Soybean seed quality and early development in cold climate conditions

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Abstract

Soybean [*Glycine max.* (L.) Merr.] development is constrained by suboptimal growing conditions during germination and emergence. In cold climate regions, biostimulants could enhance the cold tolerance of developing soybeans, as well as the quality and viability of the next generation (F2) of soybean seed. The objectives of this thesis were to determine if the F2 seed of soybean had i) better quality based on seed protein and mineral nutrient content, and ii) greater cold tolerance during germination and emergence when the parent (F1) generation was treated with biostimulant, namely two formulations provided by Via Végétale. I hypothesised that F2 seed will have higher concentrations of proteins, such as 7S and 11S, and mineral nutrients, such as K, Ca, and Mg, when the F1 parent generation was treated with biostimulant, compared to no biostimulant treatment. Furthermore, I hypothesised that F2 seed will have improved cold tolerance during germination and emergence when the F1 parent was treated with biostimulant than untreated. In 2022, biostimulant-coated soybean seeds were grown in a field for 5 months until they reached physiological maturity (R8). The F2 seeds were harvested and stored for 8 months, then tested for nutritional quality, germination and emergence. Most nutritional parameters were the same in F2 seed from biostimulant-treated and untreated soybeans, although Obelix seeds treated with VV09 formulation had a greater 11S:7S protein ratio and those treated with the VV10 formulation had greater nickel concentration than the untreated control. Germination and emergence in a controlled growing environment at 12^{o}C was the same for F2 seed obtained from the biostimulant-treated and untreated soybean. In conclusion, the tested biostimulants had limited impact on the nutritional profile and viability of soybean seed, suggesting that temporary biostimulation is unlikely to have intergenerational effects.

Résumé

Le développement du soja [Glycine max. (L.) Merr.] est limité par des conditions de croissance sous-optimales pendant la germination et l'émergence. Dans les régions à climat froid, les biostimulants pourraient améliorer la tolérance au froid du soja en développement, ainsi que la qualité et la viabilité de la génération suivante (F2) de graines de soja. Les objectifs de cette thèse étaient de déterminer si les semences F2 de soja avaient i) une meilleure qualité basée sur la teneur en protéines et en nutriments minéraux, et ii) une plus grande tolérance au froid pendant la germination et l'émergence lorsque la génération parentale (F1) était traitée avec un biostimulant, à savoir deux formulations fournies par Via Végétale. J'ai émis l'hypothèse que les graines F2 présenteront des concentrations plus élevées de protéines, telles que 7S et 11S, et de nutriments minéraux, tels que K, Ca et Mg, lorsque la génération parentale F1 a été traitée avec un biostimulant, par rapport à l'absence de traitement biostimulant. En outre, j'ai émis l'hypothèse que les graines F2 auront une meilleure tolérance au froid pendant la germination et la levée lorsque le parent F1 a été traité avec un biostimulant que s'il n'a pas été traité. En 2022, des graines de soja enrobées de biostimulant ont été cultivées dans un champ pendant 5 mois jusqu'à ce qu'elles atteignent la maturité physiologique (R8). Les graines F2 ont été récoltées et stockées pendant 8 mois, puis testées pour la qualité nutritionnelle, la germination et la levée. La plupart des paramètres nutritionnels étaient les mêmes dans les graines F2 provenant de soja traité et non traité avec des biostimulants, bien que les graines d'Obélix traitées avec la formulation VV09 aient un rapport protéines 11S:7S plus élevé et que celles traitées avec la formulation VV10 aient une concentration en nickel plus élevée que le contrôle non traité. La germination et la levée dans un environnement de culture contrôlé à 12ºC ont été les mêmes pour les graines F2 obtenues à partir du soja traité au biostimulant et du soja non traité. En conclusion, les biostimulants testés ont eu un impact limité sur le profil nutritionnel et la viabilité des graines de soja, ce

qui suggère qu'une biostimulation temporaire n'est pas susceptible d'avoir des effets intergénérationnels.

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Contribution of authors

This thesis is written in a traditional format complying with the guidelines of the McGill Graduate and Postdoctoral Studies Office and consists of an introduction which outlines the context of my research project, a literature review chapter, a chapter containing the body of the thesis, a discussion which provides direction for future research, and a final conclusion which summarises the findings of this thesis.

The first chapter contains a literature review of soybean and its relevance in French society, its susceptibility to the suboptimal growing conditions of France, and its potential to overcome adverse growing conditions with biostimulants. In addition to being presented at the end of the introduction, the two objectives and hypotheses of my thesis are also presented at the end of this chapter. The second chapter begins with the methods, which is then followed by the results of the protein and nutrient analyses addressing the first research objective, and then the results of the growth chamber experiments addressing the second research objective. The discussion addresses both hypotheses and references the literature to explain/ support the findings of this thesis. This thesis concludes with a final conclusion and summary which discusses the implications of the findings in a broader context. The introduction, the literature review chapter, the body of the thesis chapter, the discussion and the final conclusion were written by the candidate and extensively edited by Dr. Joann Whalen.

The growth chamber experiments were designed by the candidate with the guidance of Dr. Jean-Christophe Avice. The maintenance of test seeds and seedlings, the sampling of seedlings, and the sample processing of seedlings was carried out by the candidate with the help of several occasional assistants. The protein extraction results were analysed by the candidate with the help of Dr. Jean-Christophe Avice. The candidate carried out all the laboratory experiments with the guidance of Dr. Jean-Christophe Avice. The statistical

analyses for the experiments were carried out by the candidate. The data interpretation and preparation of this manuscript was done by the candidate under the supervision of Dr. Joann Whalen.

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List of abbreviations

Abbreviation	*Full Description*
BSA	Bovine serum albumin
C	Carbon
Ca	Calcium
EDTA	Ethylenediaminetetraacetic acid
F1	Parent generation of soybeans
F2	Offspring generation of soybeans
Fe	Iron
GST24	Glutathione S-transferase 24
IRMS	Isotope-ratio mass spectrometry analysis
K	Potassium
LEA	Late embryogenesis abundant
Mg	Magnesium
min	Minute
Mn	Manganese
N	Nitrogen
Ni	Nickel
Pb	Lead
rpm	Rotations per minute
S	Sulphur
s	Second
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
XRF	X-Ray fluorescence
Zn	Zinc

Introduction

Soybeans [*Glycine max.* (L.) Merr.] is a protein-rich oilseed sold primarily as a nutritional supplement in animal feeds, with a smaller volume destined for human consumption in the global market. Soybean contains roughly 41% protein on a dry weight basis (Din et al. 2021). The dominant primary storage proteins in soybean are 7S globulins, representing approximately 30% of total dry weight protein, and 11S globulins, that are about 40% of total dry weight protein (Bittencourt et al. 2005). Seeds with an 11S:7S protein ratio greater than 1.5 is generally considered to be a soybean with very high protein quality (Sexton et al. 1998, Panthee et al. 2004). Furthermore, soybeans are a source of important mineral nutrients like K, Ca and Mg that are required for animal and human nutrition (Berk, 1992). In France, 70% of organic soybean production is consumed directly by humans (Le Gall et al. 2022), which differs from the global trend of soybean production in livestock feeding. Soybean cultivated in France must therefore demonstrate a nutritional profile that is consistent with the requirements for human diets.

Soybean may be produced throughout France, but the climatic conditions are more challenging in Normandy in the northwest of France. The major obstacle in the Normandy region is cold spring temperatures that coincide with the planting period. As a warm-season annual, soybean is vulnerable to low temperatures between sowing and full emergence (Gass et al. 1996). When the air temperatures is below the optimal threshold of 25°C (BASF Canada, 2019, Szczerba et al. 2021), this can impact seed germination, emergence, physiological development and the final oilseed yield (Wuebker et al. 2001, Xu et al. 2016).

This thesis is based on the expectation that biostimulant seed treatments will counteract the negative effects of suboptimal temperatures on the early development of soybean. There is evidence that treating soybean with biostimulants can lead to greater concentrations of total protein and mineral nutrients in harvested seeds (Kocira, 2019, Kocira

et al. 2018). Soybeans treated with biostimulants are more tolerant to environmental stress, including water deficiency, heat stress, and flooding (Rosário Rosa et al. 2021, Repke et al. 2022, Andrade Silva et al. 2023). In addition, field-grown soybean treated with biostimulants had higher seed yields than untreated soybean in Poland, another cool climate region (Rymuza et al. 2023). It is unknown whether the positive in-season effects of biostimulants on this year's soybean (F1 generation) are sustained in the next generation of seeds (F2). Therefore, my thesis addresses this knowledge gap by investigating the effects of biostimulants on harvested soybean seed qualities and viability. The general objective of my thesis is to determine whether treating soybeans with biostimulants impacts the seed nutritional quality, and whether it affects the cold tolerance of F2 seed during early development. The specific objectives of this thesis were to (i) compare the protein and mineral nutrient concentrations of F2 seed, and (ii) compare the cold tolerance of F2 seed during germination and emergence. The specific hypotheses of this thesis were 1) if F2 soybeans had a persistent, 2nd generation biostimulant effect, then F2 soybeans would have contained more 7S and 11S proteins and a greater mineral nutrient concentration when the preceding generation was treated with biostimulant than untreated because biostimulants enhanced plant growth and development, increasing the protein and nutrients accumulated in soybean seed. Secondly, 2) if soybeans were exposed to suboptimal growing temperatures during germination and emergence, then seed germination and emergence success would have been greater for the F2 seed from biostimulated-treated than untreated soybean because biostimulants enhanced the adaptive responses of soybean. This could have mitigated the adverse effects of suboptimal temperatures on early plant development.

Chapter 1: Literature review

1.1 Soybean: a developing sector in French agriculture

1.1.1 Global value

Soybean [*Glycine max* (L.) Merr.] is the most produced annual oilseed crop in the world (Shahbandeh, 2023). Globally, soybean production increased nearly 1300% since the Food and Agriculture Organisation of the United Nations started recording soybean yields in 1961. Today, the estimated value of the global soybean market is $372 billion CAD (Mordor Intelligence, 2024) and the United States, Brazil, and Argentina are the leading global producers of soybean (Voora et al. 2020). In 2022/23, these three countries collectively produced roughly 314 Mt of soybeans, which accounted for more than 80% of the entire global production for that year (Shahbandeh, 2023). Global demand for soybean is expected to grow in response to the needs of an increasing world population.

1.1.1.1 Nutritional benefits of agri-food products for people and animals

For most of human history, soybean production was concentrated in China. Japan, Korea and Indonesia. In these countries, soybean was considered a source of healthy fats, and a less expensive source of protein than animal products. Before the turn of the 20th century, the United States of America (USA) grew soybean primarily as a forage crop for cattle, but in 1904 it became recognised as a high-quality source of vegetable protein and fats, resulting in a new market for soybean in human diets (USSEC, 2006). In the postwar period (1940s onward), soybean production in the USA increased markedly due to an increasing demand for domestic sources of oils and fats (Shurtleff and Aoyagi, 2007). The availability of soybean in the USA at this time is linked to international acceptance of soy-based foods.

Due to its protein content of about 40% and oil content of 10-25% (dry weight), soybean is often used to produce high quality animal feed for livestock (Medic et al. 2014). About 77% of the soybean crop produced each year is consumed by livestock in the meat and

dairy industries (Voora et al. 2020). The remaining 23% of annual soybean production is consumed by people around the world, particularly in Asia where soy is consumed daily in the form of soy sauce, tofu, and edamame. Furthermore, soy is a nutritive additive in many processed foods that are distributed worldwide.

1.1.1.2 Industrial uses of soybean oil

Soybean has non-food uses in soaps, lubricants and biofuels. The high lipid content of soybean makes it an ideal ingredient in organic soaps, waxes, and candles. Soybean oil is considered to be an eco friendly lubricant that has excellent lubricity, biodegradability, good viscosity−temperature characteristics, and low evaporation loss, although it is less stable to thermos-oxidative fluctuations and has poor cold-flow behaviour (Sharma et al. 2007). Soybean is also a suitable lipid for biofuel production because it's less expensive compared to other vegetable oils ($0.51–0.59 CAD kg^{-1}) and high oil content (Mushrush et al. 2001). Soybean is transformed into a biofuel by combining soybean oil with ethanol and salt to produce biodiesel, and a study by Huo et al. (2009) showed that the production and combustion of soybean biodiesel reduces fossil energy use by 52% and greenhouse gas emissions by 57% compared to conventional fuels. In 2022, biodiesel production was worth $139 billion CAD (Fact.Mr., 2022), with soybean oil acting as the feedstock for 50% of all production (United Soybean Board, 2022). The value of the biodiesel market is projected to reach $270 billion CAD by 2032, and it is expected that soybean oil will generate the most revenue compared to all other feedstocks (Fact.Mr., 2022).

1.1.2 Soybean production and consumption in France

1.1.2.1 Historical context and shift in French soybean production

France is credited with sparking the interest of the Western world in soybeans and the first recorded attempt of cultivation was documented in 1779 at the National Museum of Natural History in Paris (Paillieux, 1880). For nearly 200 years after that date France relied

on international imports, mostly from the USA, both for soybean and soy-based foods. However, in 1973, an embargo imposed by President Richard Nixon restricted the USA from exporting soybeans to international countries, which led to a significant shift in French agriculture to offset the abrupt decline in soybean imports (Shurtleff and Aoyagi, 2015). This shift was marked by substantial annual increases in both harvested soybean acreage and annual soybean production in France (SFigure 1). In 2018, soybean and other oilseed crops represented 10% of the total cultivation area in France (Agence Bio, 2019). Moreover, Nouvelle-Aquitaine and Occitanie, located in the southwest of France, are important oilseed cultivation regions and collectively represent nearly 50% of the country's total harvestable oilseed area (Le Gall et al. 2022). From 2012 to 2022, France's rank jumped from 32nd to 17th place in the international community, due in part to a remarkable 3.6-fold increase in production (FAOSTAT, 2024).

1.1.2.2 Current French soybean production and consumption trends

In the 2022 growing season, France cultivated soybean on 0.18 Mha , resulting in a production of 0.38 Mt and ranked as the 5th highest producer in Europe (FAOSTAT, 2024). Soybean cultivation represented roughly 10% of the total cultivation area in France, and the southwest regions of Nouvelle-Aquitaine and Occitanie produced nearly 50% of the country's total harvestable oilseed area (Le Gall et al. 2022). In terms of food use, the European Union consumed 1.5 Mt of soybean in the form of soymeal, soybean oil, and soybean seed according to the Foreign Agriculture Service of the U.S. Department of Agriculture (2024). Between 2000 and 2010, there was a consistent decrease in the human consumption of soy foods which averaged a loss 4.6% year-over-year. However, this trend has been undergoing a reversal, as the consumption of soyfoods has been increasing steadily between 2010 and 2022 by 2.5% year-over-year. At the end of 2022, the total revenue generated by French soybean exports was $137 million CAD, with Belgium, Spain, and Italy being the primary importers

(IndexBox, 2024). Looking ahead, France's annual soybean production is expected to reach 0.58 Mt by 2027, reflecting a year-over-year increase of nearly 11% over the next five years (ReportLinker Research, 2024).

1.1.2.3 Initiative to expand the French soybean industry

In June 2020, the European Union and the government of Normandy forged a new initiative called “Soja Made in Normandie", which aimed for France and Europe to regain protein sovereignty in response to the increasing demand of proteins for animal and human nutrition. The specific ambitions of the Soja Made in Normandie project included sustainably establishing a locally produced soybean sector, improving the protein autonomy of regional farms, and producing high-quality plant proteins for animal and human nutrition compliant with the regulations outlined in the “Soybean of France” charter. Nine research institutions contributed to this project through innovative technical operations built on agroecological practices (guaranteed GMO-free production, low input and low carbon footprint) and creating value throughout the chain (farmers, collectors, manufacturers, consumers). Various strategies, such as selecting adaptive soybean varieties, introducing soybean into crop rotations, and increasing resistance to environmental stress at implantation through biostimulant seed treatments were examined by these institutions to determine the most efficient method of introducing a sustainable and organic soybean sector into the Normandy region.

1.2 The susceptibility of soybean to suboptimal growing temperatures

1.2.1 The early stages of soybean development

The first phase of early soybean development begins with germination. Germination begins when the soybean seed absorbs water and the seed mass increases by 50% (Berglund et al. 2015). It takes 1–2 wk for soybean to germinate when soil temperature is 10–25℃ (BASF Canada, 2019). Warmer soil temperatures encourage faster germination, and colder

temperatures slow down germination. The first structure to emerge from the seed is the primary root, which provides a leverage point in the soil for the extension of the hypocotyl stem. The optimal soil temperature for hypocotyl stem extension is 25°C, with a minimum temperature of 10°C and a maximum temperature of 30℃ (Wuebker et al. 2001). After germination, the cotyledon must breach the soil surface for emergence and further growth. The soybean emergence phase lasts 7–10 d (BASF Canada, 2019, Ritchie et al. 1985). The cotyledon fuels soybean development until the end of emergence, losing 70% of its original weight in the process (Berglund et al. 2015). Emergence begins when the hypocotyl stem becomes the main stem and the cotyledon splits in half to reveal a pair of unifoliates. Soil crusting, which is caused by the disintegration of aggregates and binding of clay particles in hot, dry soils, acts as a physical barrier to water infiltration and seedling emergence. Heavy rainfall can also limit soybean emergence by enveloping the germinated seedlings in mud and restricting their movement once the mud dries. Emergence is complete when the first set of trifoliate leaves appear on the main stem, since the soybean now relies on photosynthesis for energy.

1.2.2 Soybean response to suboptimal growing temperatures

Soybean development is susceptible to abiotic stresses, including drought, heat, and cold temperatures. As soybean is a warm-season legume, cold temperatures during its early development negatively impact germination, emergence and consequently its further growth, development, and yield. The minimum temperature for soybean germination is 6–8 ºC, and crop emergence takes about 14 days at 10 °C and 7–10 days at 12–15 °C (Ritter and Bykova, 2021). A study by Wuebker et al. (2001) showed that soybeans germinated at an air temperature of 15°C had 33% smaller total seedling dry weight yield after 20 d compared to the soybeans germinated at 25°C. In addition, suboptimal growing temperatures at the time of planting have shown to decrease final seed yield by 15% compared to optimal temperatures

(Xu et al. 2016). The average daily temperature in April in Normandy ranges from 5–14°C ("Simulated historical climate," 2024), therefore the suboptimal growing temperature at the time of planting may pose a risk to the successful establishment of soybean.

1.2.2.1 Soybean productivity in response to cold temperatures

Cold temperatures trigger predictable physiological responses in plants, similar to the response to other unfavourable growing conditions. This response involves prioritising root development over shoot growth, leading to a redirection of resources to storage vacuoles. These vacuoles serve as reserves for molecular building blocks, crucial for synthesising plant defence compounds, including antioxidants (Alsajri et al. 2020), and thus plant storage organs, such as roots, experience an augmenting effect (Kant et al. 2015). In addition, the resource pooling also benefits essential processes within the root system, such as nutrient uptake, antigen defence, and water absorption (Alsajri et al. 2019, Alsajri et al. 2020). The outcome of this shift in resource distribution is a notable increase in the root-to-shoot ratio of soybeans.

1.3 Via Végétale: a biostimulant to improve soybean tolerance of cold temperatures

1.3.1 An overview of biostimulants

The European Biostimulant Industry Council describes biostimulants as "substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality". There are several different varieties of biostimulants, but the vast majority of publicly known biostimulants are seaweed extracts, complex organic materials containing free amino acids, humic substances, and chitin derivatives. Many studies have shown positive outcomes of treating soybeans with biostimulants, such as increasing shoot and root biomass (Jannin et al. 2013, Tandon and Dubey, 2015, Dos Santos et al. 2017), increasing seed yield (Jannin et al. 2013, Tandon and Dubey, 2015, Szparaga et al. 2018,

Rymuza et al. 2023). Furthermore, biostimulants are often reported to improve plant tolerance to environmental stress including heat stress (Campobenedetto et al. 2020, Khan et al. 2020, Repke et al. 2022), drought/ water deficiency (Shukla et al. 2018, Rosário Rosa et al. 2021, Rakkammal et al. 2023), flooding/ hypoxia (Andrade Silva et al. 2023), and cold stress (Szczerba et al. 2021, Kuczyński et al. 2022). In contrast to the abundant literature documenting these positive effects, very little is actually known about the underlying mechanisms whereby biostimulants produce these effects. Nevertheless, numerous studies have tentatively proposed a broad theory that suggests beneficial compounds within the biostimulant, particularly amino acids, serve as fundamental building blocks for more complex molecules involved in biochemical processes that support plant growth and defences.

1.3.2 Cold tolerance enhancement of Via Végétale: a case study

Via Végétale is a biostimulant containing bioavailable micronutrients that claims to improve soybean growth when applied as a seed coating. A controlled study by Besnard et al. (2021) has shown that seeds treated with Via Végétale (specific product names: VV09, VV10) had a greater tolerance to suboptimal growing temperatures of 12℃. They observed improvements in germination and metabolic awakening, which was associated with enhanced carbon and nitrogen metabolism through the overexpression of phosphoglucomutase, and enhanced environmental response through the over expression of peroxidase. In addition, the seed coated with VV09 biostimulant produced a plant that had higher leaf nitrogen content and greater nodulation. This study concluded with suggestions for further analyses to understand whether the biostimulant could promote germination, plant development, and seed quality in field-grown soybeans.

1.4 Conclusion and thesis objectives

Soybean is a warm-season legume of global economic importance that is vulnerable to cold stress. If biostimulants can improve the vigour of field-grown soybeans and consequently their F2 generation, this could translate into tolerance to cold temperatures in the F2 generation. The specific research objective was to evaluate the protein and nutrient concentration of F2 soybeans from fields that were treated with biostimulant (*Via Végétale*) or untreated. Then, the F2 soybean was germinated to test the hypothesis that F2 soybeans have more germination and greater early seedling vigour when the F1 generation was treated with *Via Végétale* than untreated under field conditions.

The knowledge gaps which concern this thesis are determining if treating field-grown soybeans with biostimulants will have an impact on the protein and nutrient profile of F_2 soybeans, and if treating field-grown soybeans with biostimulants will have an impact on the response of F_2 soybeans to the exposure of suboptimal growing conditions during early development (i.e. germination and emergence). My thesis will address these knowledge gaps by answering the following research questions:

1) Do F2 soybeans have greater protein and nutrient content when they are produced by biostimulant-treated compared to untreated field-grown soybean?
2) Do F2 soybeans have a greater cold tolerance during germination and emergence when they are produced by biostimulant-treated compared to untreated field-grown soybean?

The knowledge gaps lead to the following hypotheses:

1) If F2 soybean have a persistent, 2nd generation biostimulant effect, then F2 soybean will contain more 7S and 11S proteins and a greater mineral nutrient concentration when the preceding generation was treated with biostimulant than untreated because

biostimulants enhancement of plant growth and development will increase the protein and nutrients accumulated in soybean seed.

1) If soybeans are exposed to suboptimal growing temperatures during germination and emergence, then seed germination and emergence success will be greater for the F2 seed from biostimulated-treated than untreated soybean because biostimulants enhance the adaptive responses of soybean. This could mitigate the adverse effects of suboptimal temperatures on early plant development.

Chapter 2: Body of the thesis

2.1 Material and methods

The objectives of this thesis relate to the response of F2 soybeans to biostimulants (applied to the F1 generation), which were expected to differ among soybean cultivars (cv. Obelix and cv. Merlin). The methods for treating F1 soybeans with biostimulants and their cultivation in a field environment are described for context. However, the Results (section 2.2) focus on the analysis of F2 soybeans in laboratory and controlled growth bench experiments.

2.1.1 Field experiment for the procurement of soybeans with 2nd generation biostimulant effects

2.1.1.1 Site description

The experimental field was at the Sileban field station in Barfleur, Manche, France (49°40'N, 1°15'W). Manche is located 83 m above sea level and has a temperate oceanic climate, with an average air temperature from 3.6℃ in February to 22℃ in August, an average humidity of 89%, and about 655 mm of annual precipitation (Weather and Climate, n.d.). The Sileban soil type is a combination of Brunisol and Alocrisol, with a silty texture and 51 g soil organic matter kg-1. The field was tilled with a mechanical rototiller in April 2022 before this experiment began. During the experimental period (April 29th to September 21th 2022), the daily mean air temperature was 10–24℃ and crop water was supplied through natural rainfall.

2.1.1.2 Experimental design of the soybean field experiment

The field experiment was designed to grow treated soybean seeds (F1) until physiological maturity and then harvest the offspring seeds (F2). The inoculum, Rizoliq® TOP (Rizobacter, Buenos Aires, Argentina), was used to inoculate the seed with

Bradyrhizobium diazoefficiens strain G49 at a rate of 6×10^9 cfu kg^{-1} seed according to the manufacturer's protocol. The biostimulant was applied to the soybean seed by hand-mixing in a stainless steel bowl at a rate of 1.5 mL kg^{-1} seed. Three biostimulant treatments were included in this study: no biostimulant, VV09, and VV10. The biostimulants were obtained from Via Végétale (Le Loroux-Bottereau, France) and they contain bio-available micronutrients, although the exact composition of the biostimulants is proprietary. Rhizobacteria and biostimulants were applied to two soybean varieties (cv. Obelix and cv. Merlin). Thus, the experiment included 2 soybean varieties × 3 biostimulant treatments for a total of 6 treatment groups namely: Obelix control, Obelix VV09, Obelix VV10, Merlin control, Merlin VV09, and Merlin VV10.

Soybean was grown in a field from April 29th to September 21st, 2022. While the true number of treatment groups present in the field trial was 16 ((7 biostimulants + 1 control) × 2 varieties), it was decided by the Normandie host institution that only a subset of these groups would be examined in this thesis. The subset of these treatment groups is described in the previous paragraph. The experimental design was a two-factor randomised split-plot design with 2 soybean varieties × 8 biostimulant treatments, each with 5 replications (Fig. S1). The total plot size was 45 m × 31 m including a 1.7 m border surrounding the entire plot. The plot contained 5 blocks (approximately 27 m × 1.7 m), and each block contained 16 subplots (approximately 7 m × 1.5 m) with 0.20 m spacing around each subplot. Each treatment group was assigned to one subplot in each block, and F1 seeds were planted in each subplot at a rate of 60 seeds m^{-2}. The field was fertilised with 72 kg of N ha^{-1} in April 2022 before planting soybean and no phytosanitary treatments or methods of weed control were applied during the experiment. When the F1 soybeans reached full physiological maturity (R8), each plot was harvested manually. The harvested F2 seeds from the outermost blocks on either side of the field (i.e. Block 1 and Block 5) were discarded to control for border effects, while the F2

seeds from the three innermost blocks (i.e. Block 2, Block 3, and Block 4) were retained, and labelled according to the treatment group of the parent F1 soybean from which they were harvested. The F2 seeds were dried at room temperature (~22℃) in a windowless storage room for 14 d, and then sorted into 500 mL plastic bags. The bags containing the dried F2 seeds were stored in a windowless storage room until laboratory analysis began the following summer in June 2023 (Fig. S2).

In June 2023, powdered samples of F2 seeds from the 6 selected treatments (were prepared by aliquoting enough seeds to fill a 30 mL test tube approximately halfway and then lyophilizing the seed-filled tubes for 3 d at -50℃ to remove any traces of water. At the end of lyophilization, the seeds were transformed into a powder using a Mixer Mill 400 (Retsch, Haan Mettman, Germany) and then the powder was aliquoted into 5 mL test tubes and stored at -20℃.

2.1.2 Protein and mineral nutrient determination of F2 soybean

2.1.2.1 7S and 11S protein quantitation- Bradford method and SDS-PAGE

Protein concentration in powdered F2 seed samples was determined by the Bradford (1976) method using bovine serum albumin as a standard. Prior to measuring the absorbance on a Gel Doc™ EZ scanner (Bio-Rad Laboratories, Marne-la-Coquette, France), the 7S and 11S protein bands were isolated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue solution, according to Gallardo et al. (2002). Procedures for the preparation of buffer solutions for this analysis are provided in Table S1. Procedural steps for protein extraction and analysis are given in Table S2.

2.1.2.2 Macronutrient, micronutrient, and trace element quantification

Elemental analysis of whole F2 soybean seed for K, Mg, Si, Al, Fe, Zn, Cl, Mn, Cu, Ba, P, Ni, Ti, Ca, and Pb (all expressed as g total element/ kg) was determined, relative to a

standard citrus tissue, by x-ray fluorescence analysis on a XEPOS (Spectro Ametek, Berwyn, PA). The total C, N and S concentration in powdered F2 soybean seed was measured by a continuous flow Horizon 2 Isotope Ratio Mass Spectrometer (NU Instruments, Wrexham, United Kingdom) linked to a EA3000 C/N/S Analyzer (Euro Vector, Milan, Italy).

2.1.3 Cold tolerance determination of F2 soybean

2.1.3.1 Experimental design of the soybean seed germination test

F2 soybean germination was tested under controlled growing conditions in a KBW 720 Phytotron (Binder-GmbH, Im Mittleren, Germany) with a constant temperature of 12℃, a 16 h light:8 h dark photoperiod at a light intensity of 180 μmol s m^{-2}, and 80% humidity over the course of 5 d. The experimental unit was a 25 cm × 20 cm × 2 cm petri dish containing 400 g of soil moistened with 100 g of deionized water.

Germination success was based on Qiu et al. (2020) and Ritz et al. (2013). This included the total germination (Gmax %) after 120 h, the median germination time (G50; time required for 50% of the seeds to germinate, in h), and the germination uniformity (GU; the time it took for 90% of the seeds to germinate minus the time needed for 10% of the seeds to germinate, in h).

The germination experiment followed a basic two factor time-series design without randomisation, and comprised 6 experimental units, one for each treatment group, each containing 10 seeds. In the plastic bags containing the dried F2 soybeans, 10 seeds were selected at random. In each petri dish, 2 parallel rows of 5 seeds (10 seeds total) were planted approximately 3.5 cm apart, with at least 2 cm from the edge of the petri dish. Seeds were placed so the radical was in contact with the soil and the seed hilum was exposed to the air (Fig. S3). Deionized water (~2 mL) was sprayed evenly across the surface of the petri dish every day to keep exposed seeds moist. The location of the petri dishes on the growth chamber racks was rearranged once a day, switching to the other side of the rack and

displacing one level upwards (unless on the top rack of the growth chamber, in which case the petri dish was moved to the bottom level), to account for any inconsistencies in light distribution or temperature circulation.

2.1.3.2 Experimental design of the soybean seed emergence test

F2 soybean emergence was tested under controlled growing conditions in a KBW 720 Phytotron (Binder-GmbH, Im Mittleren, Germany) with a constant temperature of 12℃, a 16 h light:8 h dark photoperiod at a light intensity of 180 μmol s m^{-2}, and 80% humidity over the course of 12 d. 4000 g of the same soil used for the germination experiment was moistened with 1000 mL deionized water, left in a covered container for 7 d, and sieved (<5 mm) prior to use. The experimental unit was a plastic seed planter with dimensions 21 cm × 12.5 cm × 6 cm containing 800 g (wet weight) of pre-moistened soil. The planters had 15 wells (each 4 cm × 4 cm × 6 cm), with one well accommodating one seed, and thus each planter would contain a total of 15 seeds. Plastic holding trays (internal dimensions 35 cm × 25 cm × 10 cm) were used to house the planters and facilitate watering. Each holding tray could accommodate 2 planters, thus 3 trays in total were used throughout the entire duration of the emergence experiment.

Emergence was evaluated by the total emergence (Emax %) and seedling biomass after 12 d. Seedling biomass was evaluated by the root to shoot ratio, the shoot height, the fresh shoot mass, the dry shoot mass, the shoot water content, the fresh root mass, the dry root mass, and the root water content. The root to shoot ratio was determined by dividing the dry root mass by the dry shoot mass. The shoot height was determined by cutting the shoot at the soil surface and measuring the distance from the bottom cut end to the top of the cotyledon (in cm) with a ruler. The fresh shoot mass was determined immediately after the cut portion of the shoot was separated from the rest of the seedling using a balance to record the weight (in mg). The dry shoot mass was determined by using a balance to record the

weight (in mg) of the cut portion of the shoot after being oven dried at 25℃ for 14 d. The shoot water content was determined by subtracting the dry shoot mass (in mg) from the fresh shoot mass (in mg), and then multiplying the value by 100% and then dividing that value by the fresh shoot mass (in mg). The fresh root mass was determined immediately after the cut portion of the root (the remaining portion of the seedling after removing the shoot) was separated from the rest of the seedling using a balance to record the weight (in mg). The dry root mass was determined by using a balance to record the weight (in mg) of the cut portion of the root after being oven dried at 25℃ for 14 d. The root water content was determined by subtracting the dry root mass (in mg) from the fresh root mass (in mg), and then multiplying the value by 100% and then dividing that value by the fresh root mass (in mg).

The emergence experiment followed a basic two factor time-series design without randomisation, and comprised 6 experimental units, one for each treatment group. In the plastic bags containing the dried F2 soybeans, 15 seeds were selected at random. In each planter, one seed was planted 1 cm deep into the centre of each well and gently covered with extra soil. Seeds were placed so the seed hilum was oriented towards the top of the well. About 300 mL of deionized water was added to the bottom of the plastic holding tray 24 h after the emergence experiment began, and this process was repeated every other day for the remainder of the experiment. In addition, deionized water (~2 mL) was sprayed evenly across the surface of each planter every day to keep the surface soil from developing a crust. The location of the planters on the growth chamber racks was rearranged once a day, switching to the other side of the rack and displacing one level upwards (unless on the top rack of the growth chamber, in which case the planter was moved to the bottom level), to account for any inconsistencies in light distribution or temperature circulation.

2.1.4 Statistical analysis

Data normality was checked with a Shapiro-Wilk test and a Levene test was used to verify the data homoscedasticity. Normally distributed data with equal variance were analysed by a two factor analysis of variance (ANOVA) test, whereas non-normal data with unequal variance were either log transformed prior to ANOVA or evaluated by a Scheirer–Ray–Hare test. The experimental results were analysed to determine whether the two factors (i.e. soybean treatment and soybean variety), as well as any potential interactions between them, had an effect on the dependent variables. The data associated with 39 dependent variables were analysed, including the Gmax, G50, GU, Emax, root to shoot ratio, shoot height, fresh shoot mass, dry shoot mass, shoot water content, fresh root mass, dry root mass, root water content, dry matter protein content, total 7S + 11S protein content, total 7S protein content, total 11S protein content, 11S:7S protein ratio, 7S-α protein content, 7S-β protein content, 11S acidic protein content, 11S basic protein content, the macronutrient content of C, N, P, K, S, Mg, and Ca, the micronutrient content of Fe, Zn, Mn, Cu, Cl, and Ni, and the trace element content of Al, Ba, Si, Ti, and Pb. When treatment effects were significant ($P<0.05$), a Tukey Honest Significant Difference (Tukey HSD) test was used for post-hoc mean comparison. Statistical analyses were done with RStudio (version 4.3.1).

2.2 Results

No data was collected for the F1 soybeans of the field experiment, thus the following section will exclusively cover F2 soybean analyses.

2.2.1 Protein content and elemental composition of soybean grain

Total protein content was similar among soybean varieties and biostimulant treatments (Table 1). The total 7S+11S protein content is greater in Merlin than Obelix ($p<0.05$), primarily due to the greater 7S-β protein ($p<0.001$) and 11S acidic protein ($p<0.01$) in Merlin than Obelix, notwithstanding the trend for less 7S-α protein ($p<0.01$) in Merlin than

Obelix (Table 1). The 11S:7S ratio of 2.2 in grain from the Obelix variety treated with VV09 was greater than the other biostimulant treatments on Obelix grain ($p<0.05$, HSD test) but the same as the Merlin grain (Table 1). The elemental analysis revealed similar concentrations of most macronutrients (C, N, P and S), the micronutrients Cu and Cl, and non-essential trace elements like Al, Ba, Pb, Si and Ti in soybean grain, regardless of the soybean variety or biostimulant treatment (Tables 2, 3 and 4). Genetic factors controlling the uptake of nutrient cations could account for the significant ($p<0.05$ to $p<0.001$) differences in K, Mg, Ca, Fe, Mn, Zn and Ni in the grain of Obelix and Merlin varieties (Tables 4 and 5).

Table 1. Protein composition of soybean seedlings was similar among soybean varieties (Obelix, Merlin) and biostimulant coatings (none: control; biostimulants: VV09 and VV10) with the exception of the 11S:7S ratio originating from biostimulant effects. Data are the mean (± standard error), n=3.

Variety	Biostimulant	DM Protein (mg/100 mg)[x]	Total 7S+11S (%)	Total 7S (%)	Total 11S (%)	11S:7S ratio	7S-α[z] (%)	7S-β (%)	11S acidic (%)	11S basic (%)
Obelix	Control	34 (0.54)	56 (1.5)	20 (1.0)	36 (1.5)	1.8 (0.052)[a]	19 (0.39)	1.2 (0.46)	20 (0.75)	16 (0.64)
	VV09	37 (2.2)	59 (4.4)	18 (1.5)	41 (4.4)	2.2 (0.17)[b]	17 (1.2)	1.4 (0.21)	25 (2.3)	16 (1.6)
	VV10	39 (0.90)	56 (1.5)	19 (0.58)	36 (2.1)	1.9 (0.10)[a]	19 (0.52)	0.67 (0.48)	20 (2.5)	17 (0.82)
Merlin	Control	34 (6.3)	62 (6.0)	21 (1.7)	42 (5.0)	2.0 (0.12)[ab]	17 (0.54)	4.3 (0.81)	25 (2.6)	17 (2.4)
	VV09	34 (2.3)	62 (3.0)	22 (0.58)	41 (2.5)	2.0 (0.10)[ab]	17 (0.89)	3.8 (0.92)	24 (1.4)	17 (1.4)
	VV10	36 (1.0)	59 (0.58)	19 (0.0)	40 (1.0)	2.1 (0.065)[ab]	15 (0.047)	3.7 (0.30)	23 (0.022)	16 (0.63)
Source of variation	d.f.[y]									
Variety	1	NS	*	NS	NS	NS	**	***	**	NS
Biostimulant	2	NS	NS	NS	NS	*	NS	NS	NS	NS
Interaction	2	NS	NS	NS	NS	**	NS	NS	NS	NS

[x]DM Protein (mg/ 100 mg), mg of protein per 100 mg of dry matter; [y]d.f., degrees of freedom; [z] 7S-α, sum of α and α′ 7S subunits; *, **, *** Significant at $p<0.05$, $p<0.01$, $p<0.001$, respectively: NS, not significant ($p>0.05$ according to two-factor ANOVA or Scheirer–Ray–Hare analysis).

Table 2. Macronutrient composition of soybean seedlings differed among soybean varieties (Obelix, Merlin) with no effect of biostimulant coatings (none: control; biostimulants: VV09 and VV10). Data are the mean (± standard error), n=3.

Variety	Biostimulant	C (g kg^{-1})	N (g kg^{-1})	P ($\times10^{-3}$g kg^{-1})	K (g kg^{-1})	S (g kg^{-1})	Mg (g kg^{-1})	Ca ($\times10^{-3}$g kg^{-1})
Obelix	Control	550 (82)	54 (12)	7.2 (0.53)	24 (0.070)	3.3 (0.55)	2.0 (0.088)	1.5 (0.11)
	VV09	510 (4.4)	56 (5.3)	7.6 (0.48)	24 (0.24)	3.1 (0.068)	1.9 (0.066)	1.5 (0.17)
	VV10	510 (0.21)	53 (0.025)	7.2 (0.37)	24 (0.75)	3.2 (0.025)	1.9 (0.063)	1.9 (0.42)
Merlin	Control	520 (4.5)	56 (0.19)	7.1 (0.57)	20 (0.47)	3.1 (0.19)	2.1 (0.12)	1.9 (0.026)
	VV09	520 (2.5)	52 (0.017)	7.3 (0.32)	20 (0.56)	3.1 (0.017)	2.1 (0.0075)	1.8 (0.18)
	VV10	510 (1.3)	53 (0.12)	6.4 (0.32)	20 (0.58)	3.1 (0.12)	2.0 (0.097)	2.0 (0.16)
Source of variation	d.f.[y]							
Variety	1	NS	NS	NS	***	NS	**	*
Biostimulant	2	NS	NS	NS	NS	NS	NS	NS
Interaction	2	NS	NS	NS	NS	NS	NS	NS

[y]d.f., degrees of freedom; *, **, *** Significant at $p<0.05$, $p<0.01$, $p<0.001$, respectively: NS, not significant ($p>0.05$ according to two-factor ANOVA or Scheirer–Ray–Hare analysis).

Table 3. Micronutrient composition of soybean seedlings differed among soybean varieties (Obelix, Merlin) with an effect of biostimulant coatings on Ni content (none: control; biostimulants: VV09 and VV10). Data are the mean (± standard error), n=3.

Variety	Biostimulant	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Cl (mg kg^{-1})	Ni (mg kg^{-1})
Obelix	Control	140 (28)	58 (6.9)	27 (0.29)	24 (2.9)	31 (15)	5.3^{b} (0.61)
	VV09	150 (37)	61 (2.8)	26 (1.3)	26 (1.3)	25 (12)	5.2^{b} (0.48)
	VV10	130 (8.6)	59 (5.1)	29 (1.7)	25 (2.4)	53 (34)	8.1^{a} (0.37)
Merlin	Control	170 (10)	73 (1.8)	29 (1.4)	23 (1.2)	41 (17)	4.6^{b} (0.57)
	VV09	160 (4.2)	73 (2.6)	29 (1.5)	26 (1.5)	71 (95)	5.9^{ab} (0.32)
	VV10	170 (13)	69 (3.2)	29 (0.15)	23 (1.2)	41 (45)	4.4^{b} (0.032)
Source of variation	d.f.[y]						
Variety	1	*	***	*	NS	NS	*
Biostimulant	2	NS	NS	NS	NS	NS	NS
Interaction	2	NS	NS	NS	NS	NS	**

[y]d.f., degrees of freedom; *, **, *** Significant at $p<0.05$, $p<0.01$, $p<0.001$, respectively: NS, not significant ($p>0.05$ according to two-factor ANOVA or Scheirer–Ray–Hare analysis).

Table 4. Trace element composition of soybean seedlings do not differ among soybean varieties (Obelix, Merlin) or biostimulant coatings (none: control; biostimulants: VV09 and VV10). Data are the mean (± standard error), n=3.

Variety	Biostimulant	Al (mg kg^{-1})	Ba (mg kg^{-1})	Si (mg kg^{-1})	Ti (mg kg^{-1})	Pb (mg kg^{-1})
Obelix	Control	220 (89)	16 (27)	540 (710)	4.6 (7.6)	0.0 (0.0)
	VV09	180 (21)	24 (21)	180 (43)	4.4 (7.3)	0.0 (0.0)
	VV10	200 (25)	0.90 (0.0)	160 (75)	6.8 (11)	0.0 (0.0)
Merlin	Control	200 (25)	26 (21)	320 (148)	4.3 (7.1)	0.0 (0.0)
	VV09	200 (52)	12 (19)	280 (260)	4.5 (7.4)	0.0 (0.0)
	VV10	190 (13)	35 (1.5)	200 (18)	0.20 (0.0)	0.0 (0.0)
Source of variation	d.f.[y]					
Variety	1	NS	NS	NS	NS	NS
Biostimulant	2	NS	NS	NS	NS	NS
Interaction	2	NS	NS	NS	NS	NS

[y]d.f., degrees of freedom; NS, not significant (p>0.05 according to two-factor ANOVA or Scheirer–Ray–Hare analysis).

2.2.2 Soybean germination

Germination of the Obelix variety was 87–97% after 120 h, with a median germination time of 72–88 h, and a germination uniformity from 74–82 h (Table 5). The Merlin variety had a similar total germination of 87–97%, a median germination time of 80–96 h, and germination uniformity of 75–87 h (Table 5). Biostimulants VV09 and VV10 had no effect (p>0.05) on the cumulative germination over a 5 d period, relative to the control and regardless of which variety was tested (Figure 1).

Table 5. Soybean germination was the same among soybean varieties (Obelix, Merlin) and biostimulant coatings (none: control; biostimulants: VV09 and VV10), according to the total germination (Gmax %) after 120 h, median germination time (G50) and germination uniformity (GU). Data are the mean (± standard error), n=3, 10 seeds per replication.

Variety	Biostimulant	Gmax (%)	G50 (h)	GU (h)
Obelix	Control	97 (5.7)	72 (0.0)	74 (8.5)
	VV09	87 (5.7)	80 (14)	82 (9.2)
	VV10	90 (10)	88 (14)	77 (9.5)
Merlin	Control	97 (5.7)	80 (14)	75 (11)
	VV09	93 (5.7)	88 (14)	82 (6.8)
	VV10	87 (15)	96 (0.0)	87 (17)
Source of variation	d.f.[y]			
Variety	1	NS	NS	NS
Biostimulant	2	NS	NS	NS
Interaction	2	NS	NS	NS

[y]d.f., degrees of freedom; NS, not significant (p>0.05 according to two-factor ANOVA or Scheirer–Ray–Hare analysis).

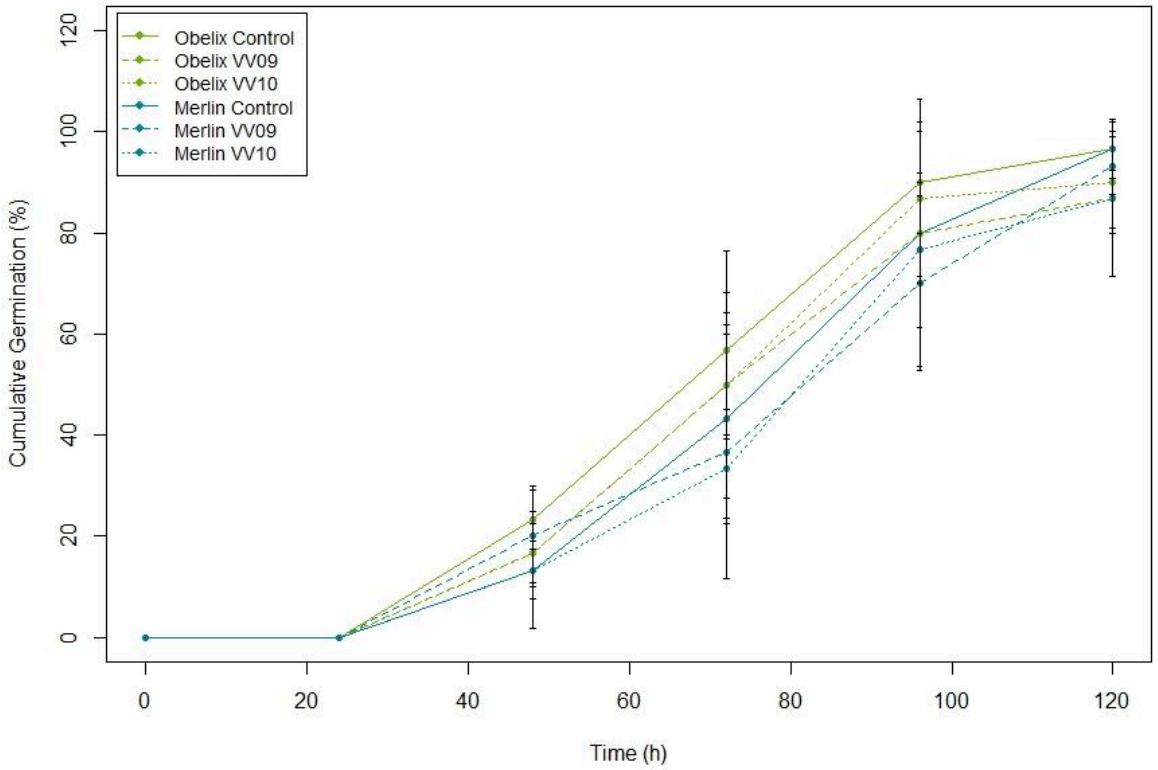

Figure 1. Germination of soybean (Obelix and Merlin varieties) from untreated (control) and biostimulant-coated (VV09, VV10) seed, from 24 to 120 h after planting. Data points are the mean (n=3) with bars for the standard deviations of the mean.

2.2.3 Soybean emergence and seedling morphology

Soybean seedlings emerged from 7 to 12 d after planting in a growth chamber at 12ºC, with 33–67% of Obelix seedlings sprouted and 67–93% of Merlin seedlings emerged after 12 d (Figure 2). At 12ºC with controlled lighting, humidity and watering, the Merlin variety produced seedlings that were significantly (p<0.01 to p<0.001) larger, being 8 to 33% taller with 17–59% greater fresh root biomass and 8–43% more dry roots than the Obelix seedlings (Table 6). While the shoot biomass was similar on a fresh weight basis and approximately 15% lower (p<0.01) in the Merlin than Obelix seedlings, this confirms the Merlin variety allocates more resources for root development during emergence, resulting in a significantly (p<0.001) greater root:shoot ratio for Merlin than Obelix seedlings up to 12 d after planting (Table 6). The seedlings from the Obelix group were damaged, discoloured, and disfigured, whereas the seedlings from the Merlin group were undamaged, dark green, and grew straight stems (Figure 3). Differences in seedling morphology were attributed entirely to varietal (genetic and gene ×environment differences), as biostimulants had no effect on soybean emergence and early development (Table 6). This is to say that the visible differences in the appearance of emerged soybeans were due to genetic differences in soybean variety which dictate their response to the environment.

Table 6. Morphology of soybean seedlings differed among soybean varieties (Obelix, Merlin) with no effect of biostimulant coatings (none: control; biostimulants: VV09 and VV10). Data are the mean (± standard error), n=15.

Variety	Biostimulant	Root:Shoot ratio	Shoot height (cm)	Fresh shoot mass (mg)	Dry shoot mass (mg)	Shoot water content (%)	Fresh root mass (mg)	Dry root mass (mg)	Root water content (%)
Obelix	Control	0.078 (0.0071)	3.2 (0.22)	636 (54)	152 (14)	80 (4.9)	115 (23)	12 (1.1)	81 (7.2)
	VV09	0.069 (0.012)	3.5 (0.34)	760 (70)	167 (16)	87 (9.6)	124 (31)	12 (2.8)	86 (7.6)
	VV10	0.073 (0.019)	3.0 (0.18)	650 (15)	162 (1.5)	88 (4.7)	128 (21)	12 (3.2)	88 (2.9)
Merlin	Control	0.16 (0.041)	4.5 (0.65)	735 (65)	138 (19)	82 (8.1)	194 (27)	21 (3.2)	83 (6.9)
	VV09	0.11 (0.021)	4.5 (0.42)	679 (114)	137 (3.8)	83 (8.1)	172 (22)	15 (2.6)	77 (14)
	VV10	0.094 (0.023)	3.8 (0.18)	627 (34)	138 (22)	87 (5.5)	155 (2.5)	13 (1.9)	89 (5.1)
Source of variation	d.f.[y]								
Variety	1	***	***	NS	**	NS	***	**	NS
Biostimulant	2	NS	NS	NS	NS	NS	NS	NS	NS
Interaction	2	NS	NS	NS	NS	NS	NS	NS	NS

[y]d.f., degrees of freedom; *, **, *** Significant at $p<0.05$, $p<0.01$, $p<0.001$, respectively: NS, not significant ($p>0.05$ according to two-factor ANOVA or Scheirer–Ray–Hare analysis).

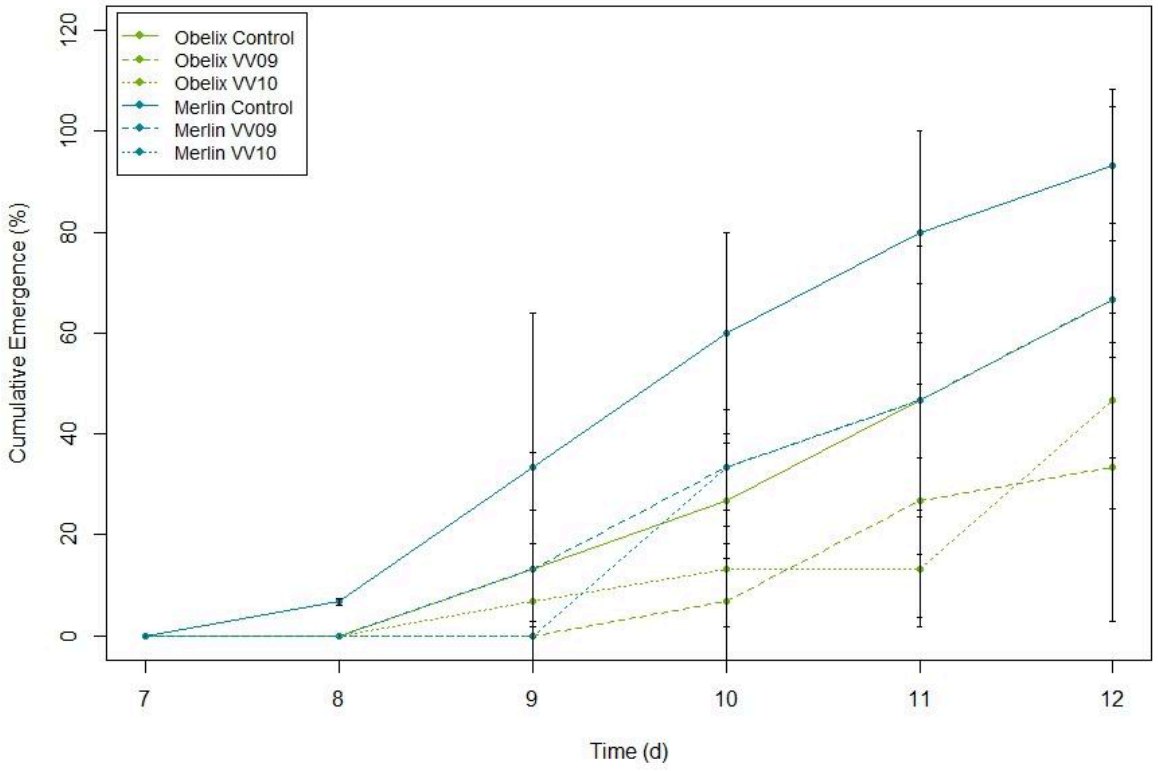

Figure 2. Emergence of soybean (Obelix and Merlin varieties) from untreated (control) and biostimulant-coated (VV09, VV10) seed from 7 to 12 d after planting. Data points are the mean (n=3) with bars for the standard deviations of the mean.

Figure 3. Side-by-side comparison of the physical states of the Obelix VV09 group (left) and the Merlin VV09 group (right)

Discussion

F2 seed produced by biostimulant-treated and untreated soybeans demonstrated negligible differences in protein and mineral nutrient content

I reject my hypothesis stating the concentration of 7S and 11S proteins and the mineral nutrient content of F2 seed produced by biostimulant-treated soybeans, would be significantly higher compared to F2 seed produced by untreated soybeans. The biostimulant treatments had no effect on the mineral nutrient content. Since Ni is not a nutritionally important mineral nutrient for humans, the minute difference in concentration will likely have inconsequential effects for plant consumers.

I did find that biostimulant treatments affected the 11S:7S ratio and Ni content in Obelix seeds. The 11S:7S protein ratio is an important indicator of protein quality in soybeans (Sexton et al. 1998). The F2 seed produced by Obelix VV09 had a greater 11S:7S protein ratio compared to Obelix VV10 (+13%) and the control (+19%). This could suggest a higher concentration of sulphur-containing amino acids like methionine and cysteine, which are 3–4 times more abundant in 11S protein compared to 7S protein (Panthee et al. 2004, Wang et al. 2022). However, this would have to be confirmed by measuring the concentration of S-containing amino acids in soybean seed, which was beyond the scope of the present study. Although the S-containing amino acid composition is affected by metabolic engineering methods that upregulate sulphur assimilation and methionine biosynthesis pathways (Li et al. 2023, Wang et al. 2022), there is little evidence to support that biostimulant seed coatings could have a similar effect. Still, sulphur availability in the growth medium is an equal or more important factor determining methionine synthesis in soybean seed (Sexton et al. 1998, Kim et al. 2014). Assuming that a seed coating could add bioavailable S to the growth medium, Via Végétale may be increasing the concentration of 11S protein by supplying extra sources of sulphur which result in the upregulation of

methionine synthesis. More detailed information about the composition of Via Végétale could be helpful in this regard.

There are other reports that biostimulants provide bioavailable sulphur or stimulate the biosynthesis of S-containing amino acids. For instance, a study by Briglia et al. (2019) used high-efficiency phenotyping and Next Generation Sequencing to demonstrate that soybeans treated with "YieldOn®" biostimulant showed a 2-fold increase in the genetic material associated with the cysteine biosynthetic process compared to untreated soybeans. Although this study does provide evidence to support the potential of biostimulants to alter gene expression in soybean, there are a number of caveats which affect the credibility of these findings to rationalise the outcome of the protein analysis in my thesis. For example, the study by Briglia et al. (2019) grew soybean in a greenhouse under controlled conditions, the biostimulant was applied as a foliar spray on V3 soybean, the sample material was soybean leaves, and with the unknown composition of Via Végétale it is impossible to make any assumptions about the ability of it to replicate any of the effects demonstrated by YieldOn®.

One limitation of this study was that F2 seed nutritional quality was based on only two criteria, namely the primary soybean protein storage components (7S and 11S) and mineral nutrient content. Further research is needed to differentiate the gains in nutritional quality of F2 seed that can be achieved by biostimulant application, since there are other soil, crop and environmental factors that modify the amino acid composition, fatty acid composition of the oil content, and the concentration of isoflavones in soybean (Sudarić et al. 2019).

F2 seed produced by biostimulant-treated and untreated soybeans experienced a similar response to suboptimal growing conditions

I reject my hypothesis stating the early development of F2 soybean is affected by biostimulants. All indicators of germination and emergence success were the same in the untreated and biostimulant-treated soybean. Despite previous experiments demonstrating that F1 seed treated with VV09 and VV10 improved soybean germination and metabolic awakening at 12°C (Besnard et al. 2021), none of these effects were observed in F2 seed. That being said, there were several sources of error in this study that may have significantly affected the outcome of the germination and emergence experiments. Firstly, there is reason to believe that the total germination and total emergence of F2 seed was suboptimal due to poor storage conditions. Germination and emergence success of soybean seed was studied by Kandil et al. (2013), considering the effects of storage temperature, storage packaging, and storage period. The best conservation method was to store seeds in a refrigerator (10℃), in cloth bags, for 3 months, based on the pre-storage total germination (Gmax) and total emergence (Emax). However, storing seeds at room temperature, in plastic bags, for 12 months affected the seed viability and produced the lowest Gmax and Emax values. Therefore, it is possible that the F2 seed viability was compromised before the germination/ emergence experiments began.

Another challenge was that 7 to 67% of the seedlings failed to emerge, resulting in n=2 to n=3 of successful replicates (out of n=3) in the emergence experiment. Although there is no consensus for a minimum number of viable plants for good statistical comparison, a study by Scott et al. (1984) suggests that experiments relying on binary responses (i.e. emergence/ non-emergence) should opt for several experimental units with smaller subpopulations ($n<50$) instead of basing overall success on one experimental unit containing a large subpopulation. These modifications would improve the resolution of treatment differences and consequently allow for a more robust statistical comparison. Still, the low temperature in the experimental design presents a physiological challenge for warm-season

crops like soybean. Yamaguchi et al. (2014) demonstrated that a low temperature coupled with high soil moisture increases the risk of seed mortality during imbibition, resulting in seed rotting before emergence. Additionally, low soil temperatures increased seedling mortality because low temperature causes irregular membrane reorganisation during imbibition, limiting the ability of embryo tissue to expand, and reducing mitochondrial respiration (Alsajri et al. 2019, Dong et al. 2021).

The study has limited capacity to make generalisations about biostimulant effects on F2 soybean seed for two additional reasons. First, this study tested 2 soybean cultivars, and physiological processes like germination and emergence success can vary greatly among cultivars. In similar controlled experiments, Szczerba et al. (2021) and Kuczyński et al. (2022) used a minimum of 4 different cultivars to assess the impact of cold temperature on early soybean development. Testing these effects on a larger number of soybean cultivars would help to increase the certainty of the results by reducing the likelihood of a cultivar's specific traits interfering with overall germination and emergence success. For example, a study using 10 cultivars has a lower likelihood of a single cultivar influencing the overall interpretation because it represents 10% of the data, while a study using 2 cultivars has a higher likelihood of a single cultivar skewing the outcome because it accounts for 50% of the data. Second, the validity of the germination and emergence outcome should be confirmed by including a negative control (i.e., the optimal temperature for soybean germination and emergence success) and a positive control (i.e., a fatal temperature that produces 0% germination and 0% emergence). Failing to include controls in optimal (30°C) and inhospitable (-10°C) conditions does not allow me to confirm seed viability in the experiments and rule out other confounding effects (e.g., moisture, lighting, soil environment, genetic factors). Moreover, I cannot accurately determine the cold tolerance of F2 seed when I do not know the variance between suboptimal, optimal and inhospitable temperatures.

Recommendations for future research

Future research is needed to verify the cold tolerance of F2 seed from biostimulant-treated soybean. An additional round of germination and emergence experiments that include a temperature range and appropriate controls is recommended as a starting point to evaluate the cold tolerance of F2 seed from several soybean cultivars. Although uncommon in northwestern France, frost events pose a significant threat to the survivability of germinating soybeans (Egli et al. 2005). A study by LeMahieu and Brinkman (1990) demonstrated the relationship between freezing temperatures and seedling mortality between sowing and full emergence, where a minor change in temperature (between -4℃ and -6°C) caused seedling survivability to decline from 75% to 56%.

Analysis of the proteome of seeds during the germination test will provide insight into the cold tolerance of F2 seed from biostimulant-treated soybean. Such analysis can detect molecular-scale responses that are not visible in seed and seedling morphology. Nouri et al. (2011) found soybean exposed to low temperatures (4℃) during imbibition down-regulated 15 protein fragments associated with the expression of proteins involved in various synthesis and cellular pathways, linked to cold stress responses. The proteome of germinated seeds may reveal whether F2 seed produced by biostimulant-treated soybeans has enhanced tolerance to cold temperatures based on protein expression. The expression of genes that positively regulate cold tolerance in soybean, such as *GmTCF1a* (Dong et al. 2021), could also be compared across treatment groups to determine if greater gene expression occurs in F2 seed produced by biostimulant-treated soybeans compared to untreated soybean. Proteome analysis could be similarly performed on soybean seedlings from the emergence experiment as well.

Finally, I suggest investigating the concentration of specific proteins associated with enhanced cold tolerance such as late embryogenesis abundant (LEA) proteins or Glutathione

S-transferase 24 (GST24) (Cheng et al. 2010). This measurement can be carried out after exposing F2 seeds to suboptimal temperatures during soybean germination and emergence. In a future emergence experiment, I would suggest studying the association between the concentration of LEA and GST24 proteins and the root to shoot ratio of seedlings. Higher root to shoot ratios indicate a soybean is responding to suboptimal growing conditions (Harris, 1992), and might be coupled with protein-level changes in the cold tolerant proteins. This possibility remains to be investigated.

Final conclusion and summary

The findings of this thesis do not support the systematic application of biostimulants with the expectation of a consistent and measurable effect on F2 seed, as the biostimulant effect on soybean protein content was not consistent enough to demonstrate a dependable trend across multiple treatments. Without reliable results, there is no way of knowing whether the findings in this thesis are a new discovery, or whether they are spurious results that happened by chance. However, integrating soybean into crop rotations offers an avenue for sustainability in Normandy by improving crop productivity while causing limited effects on the surrounding environment by increasing temporal-spatial diversity (Liu et al. 2022). Additionally, including soybeans in crop rotations is beneficial because they contribute residues with favourable C:N ratios (ProTerra, 2020). Rotating soybean with wheat, which also happens to be one of the most produced crops in Normandy (Eurostat, 2004), has demonstrated several benefits, such as increased soybean yields and enhanced nitrogen use efficiency (Gaudin et al. 2015, Janovicek et al. 2021). For soybean breeders, understanding the potential impact of biostimulants, especially VV09, on F2 soybean seed protein quality could inform future breeding programs. Additionally, farmers might benefit from biostimulants as the products have demonstrated positive effects through direct applications without causing any harm to plants (i.e. they are not toxic).

In summary, biostimulants do not appear to have the potential to contribute to the sustainable production of high quality soybeans in Normandy solely based on the results of this thesis. This thesis contributes to the scientific community's very limited knowledge of treating soybeans with biostimulants and their effects on F2 seed with respect to seed nutritional quality and cold tolerance during early development. I hope that this thesis may serve as a launch pad for similar biostimulant studies in the future, as we attempt to fully understand what they are, how they work, and what they can achieve.

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Appendix

Table S1. Buffer solution preparation for protein extraction analysis.

Buffer	Preparation Procedure
Tris HCl Buffer	Add 4.54 g of 0.75 M Trizma Base to a 100 mL volumetric flask and then fill with Milli-Q water to the line. Use 1 M HCl to adjust the pH to 7.0, and then store the finished solution at room temperature.
$MgCl_2$ Buffer	In a 1 mL test tube, combine 498 mg of 4.9 M $MgCl_2$ $6H_2O$ and 500 µL of Milli-Q water. Make 55 µL aliquots of the finished solution and store at -20℃.
RNAse l	In a 5 mL test tube, combine 5 mg of 2 mg/mL RNAse A, 1.7 mL of Tris HCl buffer, 25.5 µL of $MgCl_2$ buffer, and 2.5 mL of Milli-Q water. Make 400 µL aliquots of the finished solution and store at -20℃.
DTT	In a 2 mL test tube, combine 154 mg of 1 M Dithiothreitol (DTT) and 1 mL of Milli-Q water. Make 90 µL aliquots of the finished solution and store at 20℃.
DNAse 1	Make 65µL aliquots of Roche DNAse l (10×) and store at 20℃.
R Buffer	In a 150 mL Erlenmeyer flask, combine 888 mg of 56mM Trizma Hydrochloride, 530 mg of 44mM Trizma Base, 6 mL of 10% Triton, and 100 mL of Milli-Q water. Mix for 15 min, and then aliquot the finished solution into 5 mL test tubes and store at -20℃.
Extraction Buffer	Prepare this buffer right before the protein extraction. In a 10 mL beaker, combine 4.65 mL of R buffer, 5.45 g of urea, 2 g of thiourea, and 470 mg of CHAPS (3-[(3-cholamidopropyl)dimethylammonio] -1-propanesulfonate). Mix on a magnetic stirring plate for 30 min to allow the urea and thiourea to dissolve. When the solution has 5 min of mixing left, combine 1 tablet of cOmplete Mini EDTA-free Protease Inhibitor with 1 mL of Milli-Q water in a test tube. When the

buffer solution has finished mixing, add 86 µL of DTT, 60 µL of DNAse l, 380 µL of RNAse l, and the 1 mL of cOmplete Mini solution to the beaker to finish the extraction buffer.

Table S2. In-depth procedure for the protein extraction analysis (Gallardo et al. 2002)

Preparation Procedure
Gather the aliquots of soybean powder and place them in a styrofoam container filled with liquid nitrogen. In a 2 mL test tube, combine 20–23 mg of soybean powder and 50 µL of extraction buffer for every mg of soybean powder. Cap the test tubes and then mix them on a shaker plate (1000–1200 rpm) for 90 min at room temperature. After shaking, centrifuge the tubes (20000 rpm) for 10 min at 4°C. Recuperate the supernatant while avoiding the solid pellets, and transfer the liquid into a separate 1 mL test tube. Repeat the centrifuging and supernatant recuperation process one more time. Use the volume indicators on the 1 mL test tubes to adjust the volumes to 750 µL with the leftover extraction buffer. The concentration of protein in the samples was determined by the Bradford method (1976), whereby serial dilutions of a 2 g/ L solution of bovine serum albumin (BSA) are used to create a standard curve upon which the absorbances of the samples will correspond to the concentration of protein in the samples. The samples were loaded into the microplates in triplicate. The final steps of the protein extraction process used sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) to isolate the quantities of 7S and 11S protein in the samples. In a new 1 mL test tube, add twice the volumetric equivalent of 15 µg of protein and combine with an equal volume of 2x Laemmli sample buffer. Repeat this process for every sample. Pipette 15 µL of each sample (from the new test tubes) into 12% polyacrylamide gels (Mini-PROTEAN TGX Stain-Free Gel by BIO-RAD), and run for 30 min at 20 V. After the gels finished running, transfer to plastic containers and add enough Coomassie Blue solution (Coomassie Brilliant Blue G-250) to submerge the gels completely. Cover the containers and place onto a rotation plate under a vent hood for 24 h. The decolorization process begins by removing the Coomassie Blue solution from the container. Add enough 0.1 M Tris/ phosphoric acid (pH = 6.5) solution to

submerge the gel and then mix on the rotation plate for 3 min. Remove the solution, then add 25% methanol solution and mix on the rotation plate for 30 s. Remove the methanol solution, then add 20% ammonium sulphate solution and mix on the rotation plate for another 24 h. Transfer the gel onto a BIO-RAD White Tray and insert into the Gel Doc™ EZ Imager (BIO-RAD) to evaluate the protein bands on the gel. Finally, use the BIO-RAD Image Lab software program to quantify the 7S and 11S protein present in each sample expressed as a percentage of the total protein present in the sample. The process was carried out in triplicate, and the values were averaged.

Block 1	Block 2	Block 3	Block 4	Block 5
M5V1	M4V1	M4V1	M7V1	M1V1
M5V2	M4V2	M4V2	M7V2	M1V2
M6V1	M3V1	M7V1	M2V1	M2V1
M6V2	M3V2	M7V2	M2V2	M2V2
M4V1	M1V1	M5V1	M1V1	M3V1
M4V2	M1V2	M5V2	M1V2	M3V2
M3V1	M7V1	M3V1	M3V1	M4V1
M3V2	M7V2	M3V2	M3V2	M4V2
M8V1	M8V1	M8V1	M5V1	M5V1
M8V2	M8V2	M8V2	M5V2	M5V2
M1V1	M2V1	M6V1	M4V1	M6V1
M1V2	M2V2	M6V2	M4V2	M6V2
M2V1	M5V1	M1V1	M8V1	M7V1
M2V2	M5V2	M1V2	M8V2	M7V2
M7V1	M6V1	M2V1	M6V1	M8V1
M7V2	M6V2	M2V2	M6V2	M8V2

Fig. S1 Two factor (soybean treatment, soybean variety) randomised complete block design for the field experiment (2022) at the Sileban field working station located in Barfleur, Manche, France. The MnVn formula in each subplot corresponds to the treatment group of the soybean, where ‘M’ denotes the biostimulant and ‘V’ denotes the soybean variety (V1: Obelix, V2: Merlin). The subpopulation of treatment groups that this thesis will examine is exclusive to the biostimulants M1 (no biostimulant), M3 (VV09), and M5 (VV10). Note that only the data associated with Block 2, Block 3, and Block 4 were used for analysis. The data associated with Block 1 and Block 5 was discarded in order to account for border effects.

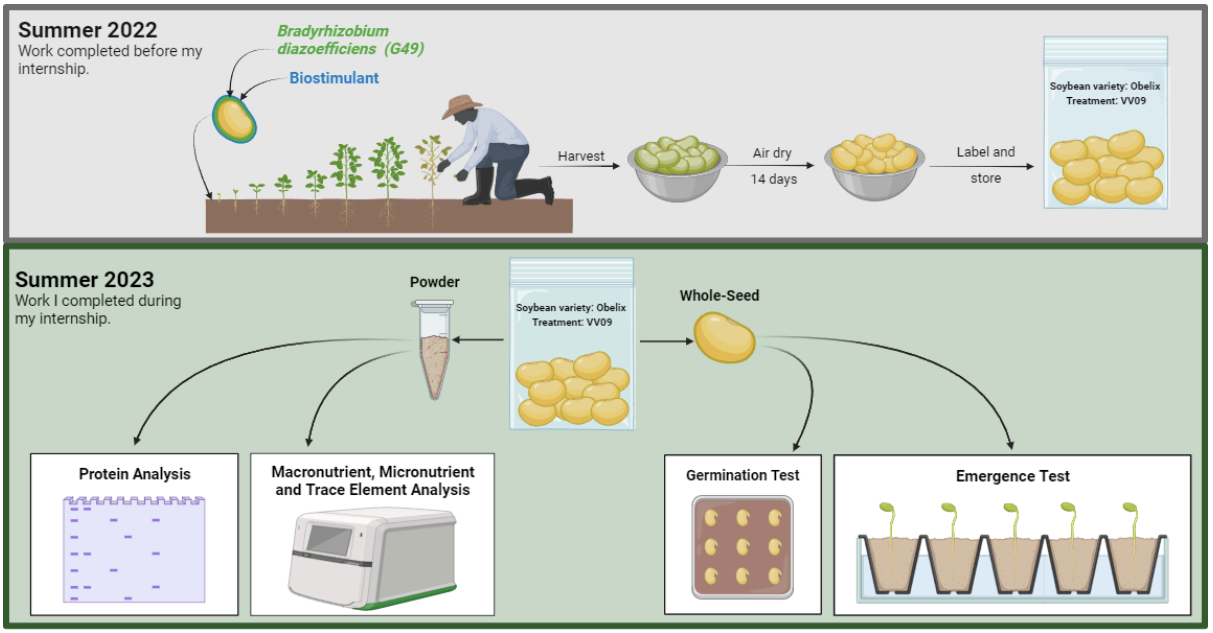

Fig. S2 This overview depicts the two phases of research associated with this thesis, and the tasks I was directly involved in. The grey upper portion of the diagram summarises the steps characterising the field experiment during the summer of 2022 (seed treatments, growing soybean to physiological maturity, harvesting the F2 seeds, drying and organising F2 seeds), and the green lower portion of the diagram summarises the steps characterising the laboratory analyses and experiments during the summer of 2023 (7S and 11S protein quantitation, soybean mineral nutrient composition, germination experiment, and emergence experiment). Created with BioRender.com

Fig. S3 Experimental unit (25 cm × 20 cm × 2 cm petri dish) for the germination experiment of F2 soybean seeds.