *C. sativa*, particularly in co-inoculations with *B. thuringiensis* ATCC 33679. Additionally, this research found that seed yields of *C. sativa* responded significantly to increasing nitrogen applications but did not respond to sulfur treatments. The fertilizers had no short-term effects on the bulk soil enzymatic activity. Our hope is for this work to be used to further develop sustainable methods of *C. sativa* production, further increasing fossil-fuel alternatives through the availability of biofuels.

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