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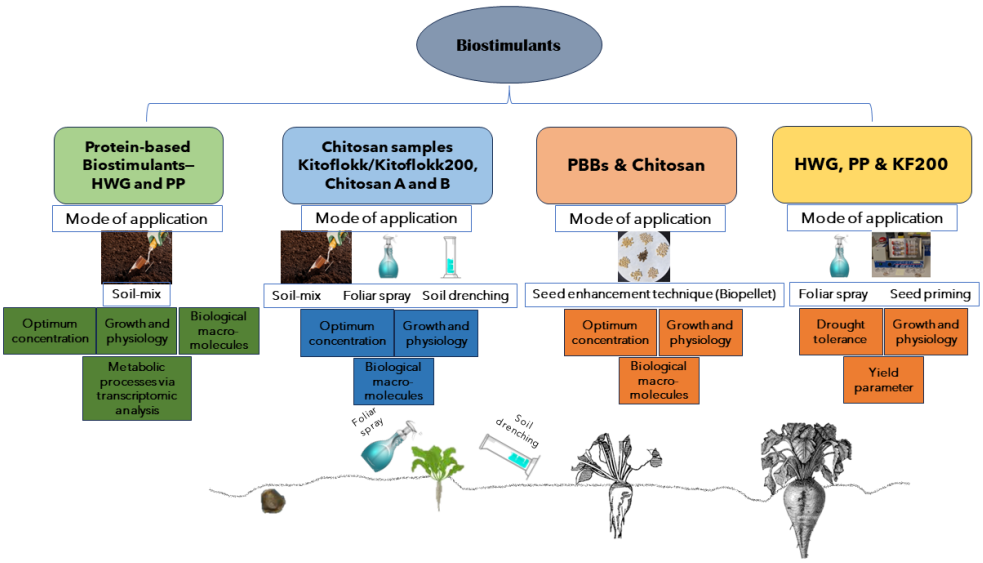
Biostimulant potential of agro-industrial side-streams - sustainable sugar beet cultivation and drought tolerance in wheat

Okanlawon Lekan Jolayemi



Biostimulant potential of agro-industrial side-streams - sustainable sugar beet cultivation and drought tolerance in wheat

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Cover: Schematic diagrams of all experiments in this thesis
(photo: Microsoft online pictures (soil mixing, spray bottle and measuring cylinder); Okanlawon Lekan Jolayemi (sugar beet bio-pellets) Ali Malik (sugar beet seed ball); Lan Yuzhou (KitoFlokk bottle) Clipground.com (sugar beet plant).

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Abstract

The biostimulant potential of three agro-industrial side-streams (hydrolysed wheat gluten (HWG), potato protein (PP/PF) and the chitosan derivative KitoFlokk™ (KF/KF200) and two chitosan types (KA and KB) was compared to that of untreated controls and nutrient solution in trials with three sugar beet genotypes (Volga, Armesa, Mustang) and two spring wheat genotypes (204, 276).

In general, 1-2 g/kg HWG or PP/PF applied as a soil mix enhanced sugar beet growth and physiology. Sugar beet also responded positively to increasing concentration (4-6 g per kg or L) of chitosan (KF/KF200, KA and KB), irrespective of application method (soil mix, soil drenching, foliar spray). In addition, bio-pellets containing 15% KB consistently improved growth and physiology of sugar beet. Transcriptome analysis attributed the biostimulant effects of HWG and PP/PF to upregulation of genes associated with metabolic processes (*e.g*. photosynthesis, protein synthesis and aromatic amino acids) compared with untreated controls. In wheat, bio-priming with 2% or 4% KF200 and foliar application of HWG, PP or 2% KF200 enhanced growth and yield components during early and late drought, with wheat genotype 276 showing better yield performance during drought than genotype 204, when primed/sprayed with biostimulants.

Therefore side-streams from wheat and potato starch processing (HWG and PF) and chitosan (KA, KB and KF/KF200) from seafood processing, have biostimulant capacity, triggering metabolic processes that improve growth and bio-macromolecules in sugar beet and drought tolerance in wheat when applied as a soil mix, soil drench, foliar spray or bio-pellet.

Keywords: Biostimulant, agro-industrial side-stream, hydrolysed wheat gluten, potato protein, chitosan, sugar beet, wheat, bio-pellet, bio-priming

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Biostimulerande potential hos agroindustriella sidoströmmar - hållbar sockerbetsodling och torktolerans i vete

Abstrakt

Den biostimulerande effekten hos tre olika sorters agroindustriella sidoströmmar (hydrolyserat vetegluten (HWG), potatisprotein (PP/PF) och kitosan: kitosanderivatet KitoFloKKTM (KF/KF200) och två sorters kitosan (KA och KB) jämfördes med obehandlade kontroller och kontroller behandlade med enbart näringslösning i försök med tre sockerbetsgenotyper (Volga, Armesa, Mustang) och två vårvetegenotyper (204, 276).

Generellt förbättrades sockerbetornas tillväxt och fysiologi när 1-2 g per kg HWG eller PP/PF tillsattes i jorden. Sockerbetorna påverkades även positivt med ökad kitosankoncentration (4-6 g per kg or L) oavsett kitosansort (KF/KF200, KA or KB) eller appliceringsmetod (jordblandning, jorddränkning, spray). Utöver detta resulterade användning av biopellets innehållande 15 % KB konsekvent till både ökad tillväxt och förbättrad fysiologi hos sockerbetor. Genom transkriptomanalys kunde de biostimulerande effekterna av HWG och PP/PF förklaras av uppreglering av gener associerade med metaboliska processer (t.ex. fotosyntes, proteinsyntes och aromatiska aminosyror) jämfört med obehandlade kontroller. För vete ökade tillväxt och skörd under både tidig och sen torka efter förbehandling av frön med 2% och 4% KF200 eller efter sprayning på bladen med HWG, PP eller 2% KF200. Behandling med biostimulanter (fröförbehandling/spray) hade störst effekt på vetegenotyp 276, vars prestanda var bättre än genotyp 204 under torka.

Sidoströmmar från stärkelseproduktion från vete och potatis (HWG och PF), samt kitosan (KA, KB och KF/KF200) från skaldjursbearbetning, har en biostimulerande kapacitet genom att starta metaboliska processer som gynnar tillväxt, biomakromolekyler i sockerbetor och torktolerans i vete när det appliceras genom jordblandning, jorddränkning, bladspray eller pellet.

Nyckelord: Biostimulant, agroindustriella sidoströmmar, hydrolyserat vetegluten, potatisprotein, kitosan, sockerbetor, vete, biopellet, bio-priming

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Sise ayẹwo agbara to wa ninu awọn ọjaja ẹnu ẹrọ isẹ-ọgbin fun imugbooro oun-ọgbin suga (Sugar beet- *Beta vulgaris*), ati alikama ti a gbin ni'gba ogbele.

Akojọpọ

Isẹ iwadi yi se ayẹwo awọn èròjà to le mú irúgbìn dàgbà ti a npe ni biostimulanti, ti a pese lat'ara alikama, ọdunkun-funfun, ati ikarahun akàn. Awọn èròjà naa ni, gluteni-alikama (HWG), proteni-ọdùnkùn (PP) ati èròjà káítósánì (KF). Èrèdí ti a fi se isẹ iwadi yi ni pe; a fẹ mọ òsùwọn ati ọna (boya nipa finfin tabi bibu si idi oun-ogbin) ti a le fi lo awọn eroja (HWG, PP ati KF) yi fun imudagba ati ilera pipe oun-ọgbin ṣuga (sugar beet) ati alikama ti a gbin ninu ọgbẹlẹ.

Isẹ iwadi yi jẹ ko ye wa pe, lilo ṣibi kan si meji (1-2 g) èròjà HWG ati PP ninu kongo iyẹpẹ kan (kg), ni oun-ọgbin suga ti se daada julọ. Òsuwọn sibi kan si meji HWG ati PP mu ki oun-ọgbin suga tobi, wọn rewa, wọn si tun ni eroja suga to pọ si. Àmọ, ni lilo KF lara oun-ọgbin suga, arii'pe, bi osuwọn KF ba se pọ to ninu iyẹpẹ tabi ninu omi (fun fínfín), beni oun-ọgbin suga se ndagba si. Isẹ iwadi miran ti a se fihan pe, a le po eroja HWG, PP ati KF pọ mọ elubo ti a fi npese koro-irugbin oun-ọgbin suga. Ohun ti a ri nipe, koro-irugbin ti o ni awọn eroja yi lara, se daada ju awọn ti ko ni lọ, paapa julọ, awọn koro-irugbin to ni eroja KF lara. Ninu iwadii miran ti a se pelu alikama ti a gbin ninu ọgbẹlẹ, a ri wipe, alikama ti a po kooro wọn mọ omi awọn eroja yi, ki a to gbin wọn, tabi eyi ti a fin pẹlu omi awọn eroja yi lẹyin ti a gbin wọn tan, se daradara ju awọn ti a ko lo eroja yi fun lọ.

Lakotan, iru awọn eroja yi pọ yanturu layikaa wa, ninu ajẹku ounjẹ, ati erè-oko to ti nbajẹ lọ lori oko tabi ninu ọjà. Bi a ba le se awari ọna ti a le gba yọ awọn èròjà yi lara irufẹ ajẹku ounje tabi erè-oko wọnyi, osese ki a mu adinku ba iwọn ajilẹ oni kẹmika ti a nlo lori oko. Eyi yoo mu adínkù ba inawo awọn agbẹ lori ajilẹ, yoo si tun mu adinku ba awọn ipenija to wa ninu lilo kemika fun isẹ ọgbin wa, lai din erè-oko wa kù.

Awọn ọrọ to se koko: biostimulanti, oun-ọgbin suga, eroja gluteni inu alikama (HWG), eroja am'aradagba inu ọdunkun funfun (PP), eroja kaitosani, irugbin-elelubo (seed pellet), irugbin-eree (seed priming).

Adiresi ounkowe: Okanlawon Lekan Jolayemi; Eka Imo Oun-ogbin,, Ile Iwe Giga Imo Ijinle Oun-Ogbin ti Ilu Sweden.

Dedication

To God Almighty, my family and everyone making the effort to have a world governed and ruled in a sustainable way.

Contents

List of publications

This thesis is based on the work contained in the following papers, which are referred to by their respective Roman numeral in the text:

I. Jolayemi O.L., Malik A.H., Ekblad T., Fredlund K., Olsson M.E. & Johansson E. (2022). Protein-based biostimulants to enhance plant growth – state-of-the-art and future directions with sugar beet as an example. *Agronomy* 12 (3211), 1-14.

II. Jolayemi O.L., Malik A.H., Vetukuri R, Ganapathi V.S., Pruthvi B. Kalyandurg, Ekblad T., Jean W.H. Yong, Olsson M.E. & Johansson E. (2023). Metabolic processes and biological macromolecules confirmed the biostimulating effects of novel protein-rich agro-industrial side streams on sugar beet plant development. *International Journal of Molecular Science* 24 (11), 9720.

III. Jolayemi O.L., Malik A.H., Farhadi M.,' Ekblad T., Bendigtsen S., Olsson M.E. & Johansson E. (2022). Evaluation of the biostimulating effects of chitosan on sugar beet phenotypic traits (manuscript).

IV. Jolayemi O.L., Malik A.H., Ekblad T., Olsson M. & Johansson E. (2022). Biostimulants as components of sugar beet seed pellets: Effect on germination, agronomic and physiological parameters (manuscript).

V. Yuzhou Lan, Jolayemi O.L., Aakash C., & Johansson E. (2022). Organic compounds sustaining wheat growth under drought – biostimulants applied by seed priming and foliar spray (manuscript).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Okanlawon Lekan Jolayemi to the papers included in this thesis was as follows:

I Designed the study with the co-authors, carried out the experimental work, data collection and data analysis. Wrote the manuscript with input from the co-authors.

II Designed the study with the co-authors, carried out the experimental work, data collection and data analysis. Wrote the manuscript with input from the co-authors.

III Designed the study with the co-authors, carried out the experimental work, data collection and data analysis. Wrote the manuscript with input from the co-authors.

IV Designed the study with the co-authors, carried out the experiment, data collection and data analysis. Wrote the manuscript with input from the co-authors.

V Designed the study with the co-authors, carried out the experimental work, data collection and data analysis. Wrote the first draft of the manuscript, with input from the co-authors.

List of tables

List of Figures

Abbreviations

AISS	Agro-industrial side-streams
BM	Basal material for sugar beet pellet
EBIC	European Biostimulants Industry Council
ELA	Easy Leaf Area (software)
HPLC	High-Performance Liquid Chromatography
HWG	Hydrolysed wheat gluten
KA	Chitosan sample A
KB	Chitosan sample B
KF	KitoFlokk™
KF200	Kitoflokk200™
NS	Nutrient solution
PBB	Protein-based biostimulants
PCA	Principal component analysis
PF	Potato protein film
PH	Protein hydrolysate
PP	Potato protein
RuBisCO	Ribulose-1,5- bisphosphate carboxylase/oxygenase
SAP	Superabsorbent polymer
SDS	Sodium dodecyl sulphate
SE-HPLC	Size exclusion-high performance liquid chromatography

1. Introduction

Biostimulants are natural bioactive ingredients that have the capacity to improve the quality and quantity of crops (Calvo *et al*., 2014; Drobek *et al*., 2019). Biostimulants target four main aspects of crop production: plant growth and development; nutrient and water use efficiency; protection against abiotic and biotic factors; and crop yield and quality (Brown & Saa, 2015). Two major classes of biostimulants, called protein-hydrolysates and chitosan, are mainly sourced from agro-industrial side-streams (AISS) (Ferraro *et al*., 2010; Martínez-Alvarez *et al*., 2015; Rajarajan *et al*., 2016).

This thesis assessed the biostimulant potential of three modified AISS, namely hydrolysed wheat gluten (HWG), potato protein (PP/PF) and the chitosan derivative KitoFlokk™/KitoFlokk-200™ (KF/KF200) for use in sustainable sugar beet cultivation and in improving tolerance to drought stress in wheat. Hydrolysed wheat gluten and PP/PF are modified products of side-streams from starch processing of wheat and potato, respectively (Capezza *et al*., 2021). Kitolokk™ is a derivative from the process of deacetylation of chitin, which is a major component of the shells of crustaceans (shrimps and crabs), and is thus another important side-stream product from aquaculture (Brown & Saa, 2015). The KF/KF200 product was first created as a flocculant for water treatment by TETA Vannresing, Norway.

This thesis evaluated the biostimulant effects of these protein-based side-streams, KF/KF200 and of two other chitosan types (denoted KA and KB) on agronomic, physiological and biological macromolecules and metabolic processes in sugar beet. It also assessed the possibility of adding HWG, PP and KB in different concentrations in a sugar beet seed enhancement technique (pelleting) and evaluated the effect of foliar application of HWG,

PP and KF200 and seed priming with KF200 on growth and yield in wheat crops grown under drought stress.

2. Background

2.1 History of biostimulants

The first definition of biostimulants, credited to Zhang and Schmidt, (1999), is as substances that are not plant nutrients, but when applied to plants in small quantities have an effect on plant growth (Jardin, 2015). The European Biostimulants Industry Council (EBIC) defines plant biostimulants as "substance(s) and/or micro-organisms 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" (Brown & Saa, 2015; Jardin, 2015). Therefore, biostimulants can be regarded as growth boosters and a sustainable nutrition source for plants. Different biostimulants studied in the past five decades include seaweed extracts, fulvic acid, humic acid, protein hydrolysates and chitosan (Jindo *et al*., 2020). There has been a more intense focus on the use of biostimulants in recent decades, as part of sustainable food production (Brown & Saa, 2015).

2.2 Classes and sources of biostimulants

Biostimulants are classified into different groups based on their active ingredient, mode of application and crop specificity (Jardin, 2015). The five main groups are seaweed extracts, fulvic and humic acids, plant growth-promoting bacteria, protein-based biostimulants and chitosan (Calvo *et al*., 2014). Most biostimulants are produced from natural raw materials, usually from plants, animals or microorganisms, which are easily available and accessible (Xu & Geelen, 2018). Many of these natural compounds are side-

stream products or, in some cases, wastes from agro-allied industries. Conversion of these side-stream products into biostimulants represents a win-win situation for both food production and the environment. However, understanding the mode of action of biostimulants is still a major challenge, mainly because of their multiple bioactive components (Khan *et al*., 2009; Ali *et al*., 2021). For instance, protein hydrolysates are composed of mixtures of polypeptides, peptides and amino acids in different proportions, depending on the source of the raw material and the degree of hydrolysis (Colla *et al*., 2015; Yakhin *et al*., 2017).

2.2.1 Protein-based biostimulants (PBBs)

Protein-based biostimulants (PBBs) are a major category of biostimulants, usually represented by protein hydrolysates and amino acids, which are products of hydrolysis of protein-rich side-streams of plant and animal raw materials (Rouphael & Colla, 2020). Every year, large volumes of agro-industrial side-streams of plant and animal origin are released into streams and rivers or dumped in landfill, thereby contributing to environmental pollution (Chalamaiah *et al*., 2012; Sharp, 2013; Xu & Geelen, 2018). Most of the side-streams from animal sources and some from plant sources have a high content of protein, which could be hydrolysed to produce biostimulants, *e.g*. protein hydrolysates (Xu & Geelen, 2018). Protein hydrolysates from plant sources include soybean (Amirkhani *et al*., 2016; Jain & Badve, 2022) and alfalfa (Schiavon *et al*., 2008; Ertani *et al*., 2009), while protein hydrolysates from animal sources include meat (Ertani *et al*., 2009; Ertani *et al*., 2013), gelatin (Wilson *et al*., 2018), bones (Carella *et al*., 2021) and pig blood (Zhou *et al*., 2022).

2.2.2 Chitosan

Chitosan is a type of biostimulant derived by removal of acetyl groups from chitin using a strong alkaline solution under high temperature (Shahrajabian *et al*., 2021). Chitin is a polysaccharide found in the shell of aquatic animals and insects that gives them structural support (Gooday, 1990). It is considered the second most abundant polysaccharide in nature, after cellulose (Bueter *et al*., 2013). Chitosan is a versatile biopolymer that has a wide variety of applications in the pharmaceutical, cosmetics, food, paper and textile industries (Jayakumar *et al*., 2010; Kalantari *et al*., 2019; Tao *et al*., 2020). It is also used as a flocculant in water purification plants, due to

its ability to adsorb metallic ions and other impurities. It has gained much attention in agricultural science in recent decades because of its role in boosting the defence of crops against abiotic and biotic stresses (Sharp, 2013). It is also reported to enhance the growth and physiology of crops, irrespective of mode of application (Pichyangkura & Chadchawan, 2015; Shahrajabian *et al*., 2021). The biostimulant activities of chitosan are strongly dependent on the degree of deacetylation and polymerisation (Shahrajabian *et al*., 2021). Crop type, stage of plant maturity and time of application can also affect the efficacy of chitosan (Shahrajabian *et al*., 2021).

2.3 Biostimulants on the global and European market

The global biostimulants market is growing very rapidly, with high research intensity compared with other industries (EBIC, 2022). Due to the rapid growth in the industry, biostimulants companies are currently reinvesting 3-10% of their turnover in research and development (EBIC, 2022). In the past decade, the global biostimulants market grew at a rate of 12% per year, to reach over 2 billion USD by 2018 (Calvo *et al.*, 2014; Brown & Saa, 2015; Jindo *et al*., 2020). The European biostimulants market accounts for almost 50% of the global market, with a market value of 0.9 billion USD in 2021. The market is expected to continue growing by about 11% per year, to reach 1.5 billion USD by 2026 (Research and Market, 2020).

The European biostimulants market is coordinated and regulated by the European Biostimulants Industry Council (EBIC), which was established 10 years ago with 10 members, but has since grown to comprise 72 members (Figure 1). More than 6 million hectares of crops were cultivated using biostimulants across Europe in 2012 (Lilliehöök, 2021). One factor driving the European biostimulants market is increasing use of biostimulants across the globe. In addition, there has been an increase in the professional network of biostimulants companies and in development of new innovative products targeting specific crop needs (EBIC, 2022). Moreover, the biostimulants market has been boosted due to the possibility to use biostimulants in both organic and conventional farming systems, the recent increases in the price of farm inputs such as fertilisers, and the increase in consumer demand for healthy and environment-friendly food products (De Pascale *et al*., 2017; Pylak *et al*., 2019; EBIC, 2022).

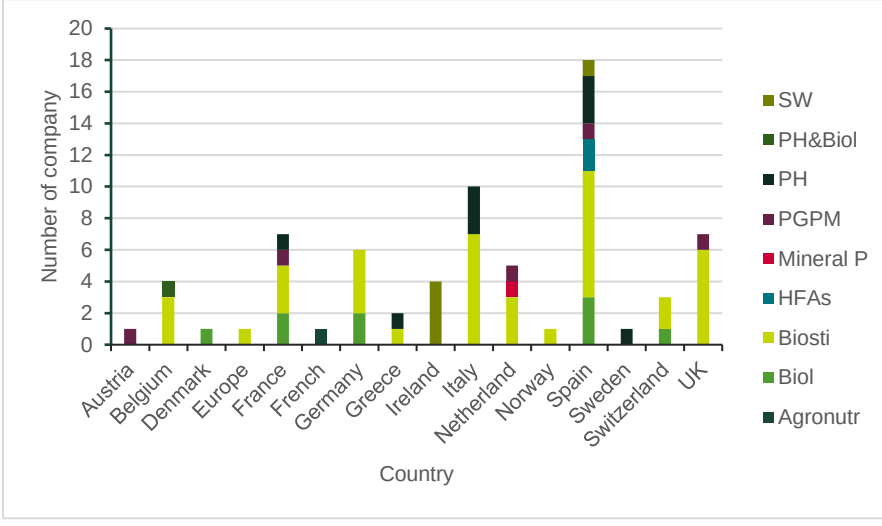

Figure 1. Members of the European Biostimulants Industry Council (EBIC), their location and products. SW: seaweed extract, PH: protein hydrolysates, PGPM: plant growth-promoting microorganisms, Mineral P: mineral phosphorus, HFAs: humic and fulvic acids, Biosti: biostimulants, Biol: biologicals.

2.4 Sugar beet history and production

Sugar beet (*Beta vulgaris* L) is a major economic crop, providing table sugar (sucrose) for the human population (Gurel *et al*., 2008). It first became prominent in the mid-19th Century, when sucrose was extracted from its roots by a German scientist, Andreas Sigidmund Marggraf (Draycott, 2008). Currently, 14% of the world's total sugar production comes from sugar beet, while the remaining 86% is from sugar cane (International Sugar Organisation, 2022; OECD/FAO, 2022). The European Union is one of world largest producers of sugar beet, with cultivation concentrated in the northern half of Europe (International Sugar Organisation, 2022).

Sugar beet is planted in late winter or early spring, and it is generally harvested in autumn or early winter. The productivity of the sugar beet crop depends significantly on high uniformity of seedling emergence in the field (Catusse *et al*., 2011). Sugar beet seeds require sophisticated seed enhancement techniques in order to optimise their germination capacity (Habib, 2010). This is because the seeds are irregular in shape and have a

hard pericarp that contains germination inhibitors (Kock *et al*., 1953; Salimi & Boelt, 2019). The pericarp also acts as a physical barrier to water and oxygen uptake, thereby retarding germination (Abts *et al*., 2013). Seed enhancement techniques are therefore employed to improve germination and establishment of sugar beet, which include seed processing (sorting or grading), polishing, priming, pelleting and coating (Taylor *et al*., 1998).

Many modern seed enhancement techniques require the use of inorganic or petroleum-based components, which are not environmentally friendly and sustainable. There is therefore a need to develop alternative products that are more eco-friendly and more sustainable. Since biostimulants are natural bioactive compounds, non-toxic and easily available, their potential as an alternative to petroleum-based products for use in seed enhancement urgently needs to be explored.

2.5 Wheat and drought tolerance

Wheat is an important food crop that has supplied humans with calories for several thousand years (Ahmad et al., 2018; Lama et al., 2022). However, recent trends in terms of climate change, and associated droughts and floods, are posing a threat to cultivation of wheat, and other crops, around the world (Nezhadahmadi *et al.*, 2013).

The best solution to the problem of drought is to develop drought-tolerant cultivars through plant breeding (Siddique *et al*., 2000). However, this is very tedious work because drought is a multigenic trait that is affected by a number of different environmental factors (Nezhadahmadi *et al*., 2013). Development of treatments that can enhance drought tolerance in major agricultural crops such as application of biostimulants, would be an easy and cheap solution.

One major function of biostimulants is to enhance tolerance to abiotic and biotic stresses, as reported and proven in multiple studies (de Vasconcelos, 2020; Francesca *et al*., 2021; Jacomassi *et al*., 2022; Taha *et al*., 2020; Wang *et al*., 2022).

3. Thesis aims and objectives

The overall aim of the studies presented in Papers I-V in this thesis was to evaluate the biostimulant potential of hydrolysed wheat gluten (HWG), potato protein (PP/PF) and the chitosan derivative KitoFlokk™ on sugar beet and wheat. The biostimulant potential of the products was assessed based on their effect on agronomy and physiology, bio-macromolecules and metabolic processes in sugar beet, and on drought tolerance in wheat. Specific objectives of the work in Papers I-V were to:

- Review the state-of-the-art and future perspectives of protein-based biostimulants, and determine the concentration for optimum growth of sugar beet (Paper I)

- Evaluate the effect of HWG and PP/PF on growth, physiological parameters, biological macromolecules and metabolic processes via transcriptome analysis of sugar beet (Paper II)

- Assess the optimum concentration of different chitosan types and the optimum mode of application for growth and physiology of sugar beet (Paper III)

- Evaluate the effect of HWG, PP and KB as components of seed pellets on growth and physiological parameters of sugar beet (Paper IV)

- Assess the effect of HWG, PP and KF in enhancing drought tolerance in wheat (Paper V).

4. Materials and methods

4.1 Brief description of the experiments in papers I-V

The experiments in Paper I-V in this thesis were performed using three sugar beet genotypes (Volga, Armesa and Mustang), two spring wheat genotypes (204 and 276), five biostimulants (HWG, PP/PF, KF/KF200, KA and KB), three modes of application (foliar spray, soil mixing and soil drenching) and two seed enhancement techniques (pelleting and seed priming). The biostimulants used in the experiments were from two different groups, namely PBBs (HWG and PP/PF, used in Papers I, II, IV and V) and chitosan (KF/KF200, KA and KB, used in Papers III, IV and V) (Table 1). Detailed descriptions of all materials and methods used can be found in the respective papers, which are included as an appendix to this thesis.

Table 1. *Details of the experiments performed in Papers 1-5*

Paper	***Description***	***Cultivar***	***Biostimulant and concentration***	***Mode of application***	***Remarks***
I	Review and preliminary experiment	Volga, Armesa and Mustang	Hydrolysed wheat gluten (HWG), potato protein film (PF) and potato protein (PP) 0-10 g/kg	Soil mix	One trial
II	Effects of protein-based biostimulant (PBB) treatment on growth, physiology and metabolic processes of sugar beet (3 trials)	Volga, Armesa and Mustang	HWG and PF 1 and 2 g/kg	Soil mix	Trial 1: Low nutrient conditions (LNC) Trial 2: High nutrient conditions (HNC) Trial 3: Equal nitrogen from HWG, PF and nutrient solution
III	Effects of chitosan on growth and physiology of sugar beet (two trials)	Armesa	Chitosan types A and B (KB and KB) and KitoFlokk™/KitoFlokk200™ (KF/KF200)	Soil mix, foliar spray, and soil drenching	Repeated two times: Trials 1 & 2
IV	Bio-pellet experiment	Volga, Armesa and Mustang	HWG, PP and KB 5% or 15% per pellet	Pellet component	In vitro germination test, Field emergence test and biotron/greenhouse trials
V	Drought tolerance in wheat	Two spring wheat genotypes	HWG, PP, KF200, nutrient solution	Seed priming and foliar spray	Seed priming: no priming as negative control and hydro-priming as positive control in two drought treatments- Early drought stress (EDS) and late drought stress (LDS) conditions Foliar spray: water spray as control in late drought stress (LDS) condition only

4.2 Data collection

Agronomic parameters of sugar beet and wheat crops that were analysed in Papers I-V included plant height, digital plant canopy area (via image analysis) and dry shoot and root biomass. Physiological parameters analysed included chlorophyll concentration, which was measured in the sugar beet and wheat trials in Papers II-V, and nitrogen content (Dumas method), which was measured only in the sugar beet trials (Papers II and III). All agronomic and physiological parameters were measured in five replicates.

The concentrations of different biological macromolecules in sugar beet, namely ribulose-1,5- bisphosphate carboxylase/oxygenase (RuBisCO), total peptide, sucrose and total sugar content, were determined in triplicate measurements using high-performance liquid chromatography (HPLC) in the sugar beet trials (Papers II-IV).

4.2.1 Agronomic parameters

Plant height was measured using a transparent 60 cm plastic ruler. Digital plant canopy area was measured using plant canopy images taken with an iPhone SE camera (12MP) and analysed using Easy Leaf Area (ELA) open software. Dry shoot and root biomass collected at the end of the experiment, *i.e.* at 8 and 12 weeks after planting for sugar beet and wheat, respectively, were determined using a digital weighing balance (Papers I-V).

4.2.2 Physiological parameters and biological macromolecules

Chlorophyll concentration was measured on fully developed young leaves of sugar beet and wheat using an Apogee chlorophyll meter (Papers II-V). Nitrogen content by the Dumas method was measured using a Flash analyser (Papers II and III).

Biological macromolecules (RuBisCO, total peptide, sucrose and total sugar content) were measured in leaf and root samples collected in zipper plastic bags and kept at -80 ^{o}C in a freezer until analysis. Prior to the measurements, the leaf and root samples were freeze-dried for 72 hours and ground using IKA A-10 basic analytical mill. Protein and sugars were extracted using different buffer solutions, following standard laboratory procedures, and analysed by HPLC.

4.3 Data analysis

All data on agronomic and physiological parameters and bio-macromolecules were subjected to analysis of variance, to detect differences in means (Paper II-V). Where differences were observed, line graphs, boxplots or bar charts were generated using “PivotChart” on the "Insert” tab in Microsoft Excel (Papers I-IV). Means were calculated using 3-5 replicates, depending on the parameter, and were separated using ± standard error. In some cases, principal component analysis (PCA) was performed and PCA score plots and loading plots were generated using Minitab 2.1 (Papers II, IV and V).

5. Results and discussion

5.1 Agro-industrial side-streams as a reservoir of bioactive ingredients.

One of the main aims of this thesis was to determine the current and future potential of agro-industrial side-streams (AISS) for use as PBBs. Some AISS end up as supplements in animal feed and other low valued products. However, where there is low application or low storage capacity for AISS, indiscriminate disposal of AISS is inevitable, thereby causing huge environmental problems (Xu & Geelen, 2018). Incidentally, most agro-industrial wastes of animal origin and some of plant origin are rich in protein and other bioactive ingredients, and thus have the capacity to act as biostimulants (PBBs) (Jorobekova & Kydralieva, 2019; Moreno-Hernández *et al*., 2020). Recycling agro-industrial wastes into PBBs would solve the environmental problems arising from their disposal, while at the same time producing safe food for human consumption (Ali *et al*., 2021).

Many bioactive ingredients have been reported to be effective in stimulating plant growth and defence against abiotic and biotic stresses, and some are already being sold in the market. In Paper I, HWG and PP/PF were shown to have promising biostimulatory effects on sugar beet growth and can be further explored for other crops and in terms of their economic viability. There is also the possibility of developing protein-based super absorbent polymers (SAPs), which have high-water holding capacity, for use in mitigating drought stress in crops (Capezza *et al*., 2021). Protein-based SAPs will also contain bioactive molecules, such as peptides and free amino acids, in their network, and these can serve as a source of nutrients for crops under drought conditions.

5.2 Protein-based biostimulants as growth promoters and bio-macromolecules

Evaluation of HWG and PP/PF as PBBs in Paper I, using sugar beet as the test crop, revealed that a concentration of 1-2 g/kg resulted in optimum sugar beet growth (Figure 2). All agronomic parameters (plant height, plant canopy area and total fresh biomass) of the three sugar beet genotypes used (Volga, Armesa and Mustang) were at their best when 1-2 g/kg HWG, PF or PP were applied (Figure 2).

This optimum concentration range (1 or 2 g/kg HWG and PF) was further evaluated, either individually or in combination with nutrient solution, in Paper II. Generally, it was found that 1 and 2 g/kg HWG and PF enhanced all growth and physiological parameters of the three sugar beet cultivars compared with the untreated control (Figure 3). However, addition of nutrient solution to 1 or 2 g/kg HWG and PF compared with only applying HWG or PF did not give any particular improvement in growth and physiology of sugar beet in trial 1 (low nutrient conditions, LNC) or trial 2 (high nutrient conditions, HNC) in Paper II (Figure 3A, Figure 4). Treatment with HWG and PF also enhanced dry root biomass of sugar beet compared with nutrient solution and the control in trial 1, but only 2 g/kg HWG enhanced dry root biomass in trial 2 compared with the other treatments (Figure 3B). Most HWG and PF treatments in both trials 1 and 2 increased the content of RuBisCO compared with nutrient solution and the control (Figure 3C). The size and intensity of the bands on sodium dodecyl sulphate (SDS) gel confirmed the enhancing effect of the HWG and PF treatments on protein content in sugar beet leaves. The gel images revealed that all HWG and PF treatments except PF1 and PF1 plus nutrient solution (PF1+NS) in trial 1, enhanced both large and small subunits of RuBisCO in leaves of sugar beet genotype (cv.) Mustang (Figure 5).

Therefore, in most cases, using a combination of HWG or PF with nutrient solution did not have much added effect on growth and physiological parameters of sugar beet, indicating that PBBs can be used as the sole nutrient source for the crop, especially if nutrients are not in limited supply.

This means that the use of PBBs in combination with nutrient solution would reduce the amount of chemical nutrients in the environment.

The results in Paper II confirmed findings in previous studies evaluating the effect of PBBs on crop growth and physiology (Colla *et al*., 2015; Cristiano, 2018; Colla *et al*., 2020). For example, protein hydrolysate derived from legume has been found to boost agronomic traits of lettuce in a similar way to seaweed and nitrogen fertiliser (Di Mola *et al.*, 2019). Therefore, it has been suggested that PBBs can be used as source of nutrients for low fertility soils, thereby acting as a component in sustainable food production (Di Mola *et al*., 2019; Colla *et al*. 2020).

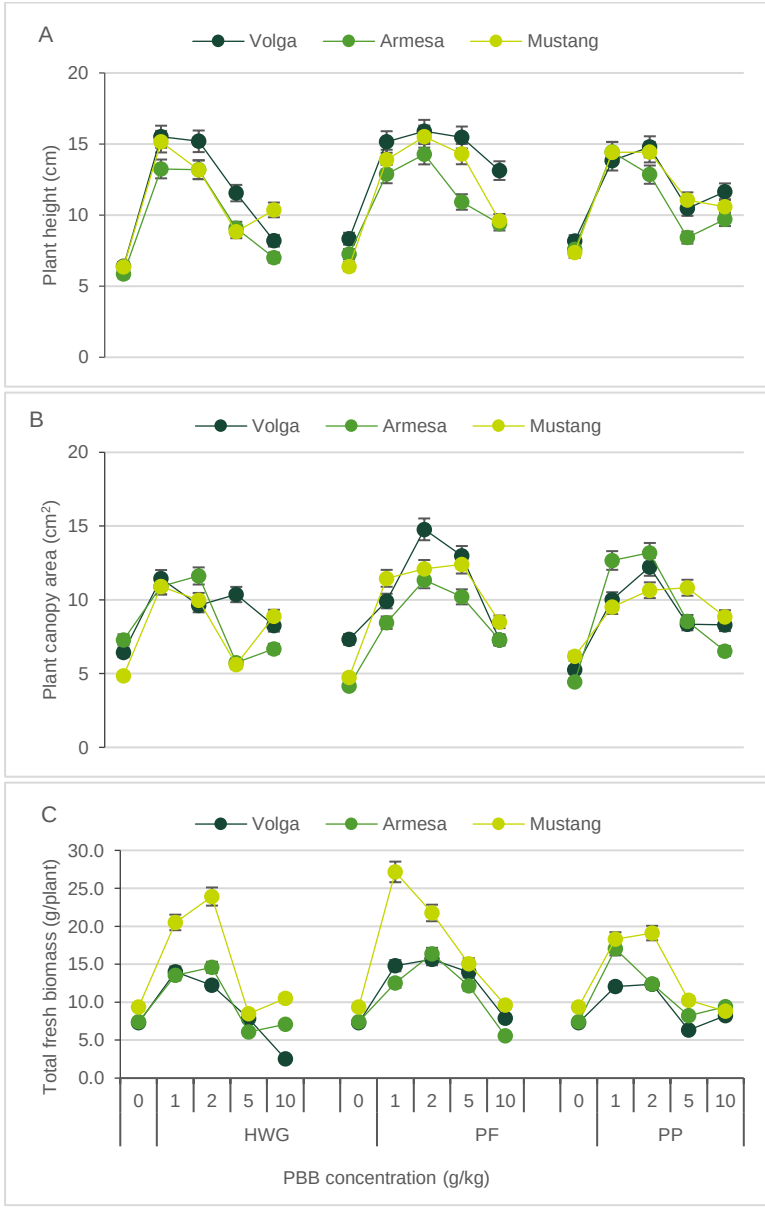

Figure 2. Agronomic parameters of sugar beet under protein-based biostimulant (PBB) treatments. (A) Plant height, (B) plant canopy area and (C) total fresh biomass of sugar beet cultivars at different concentrations of hydrolysed wheat gluten (HWG), potato protein film (PF) and potato protein (PP). Each point is an average of five plants; means separated using ± standard error.

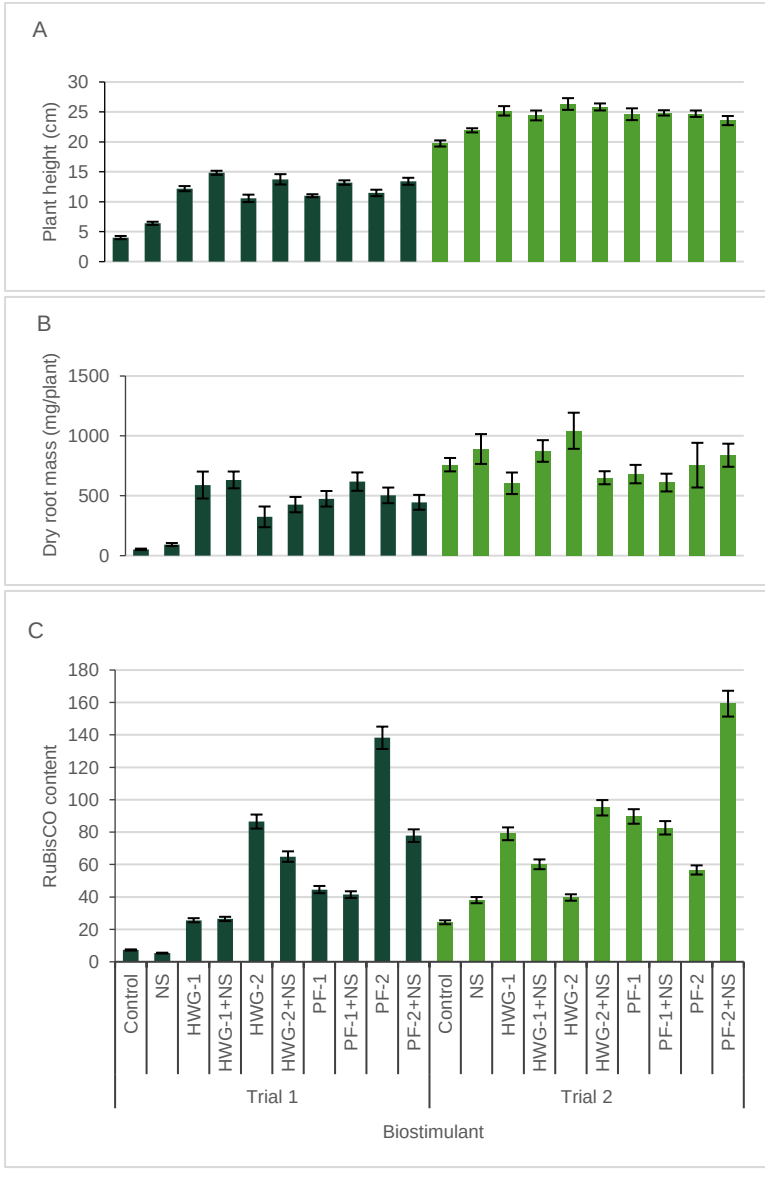

Figure 3. Effect of protein-based biostimulant (PBB) treatments on the agro-physiology of sugar beet. (A) Plant height, (B) dry root mass and (C) RuBisCO content of sugar beet under hydrolysed wheat gluten (HWG) and potato protein film (PF) treatments, applied alone or in combination with nutrient solution (NS). Results from two trials, where each bar is an average of five plants; means separated using ± standard error.

Figure 4. Sugar beet plants under protein-based biostimulant treatments in low nutrient conditions. Left to right: control, nutrient solution (NS), hydrolysed wheat gluten (HWG), HWG+NS, potato protein film (PF), PF+NS.

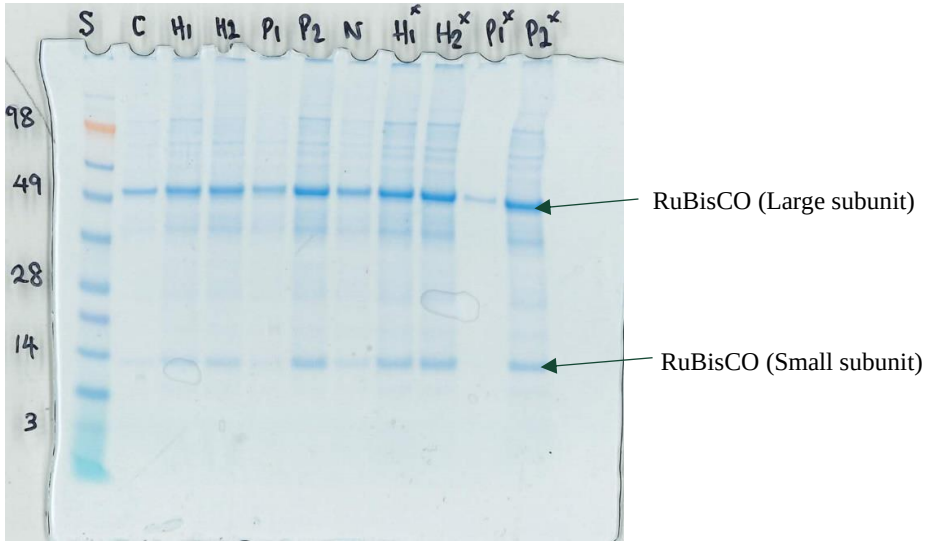

Figure 5. Sodium dodecyl sulphate (SDS) gel image of sugar beet (cv. Mustang) leaf showing protein expression in different protein-based biostimulant treatments. Left to right: S: protein standard, C: control, H1: hydrolysed wheat gluten (HWG) 1 g/kg, H2: HWG 2 g/kg, P1: potato protein film (PF) 1g/kg, P2: PF 2 g/kg, N: nutrient solution (NS), H1*: HWG1+NS, H2*: HWG2+NS, P1*: PF1+NS, P2*: PF2+NS.

5.3 Equal nitrogen dose: Protein-based biostimulants *vs* nutrient solution

In Paper II, equal nitrogen dose from PBBs (HWG and PP/PF) and nutrient solution generally enhanced all agronomic, physiological and bio-macromolecular parameters in sugar beet compared with the untreated control (Figure 6). However, there were some slight differences in the effect of equal nitrogen from HWG, PF and nutrient solution on the sugar beet genotypes. For instance, equal nitrogen dose supplied by nutrient solution, HWG and PF treatments increased plant height in all three genotypes of sugar beet in a similar way compared with the control (Figure 6A, Figure 7). However, the nutrient solution treatment gave greater plant canopy area in cvs. Volga and Mustang, whereas the HWG treatment enhanced chlorophyll concentration in all three sugar beet genotypes (Figure 6A and B). Furthermore, treatment with nutrient solution promoted root diameter and dry root biomass compared with the HWG and PF treatments (Figure 6C). The RuBisCO content in cvs. Volga and Armesa was enhanced only by the PF treatment, whereas both HWG and PF increased RuBisCO content in cv. Mustang (Figure 6D). In addition, HWG increased total peptide content in all three sugar beet genotypes compared with the levels reached in the other treatments (Figure 6D).

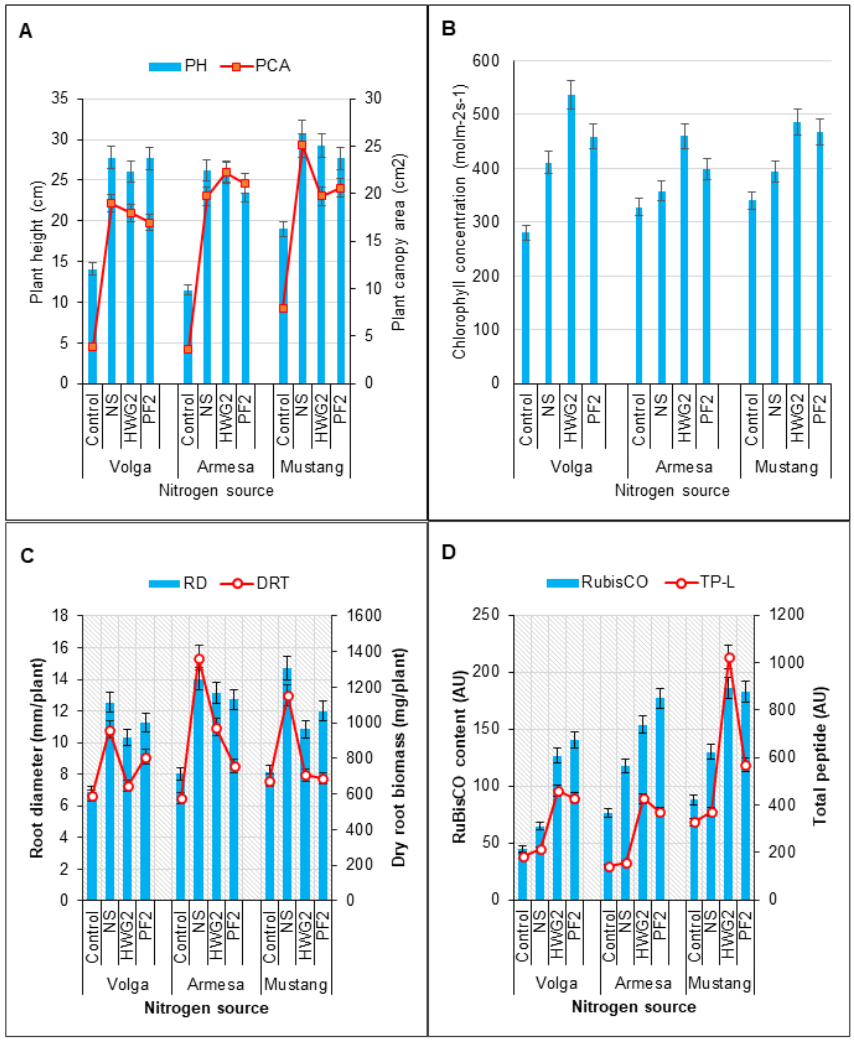

Figure 6. Agronomic, physiological and bio-macromolecular parameters of sugar beet under equal nitrogen treatment from protein-based biostimulants (hydrolysed wheat gluten (HWG), potato protein film (PF)) and nutrient solution (NS). (A) Plant height (PH) and plant canopy area (PCA), (B) chlorophyll, (C) root diameter (RD) and dry root biomass (DRT) and (D) RuBisCO and total peptide content (TP-L). Each bar is mean of five replicates in A-C and mean of three replicates in D; means separated using ± 0.05.

Figure 7. Eight-week-old sugar beet plants (A) in the untreated control and treated with equal nitrogen content from (B) hydrolysed wheat gluten (HWG), (C) potato protein (PP) and (D) nutrient solution.

Thus the data obtained in Paper II indicated that use of PBBs had comparable or in some cases superior fertiliser effects to nutrient solution on agronomic, physiological and bio-macromolecular parameters of sugar beet. This means that PBBs can be used to reduce or replace inorganic nitrogen in crop production.

The results in Paper II confirmed findings by Dudas *et al*. (2016), Rehim *et al*. (2021) and Ottaiano *et al*. (2021) that the use of protein-based biostimulants to replace inorganic fertilisers is promising for sustainable crop production. Dudas *et al*. (2016) observed an equal effect of biostimulant and inorganic fertiliser on growth, biomass and physiological traits in winter lettuce, while Rehim *et al*. (2021) observed increases in growth traits of radish crops with biostimulant compared with inorganic fertiliser. Ottaiano *et al*. (2021) investigated the effect of a biostimulant on lettuce and observed an increase in growth with the biostimulant and with inorganic fertiliser. However, the biostimulant also enhanced chlorophyll concentration and nitrogen use efficiency in the lettuce crop, while inorganic fertiliser did not (Ottaiano *et al*., 2021. These results suggest that biostimulants can be used in sustainable food production and can possibly replace inorganic fertiliser in low-stress conditions (Rehim *et al*., 2021; Ottaiano *et al*., 2021).

5.4 Effects of protein-based biostimulants on metabolic processes

Transcriptome analysis confirmed the enhancing effects of HWG and PF on agronomic and physiological parameters of sugar beet observed in Paper II. The analysis revealed that genes associated with some important metabolic processes were either upregulated or downregulated in leaves and roots of sugar beet plants treated with HWG or PF (Figure 8). Interestingly, genes associated with major metabolic processes influencing growth were upregulated in leaves and roots of plants treated with either HWG or PF (Figure 8). For instance, genes associated with ribosome and photosynthesis metabolism, which are directly related to crop growth, were upregulated in leaves of sugar beet treated with either HWG or PF (Figure 8A and B). This upregulation of ribosome genes observed in HWG- or PF-treated plants can explain the improvements seen in nitrogen-related parameters (nitrogen content, total peptide content, RuBisCO content) in Paper II. Furthermore, genes associated with aromatic amino acids, such as tryptophan, phenylalanine and tyrosine, were upregulated in leaves of plants treated with either HWG or PF (Figure 8A and B). These amino acids are either precursors of phytohormones (tryptophan) or are involved in plant defence mechanisms (tyrosine and phenylalanine).

On the other hand, genes associated with biosynthesis of secondary metabolites were greatly downregulated in roots of sugar beet plants treated with HWG and PF. These genes accounted for 50-60% of the downregulated genes (Figure 8A and B). Interestingly, genes associated with different nuclear activities were upregulated in the roots of plants treated with PF (Paper II). These nuclear activities included DNA replication, nucleocytoplasmic transport, base excision repair, mismatch repair and nucleotide excision repair (Figure 8B). Such activities are reported to be connected to stress-related responses, confirming previous claims that PF treatment can enhance tolerance to biotic or abiotic stresses in sugar beet (Xu *et al*., 2020).

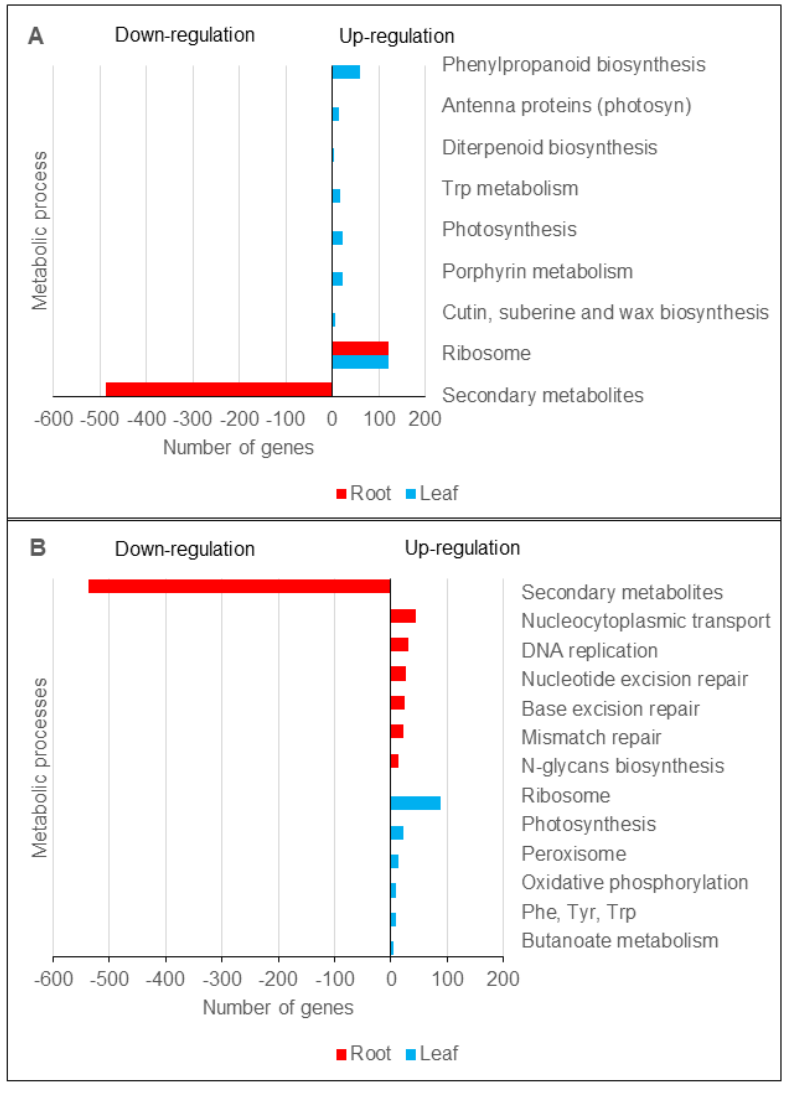

Figure 8. Metabolic processes identified in transcriptome analysis of leaves and roots of sugar beet plants treated with (A) hydrolysed wheat gluten and (B) potato protein film compared with an untreated control.

5.5 Effects of chitosan on growth and physiology of sugar beet

In general, growth and physiological parameters of sugar beet were enhanced along concentration gradients by all chitosan types tested in Paper III (KF/KF200, KA and KB), irrespective of the mode of application (soil mix, foliar spray, soil drenching) (Figure 9). In soil mix treatment, higher concentration (4 or 6 g/kg) of KB and KF enhanced chlorophyll concentration compared to other treatments including untreated control (Figure 9A). In addition, 6 g/kg KF promoted dry root biomass of sugar beet whereas, KB treatments did not affect dry root biomass (Figure 9A). Foliar application of KB at 6 g/L and KF200 at 1 or 2% enhanced both chlorophyll concentration and dry root biomass of sugar beet. On the other hand, low concentration (2 g/L) of KA led to higher dry root biomass (Figure 9B). In similar manner, soil drenching treatments of KA, KB and KF200 enhanced both chlorophyll concentration and dry root biomass (Figure 9C). However, in general higher concentration of KF (6 g/kg) or KF200 (2%) gave the best dry root mass under soil mix and soil drenching treatments (Figure 9). However, low concentration of KA led to higher dry root biomass under foliar spray (Figure 9B).

These results are similar to previous findings on the effect of chitosan in other crops. For example, Ohta *et al*. (1999) reported enhanced vegetative growth and reduced number of days to flowering in *Eustoma grandiflorum* grown in chitosan-treated soil, while Chookhongkha *et al*. (2012) found that micro-propagated chilli raised in chitosan-treated soil showed better growth than plants in untreated soil. Foliar application of chitosan has also been shown to improve agronomic and physiological parameters of strawberry (Abdel-Mawgoud *et al*., 2010), rice (Van-Toan & Hanh, 2013), okra (Mondal *et al*., 2012), chilli (Dzung *et al.*, 2017) and tomato (Reyes-Perez *et al*., 2020). In addition, soil drenching with chitosan has been found to promote agronomic and physiological parameters of rice (Boonlertnirun *et al*., 2007).

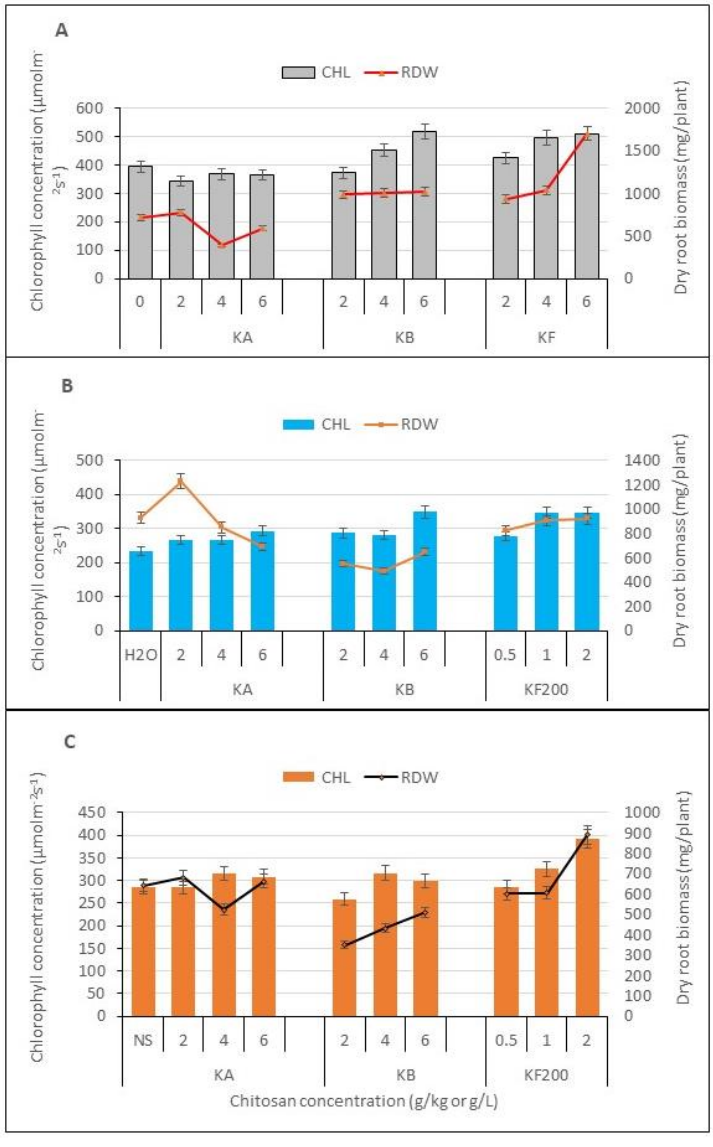

Figure 9. Effect of chitosan application by different methods (A) soil-mix, (B) foliar spray (C) soil drenching on chlorophyll concentration and dry root biomass. KA: chitosan type A, KB: chitosan type B, KF: KitoFlokk^{TM}. Each point is an average of five plants; means separated using ± standard error.

5.6 Effects of bio-pellets (HWG, PP and KB) on germination capacity of sugar beet

In Paper IV, HWG, PP and KB were successfully added at the rate of 5 or 15 % of basal material (BM) for sugar beet pellet (Figure 10). Under *in vitro* conditions, treatments HWG5, HWG15 and KB15 generally led to reduced percentage germination in all three sugar beet cultivars tested, with cv. Volga showing 77-86% germination, cv. Armesa 78.5-90% and cv. Mustang 89-94.5% under these conditions (Figure 11). Thus germination of Mustang was best under *in vitro* conditions, as it resulted in approximately 90% germination and above (Figure 11). Emergence of sugar beet from bio-pelleted seed under field conditions in Paper IV was 78-84% in Volga, 85-92% in Armesa and 84-90% in Mustang (Figure 12). The general rule is that all sugar beet cultivars should have emergence of >90%, but only cv. Armesa met this requirement. Multiple factors may have been responsible for the shortfall in emergence, ranging from climate to soil conditions and time of the year when the bio-pellets were sown.

Figure 10. Sugar beet pellets supplemented with 5 or 15 % hydrolysed wheat gluten (HWG), potato protein (PP) or chitosan B (KB). Naked seed of sugar beet cultivar Armesa is shown in the centre of the image.

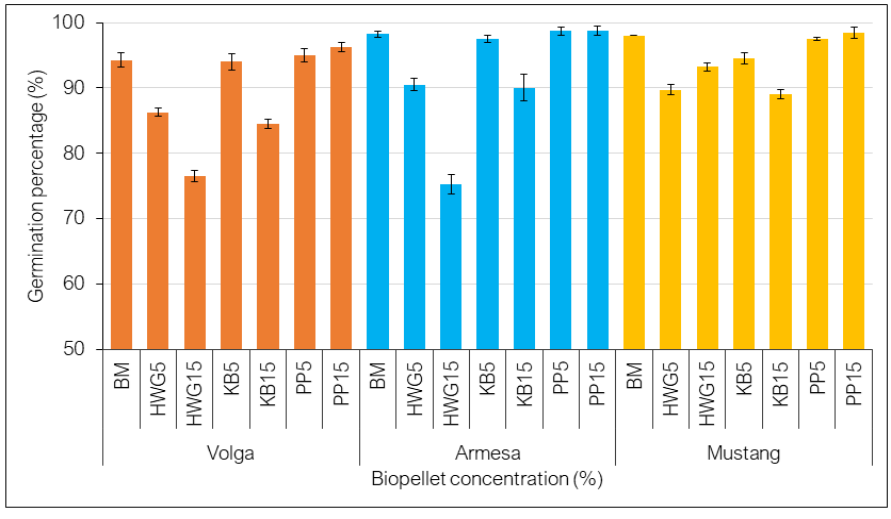

Figure 11. Percentage germination of bio-pelleted seed of sugar beet cultivars Volga, Armesa and Mustang under *in vitro* conditions. Pellets were unsupplemented (BM) or supplemented with 5 or 15 % hydrolysed wheat gluten (HWG), potato protein (PP) or chitosan B (KB). Each bar is mean of 100 seeds; means separated using ±standard error.

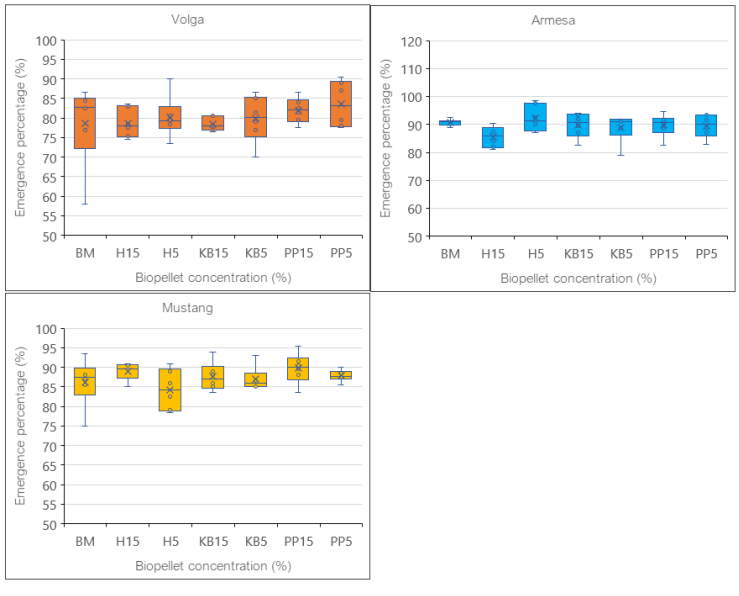

Figure 12. Percentage emergence of bio-pelleted seed of sugar beet cultivars Volga, Armesa and Mustang under field conditions. Each box is mean of 100 seeds; means separated using ±standard error. For treatment abbreviations, see Figure 11.

In biotron conditions, number of days to emergence of the three sugar beet cultivars was either enhanced or delayed, depending on the cultivar and the bio-pellet treatment (Paper IV). On average, it was 13.6-19.2 days in cv. Volga, 13.4-18.2 days in cv. Armesa and 13.4-28.8 days in cv. Mustang (Figure 13). In cv. Volga, all bio-pellet treatments apart from HWG5 delayed emergence by almost four days compared with basal material (BM) (Figure 13). In cv. Armesa, treatments BM, KB5 and PP5 delayed emergence by at least four days compared with the other treatments (Figure 13). In the PP15 treatment, cv. Mustang emerged almost 29 days after sowing, which was 10-15 days later than in the other treatments (Figure 13).

These results are in line with findings in other studies investigating the effect of bio-pellets on germination of different crops. Some have observed reduced germination percentage in seeds pelleted with different bio-based materials under *in vitro* conditions, while others have observed increased germination rate. For instance, a reduction in germination percentage has been reported for broccoli seeds coated with soy flour, perennial ryegrass seeds coated with soy flour and durum wheat seeds coated with microbial agent (Amirkhani *et al*., 2016; Qiu *et al*., 2020; Vitti *et al*., 2022). In contrast, increased germination has been reported for red clover seeds coated with soy flour, spring wheat, barley and sugar beet seeds coated with fulvic acid film, and sesame and bean seeds coated with chitosan (Qiu *et al*., 2020; Braziene *et al*., 2021; Godínez-Garrido *et al*., 2022). This indicates that the germination capacity of pelleted seeds is dependent on factors such as crop species, pelleting material, nature of binder and growth environment factors.

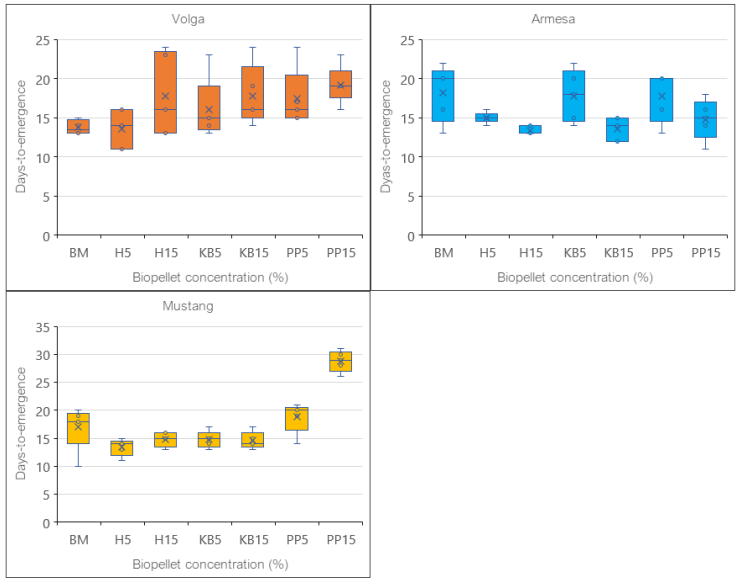

Figure 13. Boxplots of number of days to emergence of bio-pelleted seed of sugar beet cultivars Volga, Armesa and Mustang under biotron conditions. Each bar is mean of five replicates; means separated using ±standard error. For treatment abbreviations, see Figure 11.

5.7 Bio-pelleting: A sustainable seed enhancement technique for sugar beet

Sugar beet seeds are usually processed as pellets, which helps to facilitate planting using machines and promotes uniform germination. Supplementing seed pellet recipes with biostimulants such as HWG, PP and chitosan (bio-pellets) has shown to be promising, as this process led to enhanced growth and physiology of sugar beet compared to unsupplemented (BM) treatment (Figure 14) (Paper IV). Although, sugar beet genotypes responded differently to bio-pellet treatments, however, there were improvement in growth and physiology when bio-pellet treatments were applied (Figure 14). For instance, H5, KB15 and PP15 enhanced plant height of Volga, while KB15 enhanced plant height of Mustang compared to BM (Figure 14A). Chlorophyll content was promoted when H15 or KB5 and KB5 or KB 15 were applied to Armesa and Mustang respectively (Figure 14B). In addition, dry root biomass was enhanced in Volga by the application of KB5, in

Armesa by the application of PP15 and KB15, whereas in Mustang by KB15 (Figure 14) (Paper IV).

In general, KB treatments (KB5 and KB15) consistently improved most agronomic and physiological parameters of all three sugar beet genotypes compared with the BM treatment (control). These results are similar to previous findings on the effect of bio-pelleting on growth of other crops. For instance, red clover and perennial ryegrass have been found to grow better than a control when the seed is pelleted with soy flour (Qiu *et al*., 2020), while coating broccoli seeds with soy flour enhances seedling vigour, plant height and yield compared with a control (Amirkhani *et al*., 2016). Similarly, Salachna & Pietrak (2021) observed increased growth and yield parameters of pineapple lily seeds coated with different chitosan derivatives.

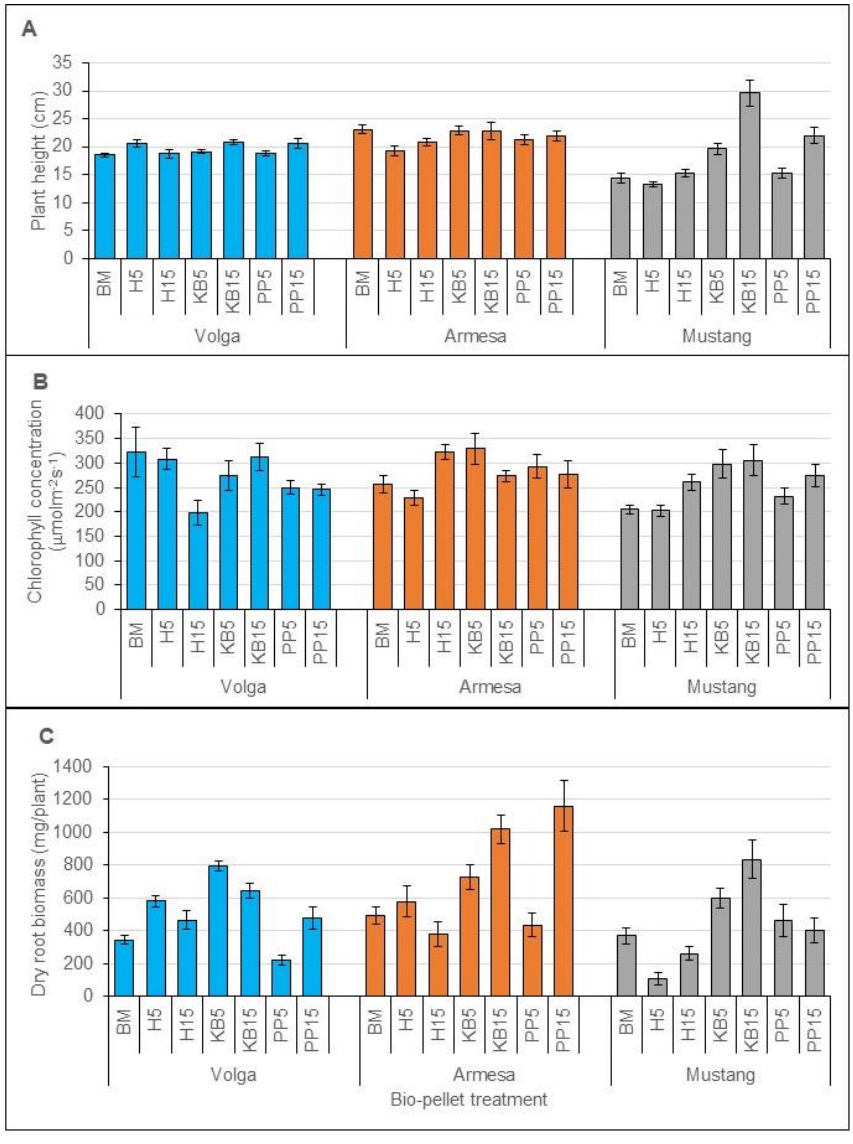

Figure 14. Effect of bio-pellet treatments on growth and physiological parameters of sugar beet cultivars Volga, Armesa and Mustang. (A) plant height, (B) chlorophyll concentration, (C) dry root biomass. Each bar is mean of five replicates; means separated using ± standard error.

5.8 Bio-priming and drought tolerance in wheat

The PCA score plot showed that the two spring wheat genotypes (old genotype 204 and modern genotype 276) tested in Paper V responded differently to the different drought conditions – early drought stress (EDS), late drought stress (LDS) and control (Figure 15A). The LDS treatment mostly affected growth and yield parameters of wheat, and were aligned with the negative axis of principal component 2 (PC2) (Figure 15A). However, in both drought treatments and in the control, the modern genotype 276 showed superior yield performance compared with genotype 204, as indicated by its position above that of genotype 204 in all cases (Figure 15A). In the EDS treatment, KF4 priming improved number of days to anthesis (DTA), days to heading (DTH), spikes per plant (SPP) and productive spikes per-panicle (PSPP) compared with the other treatments (Figure 15). Under LDS conditions, priming with KF4 followed by KF2 was superior to hydro-priming (H2O) and no priming in enhancing the above-mentioned traits (Figure 15A).

In general, genotype 204 had more grains per panicle (GPP) in control and LDS conditions than genotype 276. In contrast, genotype 276 had greater thousand grain weight across all drought treatments and the control compared with genotype 204 (Figure 16). Bio-priming with KF2 or KF4 did not improve thousand grain weight in non-stress conditions. On the other hand, bio-priming with KF4 enhanced thousand grain weight of genotypes 204 and 276 under LDS conditions (Figure 16) (Paper V). The superiority of genotype 276 (modern Swedish breeding line) over 204 (old Swedish cultivar) can be linked to improvements achieved through plant breeding.

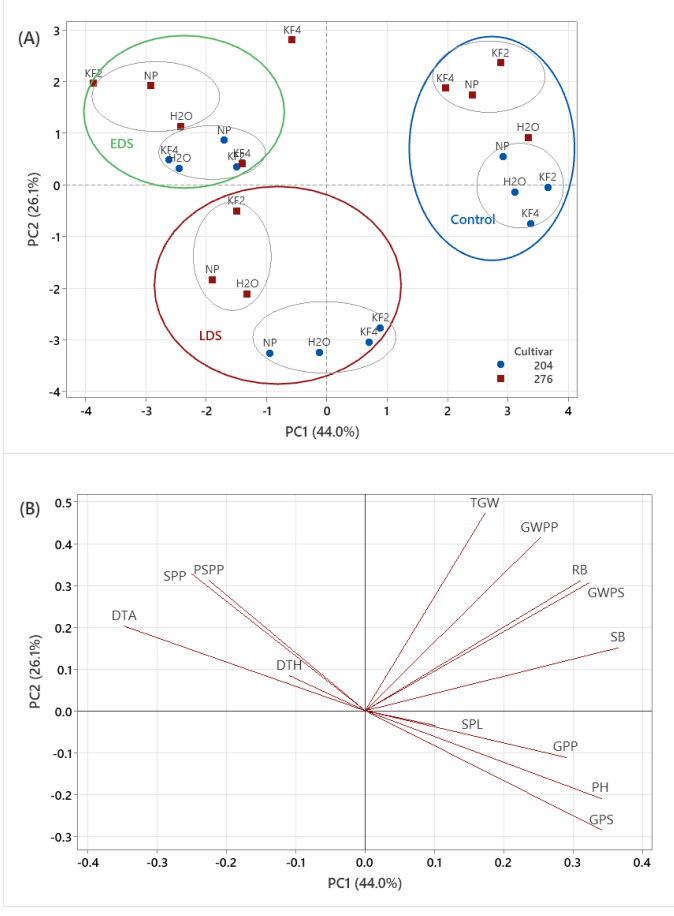

Figure 15. Principal component analysis (A) score plot and (B) loading plot of agronomic and yield parameters in different drought conditions of wheat genotypes 276 and 204 bio-primed with chitosan (KitoFlokk200). EDS: early drought stress, LDS: late drought stress, H2O: water, NP: no priming, KF2: 2% KitoFlokk200, KF4: 4% KitoFlokk200, PH: plant height, DTH: days to heading, DTA: days to anthesis, SPL: spike length, SPP: spikes per plant, PSPP: productive spikes per plant, GPP: grains per plant, GPS: grains per spike, TGW: thousand grain weight, GWPP: grain weight per plant, GWPS: grain weight per spike, SB: dry shoot biomass, RB: dry root biomass.

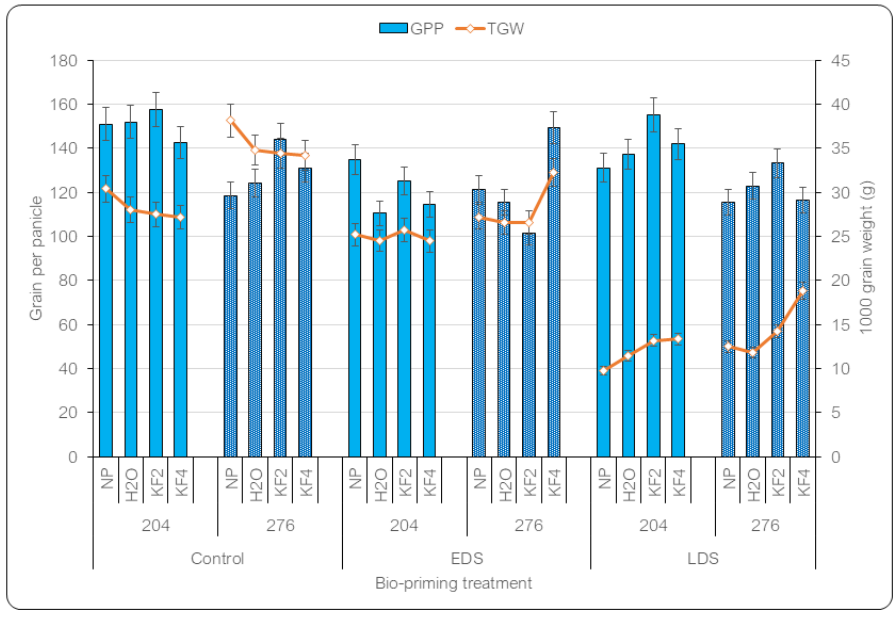

Figure 16. Effect of bio-priming on yield traits of wheat genotypes 204 and 276. Each bar is mean of five replicates; means separated using ± standard error. EDS: early drought stress, LDS: late drought stress, H2O: water, NP: no priming, KF2: 2% KitoFlokk200, KF4: 4% KitoFlokk200, GPP: grains per panicle, TGW: thousand grain weight.

5.9 Foliar application of biostimulants and drought tolerance in wheat

In the foliar spraying experiment in Paper V, the PCA score plot showed that wheat genotypes 204 and 276 were clearly separated along drought conditions (control and LDS) (Figure 17A). In the old Swedish genotype 204 under late drought, foliar application of biostimulant, especially HWG and PP, increased the number of grains per panicle and per spike, and also plant height (Figure 17). On the other hand, in genotype 276 under late drought, foliar application of nutrient solution and PP improved days to heading and anthesis, spikes per panicle and especially thousand grain weight (Figure 17).

In general, grains per panicle and thousand grain weight varied inversely in genotype 204 and genotype 276, irrespective of drought stress treatment (Figure 18). Under well-watered conditions, foliar spraying with water (H2O) and HWG increased the number of grains per panicle in genotype 204, while thousand grain weight was increased by foliar spraying with KF,

nutrient solution and PP (Paper V). Foliar spraying with biostimulant did not affect grains per panicle in genotype 276, but foliar spraying with nutrient solution and PP increased thousand grain weight (Figure 18). Foliar spraying with HWG and nutrient solution increased thousand grain weight in genotype 276 under late drought (Figure 18).

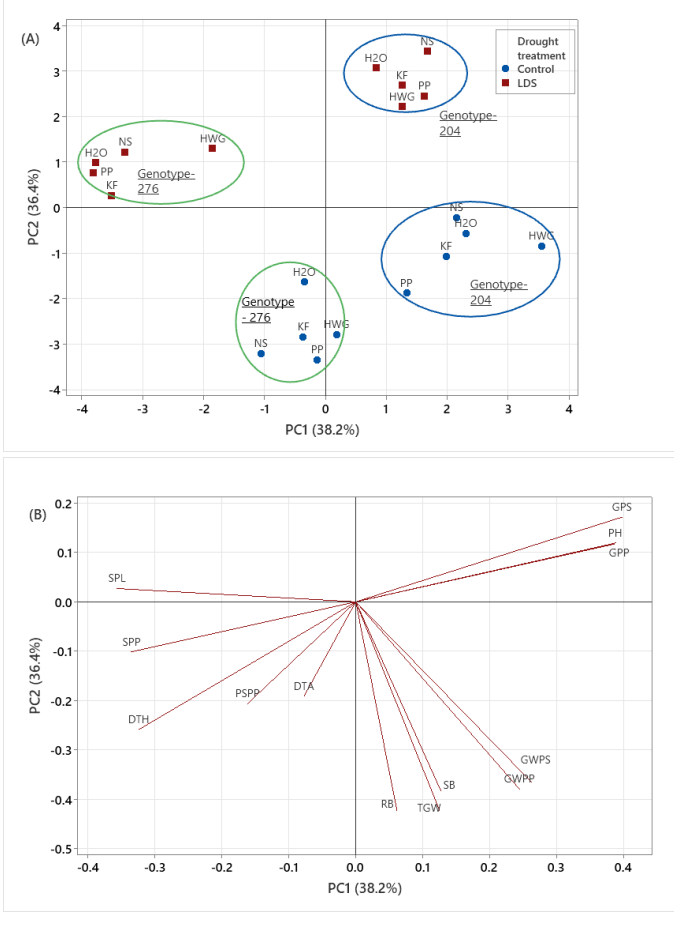

Figure 17. Principal component analysis (A) score plot and (B) loading plot of agronomic and yield parameters under late drought conditions of wheat genotypes 276 and 204 treated with biostimulants (HWG, PP, KF) by foliar spraying. LDS: late drought stress, H2O: water, NS: nutrient solution, KF: 2% KitoFlokk200, HWG: hydrolysed wheat gluten, PP: potato protein, PH: plant height, DTH: days to heading, DTA: days to anthesis, SPL: spike length, SPP: spikes per plant, PSPP: productive spikes per plant, GPP: grains per plant, GPS: grains per spike, TGW: thousand grain weight, GWPP: grain

weight per plant, GWPS: grain weight per spike, SB: dry shoot biomass, RB: dry root biomass.

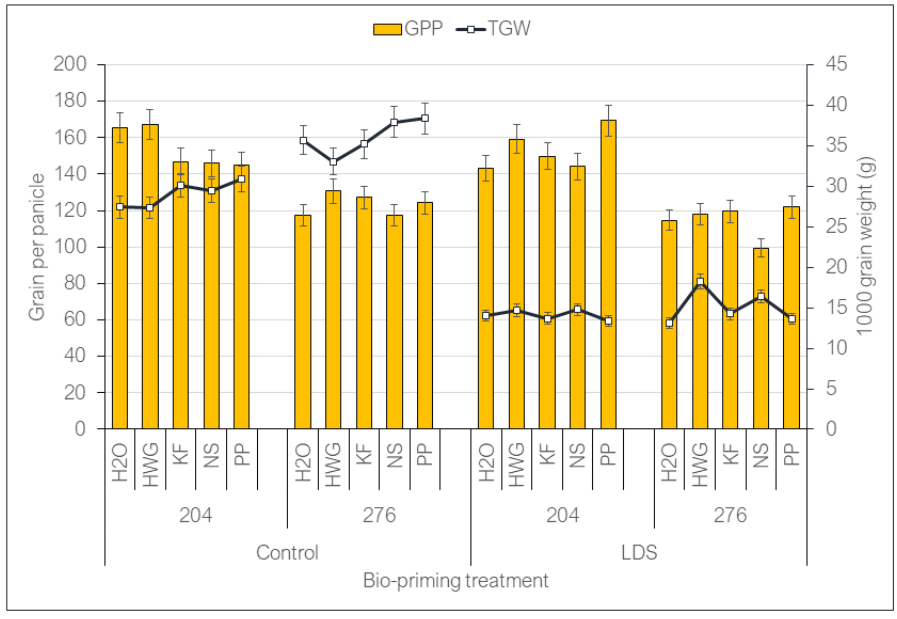

Figure 18. Impact of foliar spraying with biostimulants (HWG, PP, KF) on yield traits of wheat genotypes 204 and 276 under late drought stress (LDS). Each bar is mean of five replicates; means separated using ±standard error. H2O: water, HWG: hydrolysed wheat gluten, KF: 2% KitoFlokk200, NS: nutrient solution, PP: potato protein, GPP: grain per panicle, TGW: 1000 grain weight.

Our results have further established the drought tolerance enhancement of biostimulants, which have been reported by others. For instance, Francesca et al., (2021) and Richardson et al., (2004) reported growth, physiological and yield parameters of tomato and paper birch respectively under drought conditions. Furthermore, seaweed extract-based biostimulants improved sugar cane agronomic and yield components under drought stress (Jacomassi et al., 2022). Enhanced growth and physiological parameters of tomato and grape vine have also been reported under drought stress conditions (Irani et al., 2021; Wang et al., 2022). The enhancement of drought tolerance in these crops is possible irrespective of the methods of application; foliar spray, soil drenching etc.

6. Conclusions

The biostimulant potential of agro-industrial side-streams from wheat, potato and chitin products was evaluated in this thesis in terms of three of the four major functions of biostimulants (growth enhancement; yield and quality improvement; tolerance to stresses), based on their effects on sugar beet and wheat. The results in Papers I-V demonstrated that all these products have biostimulant potential. The main conclusions were as follows:

- Hydrolysed wheat gluten and potato protein products (PP/PF) at 1 or 2 g/kg and KitoFlokk^{TM} (KF/KF200) at 4-6 g/kg or g/L, under different modes of application, improved agronomic and physiological parameters in sugar beet. The levels of some biological macromolecules in the plants, such as sucrose, total sugar, total peptide and RuBisCO, were also enhanced at these biostimulant concentrations.

- Hydrolysed wheat gluten, potato protein products (PP/PF) and KitoFlokk^{TM} (KF/KF200) used as biostimulants can be applied directly to soil (soil mixing or soil drenching) or to plants (foliar spraying) and used in seed treatments (priming, pelleting).

- Hydrolysed wheat gluten (HWG), potato protein products (PP/PF) and KitoFlokk^{TM} (KF/KF200) improved tolerance of wheat to early and late drought stress. All growth and yield parameters of the two wheat genotypes tested (one modern, one old) were improved by seed priming using chitosan products (KF2, KF4) and foliar spraying using HWG, PP and KF2.

- Applying HWG and PP increased accumulation of sucrose, RuBisCO and total peptides in leaves of sugar beet plants. Transcriptome analysis

revealed that genes associated with biosynthesis of these bio-macromolecules were upregulated

- Chitosan derivatives (KB and KF) enhanced sugar beet growth and physiology along a concentration gradient

- Use of HWG and PP alone as biostimulants improved growth and physiology of sugar beet with little addition of mineral nutrients, reducing the amount of mineral fertiliser required by the crop

- The effect of chitosan derivatives (KB and KF) on growth and physiology of sugar beet was visible up to a rate of 6 g/kg. KB was more effective as a soil mix, while KF200 was more effective as a soil drench and foliar spray

- Use of HWG, PP and KB in seed bio-pelleting improved the agronomic and physiological parameters of sugar beet. Including KB at a rate of 5 or 15% was most effective

- Bio-priming with KF4 increased thousand grain weight of wheat genotypes 204 and 276 under early and late drought stress. Foliar spraying with HWG and nutrient solution increased thousand grain weight of both wheat genotypes under late drought stress.

7. Future plans

The analyses reported in this thesis can be extended by determining the optimum concentration and mode of application of the biostimulants for improving growth and physiology in other crops. It would also be interesting to identify the mode of action of the bioactive ingredients (HWG, PP/PF and KF/KF200) in other crops, using molecular tools (proteomics, transcriptomics, genomics *etc*.). It is very important to assess the financial viability of developing these bioactive ingredients into commercial biostimulants, from raw material to finished product, using life cycle assessment (LCA). Interestingly, the company manufacturing HWG (A. Constatino & Co.) is currently packaging and selling it as a plant biostimulant at commercial level and KF/KF200 is commercially available for use as a flocculant in water treatment. This means that HWG and KF are already sustainable and economically viable. On the other hand, PP is still being produced for internal development use only. Our research group has been using it for the development of bioplastics, biofoams and superabsorbent polymers (SAPs), and these other areas of research interest could lead to limited availability of PP for use as a biostimulant. Therefore, there is a need for economic assessments on the availability and sustainability of PP.

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Popular science summary

Agricultural wastes for food production

Agriculture generates tons of waste, which are normally used as supplements in animal feed and other low value products. However, in cases where there are no local application or means of storing these wastes, they may end up constituting environmental pollution. Most of these agro-industrial wastes are in fact reservoirs of important bioactive ingredients and could be developed into valuable materials for human use, thus avoiding harmful impacts on the environment. Useful recycled products obtained from agro-industrial wastes include bioplastics, biofoams and biofuel. Another way to add value to agro-industrial wastes is to convert them into biostimulants that can be used in sustainable and eco-friendly crop production. This thesis explored the potential of hydrolysed gluten obtained from wheat, protein obtained from potato and chitosan obtained from shellfish as protein-based biostimulants in sustainable sugar beet production and as drought tolerance enhancers in wheat production.

The results showed that applying these bioactive ingredients as a soil mix, foliar spray or soil drench, or in seed priming and pelleting, generally helped to improve the quantity and quality of different sugar beet traits and growth and yield of wheat grown under drought stress. Treated sugar beet plants grew better, faster, healthier and stronger, and produced larger tubers, and consequently more sugar from their tubers. Wheat treated with these bioactive ingredients, in particular through seed priming and foliar spray, performed better under early and late drought stress.

Therefore, converting agro-industrial wastes into bioactive ingredients for use in cropping can increase food production in ways that are better for human health, agricultural productivity and the safety of the environment.

Populärvetenskaplig sammanfattning

Jordbrukets rester för produktion av livsmedel

Jordbruket genererar en stor mängd avfall som normalt används som djurfoder och andra produkter av lågt ekonomiskt värde. I de fall där lokala användningsområden eller lagringsmöjligheter saknas kan detta avfall dock leda till miljöföroreningar. Många sorters agroindustriella avfall är i själva verket en stor tillgång, eftersom de ofta innehåller viktiga bioaktiva substanser. Dessa skulle kunna utvinnas och bli värdefulla produkter och på så sätt skulle avfallets negativa effekter på miljön kunna undvikas. Användbara produkter återvunna från agroindustriellt avfall inkluderar till exempel bioplast, bioskum och biobränsle. Ett annat sätt att ge avfallet mervärde är genom att omvandla det till biostimulanter som skulle kunna användas i en hållbar och miljövänlig växtodling. I denna avhandling undersöktes möjligheterna att använda hydrolyserat vetegluten, potatisprotein och kitosan från skaldjur som proteinbaserade biostimulanter för en hållbar sockerbetsodling och för ökad torktolerans hos vete.

Resultaten visade att tillsats av dessa bioaktiva ingredienser genom jordblandning, bladspray, jorddränkning, fröförbehandling (*seed-priming*) eller pelletering, generellt hjälpte till att förbättra tillväxten och många kvalitetsegenskaper hos sockerbetor. De bioaktiva ingredienserna gav även en ökad tillväxt och avkastning hos vete som utsatts för torka. Behandlade sockerbetsplantor växte bättre och snabbare, var friskare och starkare och producerade större knölar, och producerade följaktligen mer socker. Vete som behandlats med dessa bioaktiva ingredienser, i synnerhet genom fröförbehandling och bladspray, presterade bättre under tidig och sen torka.

Därför kan omvandling av agroindustriellt avfall till bioaktiva ingredienser för användning i växtodling bidra till en ökad livsmedelsproduktion på sätt som är bättre för människors hälsa, jordbrukets produktivitet och miljön.

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Review

Protein-Based Biostimulants to Enhance Plant Growth—State-of-the-Art and Future Direction with Sugar Beet as an Example

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Abstract: Protein-based biostimulants (PBBs) are derived from the hydrolysis of protein-rich raw materials of plant and/or animal origins, usually by-products or wastes from agro-industries. The active ingredients (AIs) produced by hydrolysis have the capacity to influence physiological and metabolic processes in plants, leading to enhanced growth, nutrient and water-use efficiency, tolerance to abiotic and biotic stresses, and improved crop yield and quality. This paper reviews the state-of-the-art and future opportunities for use of PBBs, based on potential effects on the soil, crops, and sustainability (social, economic, environmental). In this case, two examples of PBBs (hydrolyzed wheat gluten and potato protein) and their effects on the early growth of three sugar beet varieties are described and discussed. Both PBBs have a significant stimulating effect on early sugar beet growth and development. The opportunity to develop PBBs into superabsorbent polymers (SAPs) is discussed. To conclude, PBBs/SAPs developed from agro-industrial wastes have the potential for sustainably supplying water and nutrients in agricultural systems and for enhancing plant growth and development over a substantial period.

Keywords: biostimulant; hydrolyzed wheat gluten; potato protein; sugar beet; sustainable development; agro-industrial wastes; superabsorbent polymers

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1. Introduction to Biostimulants—Definition and Categories

Biostimulants are natural products that originate from plants, animals, or microorganisms and, when applied to plants (foliage or rhizosphere) in small quantities, stimulate natural processes that enhance growth, crop quality, nutrient-use efficiency, and tolerance to biotic and abiotic stresses [1–7]. Thus, the use of biostimulants can facilitate a reduced use of agrochemicals (especially fertilizers) in agriculture, without compromising crop productivity and quality, while also providing protection against abiotic and biotic stresses [1,3,8]. The use of biostimulants may not necessarily provide nutrients directly to plants or target pathogens, rather they regulate physiological processes that lead to enhanced growth and tolerance to abiotic and biotic stresses [2,3,5]. The current high use of agrochemicals in agriculture and food production poses risks to human health and the environment [1,2]. Biostimulants could form part of a solution to mitigate such risks deriving from the use of agrochemicals [8,9].

The use of biostimulants in commercial cropping settings is becoming increasingly popular, as the high content of bioactive components offers different benefits, whereas their mode of action is largely yet unknown [10]. They promote crop growth and reduce the impacts of agriculture on human health and the environment [10] and can thus be an important component in climate-smart agriculture (CSA) [7,11]. CSA is an approach that pushes for green and climate-resilient agri-food systems. Biostimulants are categorized

into seaweed extracts, humic and fulvic acids, beneficial chemical elements (e.g., silicon, selenium, sodium, cobalt, aluminum), chitin and chitosan derivatives, beneficial microorganisms, inorganic salts (phosphite), and protein-based biostimulants (peptides and amino acids) [5,11,12].

The protein-based biostimulants (PBBs) are an important group because of the abundance and accessibility of protein-rich side-streams from agro-industries that can be used as raw materials for PBB production [13,14]. PBBs are commonly derived from individual organic materials or a combination of organic materials, most commonly obtained from agro-industries [15]. These industries often generate tons of waste and side-stream products, most of which have a high content of proteins and other bioactive compounds [3,13]. According to available statistics, the wastes generated annually by agriculture and agro-industries include 9000 tons of dairy protein [16], 3 million tons of seafood waste [17], and 8 million tons of livestock protein [18]. Converting these wastes into useful products, e.g., biostimulants, would thus address sustainability issues by contributing to a reduction in the environmental footprint, providing economic benefits from the use of novel products, and improving human and environmental health and food quality [3,9,19].

The aims of this review were to describe the current state-of-the-art of PBBs and to outline possible future directions for the research and development of PBBs. Many research groups are currently evaluating opportunities to use agricultural and food wastes/side-streams as alternative products, with the development of novel biostimulants being one option [15]. However, the current knowledge of the use of PBBs, especially from wheat and potato industries side streams, has not been summarized until now, and future perspectives in relation to their uses have not been given. Therefore, in this paper, we review current knowledge on (i) how PBBs are produced, (ii) the effects of PBBs on soil and crops, and (iii) the sustainability (social, economic, environmental) of use of PBBs in agriculture. As examples, we evaluate and discuss the effects of two types of PBBs on sugar beet growth. Based on the findings, we consider future directions for research.

2. Protein-Based Biostimulants

2.1. Production

Protein-based biostimulants are basically mixtures of peptides and amino acids [2,20]. Most PBB products are derived from protein-rich substances (plant or animal origins) that have been enzymatically or chemically treated or subjected to thermal hydrolysis. The products are, therefore, often referred to as protein hydrolysates (PHs) [2,3,11,21]. They contain peptides and free essential and non-essential amino acids present in different quantities, depending on the protein source, processing methods utilized and degree of hydrolysis [2,13] (Figure 1). The active ingredients (peptides and amino acids) in the PHs, contribute to an increased uptake of beneficial elements into plant tissues via the leaves or roots [3,11]. Currently, more than 90% of commercially available PHs are derived from chemical hydrolysis of animal proteins, e.g., collagen, fish by-products, blood meal, chicken feathers, etc. [20,22]. Commercially available animal-derived PH products include Siapton [3], Pepton [23], and Hydrostim [24]. However, there are restrictions on the use of PBBs derived from animal by-products in the European Union (EU), where animal-derived products can only be used as raw material for biostimulants at the endpoint of the manufacturing chain, and with a particular focus on the safety of humans, animals, and the environment [25]. Under current EU regulations, biostimulants from animal-derived products may also not be applied directly to edible plant parts and the maximum concentration of heavy metals must be non-detectable [25]. Sweden, as an EU country, is following EU regulations. Commercial plant-derived PHs, e.g., Coveron [26] and Trainer [21], are also available in different forms (liquid, water-soluble powder, granules) and can be applied as foliar spray, seed, root, or soil treatments [11,22]. However, plant biostimulants are a recently emerging field of research with an increasing number of publications from 2015 and onwards [5] and the research on PBBs and PHs is keeping track of that development [2–4]. As an increasing number of commercial plant- and animal protein-based biostimulants will enter the market

as a result of the increasing research activities, additional regulations on the use of the products are expected.

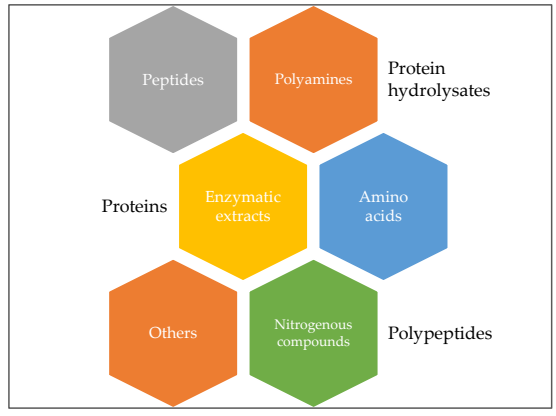

Figure 1. Possible active ingredients in protein-based biostimulants (PBBs). Compounds in white and black are large molecules, while active ingredients in colored hexagons are low molecular weight components of proteins [27].

2.2. *Effects of PBBs on Soil and on Agronomic, Physiological, and Molecular Plant Parameters*

Research on PBBs and their commercial use in agricultural and horticultural applications are of a rather recent origin, with most development having taken place during the past two decades [11]. Most of the PBBs evaluated to date have been shown to have a broad-spectrum effect on the biochemical properties and microbial community of the soil. They have also been found to have a significant effect on plant growth and health, as summarized in Table 1. As a result, PBBs have been used in soil bioremediation activities, for soil restoration, and for preventing soil erosion [28]. Studies have indicated that a high nitrogen content (>50%) and a high percentage (>60%) of peptides with low molecular weight (<3 kDa) are beneficial in PBBs used as an amendment to semi-arid soil [28].

Furthermore, PBBs have a positive impact on the metabolic processes of plants, as they enhance root and shoot growth, photosynthesis rate, and crop quality [3,12,22,29,30]. PBBs are reported to regulate biochemical processes that boost the tolerance of crops against abiotic stresses (drought, salinity, and heavy metals) [12]. They have also been found to stimulate nutrient uptake and nutrient use efficiency in crops, largely due to their growth-enhancing effects on roots [12]. In addition, PBBs can have indirect effects on plants by enhancing uptake and efficient use of macro- and micronutrients [12,30]. Most biostimulating effects have been linked to the presence of soluble peptides and free amino acids in PHs, which in many cases, act as precursors for the biosynthesis of phytohormones (plant-growth regulators) and other metabolically important bioactive compounds which then contribute to the plant-growth enhancement [3,8,22]. These soluble peptides and free amino acids are easily absorbed by soil microorganisms, which helps to improve soil structure, soil organic matter content, and nutrient availability [28].

Enhanced shoot and root growth have been reported, e.g., in kiwi and snapdragon plants to which PBBs were applied at a low dosage [26,31,32]. Increased coleoptile length in maize has been reported, although a relatively high concentration of PBBs was needed to obtain that effect [11]. Soy PBB incorporated into broccoli seed pellets has been found to enhance plant height [6]. Similarly, enhanced plant height and plant canopy area have been obtained in maize [33] and tomato [34] through the use of PBBs. Moreover, the use of PBBs has been found to enhance the biomass production of broccoli, maize, lettuce [6,30,35],

banana, and rocket [36,37]. In one study, plant height and total biomass of hibiscus plants were increased by applying PBBs from two urban biowaste materials [38].

In addition to the effect on plant growth, PBBs have been found to be involved in several molecular and physiological processes in plants [12]. For example, the nitrogen content in maize and cucumber plants has been found to be increased by treatment with plant-derived PHs and hydrolyzed collagen, respectively [39,40]. Furthermore, PBB treatment of maize has been shown to induce the secretion of enzymes involved in carbon and nitrogen metabolism [33,41].

Several studies have demonstrated that PBBs can improve crop tolerance to abiotic stresses [22], e.g., calcium protein hydrolysate has been found to reduce chloride uptake in Oriental persimmon (*Diospyros kaki* L.) [42]. Furthermore, gelatin-treated cucumber plants have been shown to exhibit higher salinity tolerance than untreated plants [43], while the foliar application of PH to lettuce can enhance the tolerance to low temperatures [44]. Paul et al. [45] observed an increased growth in tomatoes treated with PHs under drought stress. Others have observed a reduction in anti-nutritional content (nitrate) in leaves of lettuce treated with PH (both foliar and root application) compared with untreated plants [46]. The ability of plants to tolerate abiotic stresses following PH treatment has been attributed to genes being induced that contribute to enhanced growth, improved nutrient status, greater cell structure stability, osmolite and antioxidant accumulation, and enzyme activation by PHs [22].

Generally, the effect of PBBs depends on the source and characteristics of the PBB, the crop (species and cultivars) on which the PBB is utilized, the age or growth stage of the crop, growing conditions, PBB concentration, timing and mode of application (soil, seed, or foliar treatment), PBB solubility, and leaf permeability [22].

There have only been a few comparative studies on the efficiency of PBBs and other categories of biostimulants and chemical fertilizers [47–50]. One major principle of biostimulants is that they help to reduce the quantity of fertilizer required, rather than replacing chemical fertilizers [51]. Dudas [47] achieved enhanced growth and biochemical concentrations in lettuce by using biostimulants and fertilizer, compared with an untreated control. The specific effects of PBBs and other categories of biostimulants are largely based on the different bioactive components present in their molecules [49,50,52]. These specific effects include the enhancement of antioxidant content, antibiotic effect, abiotic tolerance, etc. [20]. The comparative efficiency of different categories of biostimulants in relation to chemical fertilizers can be established using different omics approaches [53].

Table 1. Reported effect on crop performance of different protein hydrolysates (PHs) used as protein-based biostimulants (PBBs).

SN	PBB	Effect	Source
1	PH, plant source	Improves yield and quality of perennial wall rocket	Caruso et al. [21] https://www.mdpi.com/2223-7747/8/7/208 Accessed 23 July 2022
2	PH, plant source	Enhances plant physiology and stimulates soil microbiome	Colla et al. [22] https://www.frontiersin.org/article/10.3389/fpls.2017.02202 Accessed 23 July 2022
3	PH, animal source (Pepton)	Improves salicylic acid and growth of tomato roots under abiotic stress	Casadesus et al. [23] https://www.mdpi.com/2223-7747/8/7/208 Accessed 23 July 2022
4	PH, animal source	Improves growth and microelement concentration in hydroponically grown maize	Ertani et al. [31] https://doi.org/10.1002/jpln.201200020 Accessed 23 July 2022

Table 1. *Cont.*

SN	PBB	Effect	Source
5	PH, plant source	Enhances growth and nitrogen metabolism of maize	Ertani et al. [33] https://doi.org/10.1002/jpln.200800174 Accessed 23 July 2022
6	PH, plant source	Improves agronomic, physiological and yield parameters of baby rocket plant	Di-Mola et al. [37] https://doi.org/10.3390/plants8110522 Accessed 23 July 2022
7	PH, plant source (Trainer)	Improves performance of maize and lettuce	Colla et al. [39] https://doi.org/10.17660/ActaHortic.2013.1009.21 Accessed 23 July 2022
8	PH, animal source (gelatin)	Improves plant performance	Wilson et al. [40] https://www.frontiersin.org/article/10.3389/fpls.2018.01006 Accessed 23 July 2022
9	PH, plant source	Enhances gene expression, enzymes and nitrogen metabolism in maize	Schiavon et al. [41] https://doi.org/10.1021/jf802362g Accessed 23 July 2022
10	Calcium, PH	Improves salinity tolerance and leaf necrosis in *Diospyros kaki* L.	Visconti et al. [42] https://doi.org/10.1016/j.scienta.2015.01.028 Accessed 23 July 2022

2.3. Social, Economic, and Environmental Aspects of PBBs

Through their direct and indirect effects on crop yield and quality, nutrient-use efficiency, and tolerance to biotic and abiotic stresses, PBBs have the potential to contribute to socioeconomic development [54–56]. First, the production and use of protein-rich side-streams from agro-industries create novel jobs and novel products, which in turn provide novel income opportunities [54]. The sustainable use of more side-streams from agro-industries contributes to (i) social development in societies involved in the business, (ii) economic development and growth through product development, and (iii) environmental benefits from more complete use of natural resources [56]. Farm income is increased due to increases in crop yield and quality resulting from use of PBBs [56,57]. Economic benefits from the use of plant-based biostimulants, due to the increase in the yield have been reported for a range of crops, including perennial wall rocket and lamb's lettuce [58,59]. However, the economic return of using plant side-streams for additional products is always decreasing as soon as an extra harvesting or processing step is introduced into their production [57]. Thus, a benefit of using PBBs from side streams of the food industry (e.g., wheat gluten or potato protein) is that these substances are readily available at a reasonable price from the industry [14]. Furthermore, the use of PBBs may lead to the production of healthier crops and more nutritious food, which will enhance the health of consumers [56]. PBBs might improve land-use efficiency by enhancing crop yield, quality, and profitability per acre [60].

The economic efficiency of various types of biostimulants has been limitedly evaluated. Most studies report a certain increase in crop yield or plant development, often given in % of increase as related to a control treatment. For PBBs, the comparison of effects between different types or to other types of biostimulants, e.g., other biological, chemical or fertilizer compounds, is mainly lacking; although, a high effect has been reported in few studies [61].

The use of PBBs may also lead to the production of healthier crops and more nutritious food, improving consumer health [56]. Additionally, PBBs may improve land use efficiency by enhancing crop yield, quality, and profitability per acre [58].

The use of biostimulants has been proven to have positive effects on the environment by improving the nutrient-use efficiency of crops, thereby reducing the quantities of agrochemicals needed in food production by up to 50% [3,4]. PBBs also improve soil

health, by boosting the communities of beneficial soil microorganisms present [54] and by strengthening soil structure and increasing soil water-holding capacity, thus preventing soil erosion [55]. The small quantity of biostimulants required for crop growth and development improvements means that there are no residues left in crops and soil [2,37]. There is, thus, a limited risk of PBBs causing environmental problems in food, soil, or water bodies [2,37,62–66]. The fact that most PBBs are highly biodegradable also results in the safety of life on land and in water [3,9]. Thus, the use of PBBs could result in improved surface water quality and lower carbon emissions [29].

The recycling and conversion of protein-rich wastes or side streams products from agriculture and agro-allied industries into PBBs, pave the way for a more resilient use of natural resources [3,67–71]. The food industry is one of the major contributors to greenhouse gas emissions contributing to climate change, and the increased use of side streams from food production is seen as important to mitigate climate change [68]. This leads to a strong focus in the plant biologicals industries to continue to develop novel natural active ingredients (biostimulants) from agro-industrial wastes [70].

3. Hydrolyzed Wheat Gluten (HWG) and Potato Protein (PP) as Possible PBBs

3.1. Hydrolyzed Wheat Gluten (HWG)

Wheat gluten is defined as the rubbery mass of proteins, obtained when wheat flour is washed with water to remove starch and other water-soluble components [72–74]. Wheat gluten is available in large quantities and at low cost as a result of large-scale industrial starch extraction from wheat flour [71–80]. Some industrially produced gluten is used as a co-product for several purposes, e.g., within the baking industry [78]. However, the quantities of wheat gluten produced leave much scope for additional uses [72,81–83]. To increase the applicability of wheat gluten, structural modification to enhance its functional properties is often required [71,72], as it is highly polymerized in its native state [14,74,77,81]. The most common way to modify its structure is by enzymatic or thermal treatment or chemical hydrolysis, or a combination of these processes [71,72,78].

Like many other plant PHs, HWG has a wide set of applications in the food industry, particularly as an ingredient since it resembles glutamate in terms of taste [71,78]. As HWG is a hydrolyzed protein-rich co-stream from starch production, it most likely (based on the above discussion) has properties that make it suitable as a biostimulant within agriculture and horticulture [72]. However, to our knowledge, HWG has, until now, not been evaluated as a source to be used in agricultural applications. Similar to other PHs (biostimulants), the hydrolysis of wheat gluten results in a breakdown of the protein into peptides and a large amount of free amino acids, which are beneficial for plant growth and health [71,76–80].

3.2. Potato Protein (PP)

Potato fruit juice (PFJ) is a massive protein-rich side-stream generated in starch extraction from potatoes [14]. In 2018, the amount of PFJ obtained after starch extraction represented around ~1% (3.5 million tons) of the total global potato production (>360 million tons) [81,84,85]. In the past, PFJ was regarded as waste and was released into nearby streams and other water bodies, resulting in environmental pollution [82,83]. However, potato protein (PP) is a potentially valuable product that can be produced from PFJ through acidification and harsh thermal processing [14,86]. These processes result in intensive coagulation and protein recovery [14,82]. In theory, a total of 200,000 tons of PP could be generated from the 3.5 million tons of PFJ made available annually worldwide [73,79]. Some studies have indicated that PP is one of the largest under-utilized agro-industrial protein-rich side-streams in the world [14]. PP has the potential to act as a ready source of organic nitrogen for crops, as the protein content of PP is >80% [14]. A sustainable way of using PP would be through its application as a PBB. To our knowledge, PP has been limitedly evaluated for its use in agriculture, although, trials to use it as a functional food component are ongoing [87,88].

4. Biostimulating Effect of HWG and PP; Sugar Beet as an Example

A range of bioactive molecules, such as biochar, humic and fulvic acids, chitosan, phosphites, essential amino acids, soil bacteria, phytoextracts, and extracts of algae or other plant parts, have been evaluated as biostimulants on sugar beet as well as other crops [6,89–91]. In a previous study by our research group, we observed a biostimulating effect of HWG and PP on young sugar beet plants (cultivars Volga, Armesa, and Mustang) when applied to soil in different concentrations (0–10 g/kg soil) [61].

The results we obtained for sugar beet showed slight variations in genotypic responses to HWG and PP treatment (1–10 g/kg) [84], but in most cases, we observed the enhancement of plant growth (plant height, fresh weight, plant canopy area) compared with an untreated control (Table 2, Figure 2). Applying lower concentrations (1 and 2 g/kg soil) of either HWG or PP resulted in the tallest plants across all three cultivars of sugar beet tested (Table 2). The HWG and PP treatments also increased the total fresh weight of the three sugar beet cultivars [61], with an increase of 88–150% compared with the control across all three cultivars (Table 2). As seen for plant height, the greatest increases in fresh weight were obtained for application rates of 1 and 2 g/kg of soil [61]. Furthermore, HWG and PP at 1 or 2 g/kg enhanced the plant canopy area of all three cultivars compared with the other doses tested (Figure 2). Thus, an enhancing effect on early growth and establishment of young sugar beet plants was achieved at a relatively low concentration of PBB, irrespective of whether the PBB was added as bottom-dressing or as a soil mixture [61]. The increase in growth of the young sugar beet plants following the use of PBB was substantial and well in accordance with the effects of other types of biostimulants used on other crops [61]. The decrease in plant height, plant canopy area, and fresh weight of sugar beet observed in PBB treatments (HWG and PP) at higher concentrations (5 and 10 g/kg of soil) might have been due to toxicity effects due to high N concentrations from the PBBs.

Table 2. Plant height and total fresh weight of sugar beet cultivars (Volga, Armesa, Mustang) treated with hydrolyzed wheat gluten (HWG) and potato protein (PP). Source: Jolayemi et al. [61].

	Conc. (g/kg)	Sugar Beet Plant Height (cm)			Total Fresh Weight (g/plant)		
		Volga	Armesa	Mustang	Volga	Armesa	Mustang
Control	0	7.6d	6.9c	6.7c	2.43d	2.47c	3.13e
HWG	1	15.5a	13.3a	15.2a	7.00a	6.77a	10.25b
	2	15.2a	13.2a	13.2a	6.11b	7.30a	11.96a
	5	11.6b	9.1b	8.8b	3.92c	3.04b	4.24d
	10	8.2c	7.0bc	10.4b	1.26e	3.54b	5.25c
	Mean	12.6a	10.6b	11.9a	4.57c	5.16b	7.92a
PP	1	13.8a	14.4a	14.4a	6.03a	8.51a	9.15a
	2	14.8ab	12.9b	14.4a	6.17a	6.19b	9.55a
	5	10.5c	8.4c	11.1b	3.17c	4.12d	5.12b
	10	11.7bc	9.7c	10.6b	4.11b	4.69c	4.41c
	Mean	12.7a	11.4b	12.6a	4.87c	5.88b	7.06a

Each value is an average of five plants. Means were separated using Tukey's method. Means within columns with different superscript letters differ significantly ($p < 0.05$).

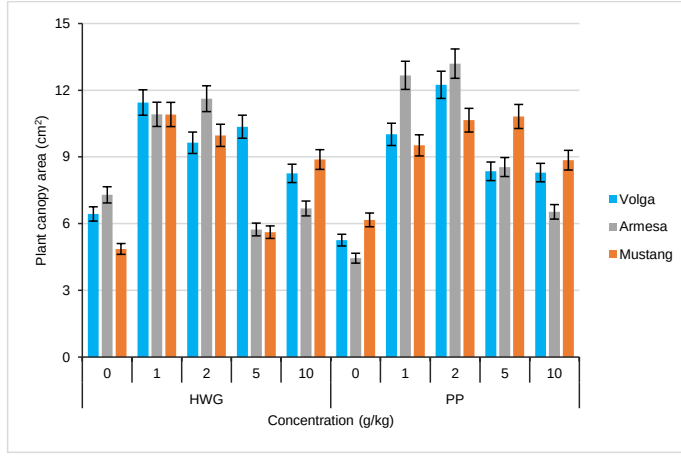

Figure 2. Effect of varying concentrations of hydrolyzed wheat gluten (HWG) and potato protein (PP) on plant canopy area in the sugar beet cultivars Volga, Armesa, and Mustang.

PBBs are known to consist of a mixture of peptides and amino acids [2], which should also be the case for HWG, a hydrolyzed protein source [70]. On the other hand, PP is known to contain 40% patatin, which is mainly water-soluble [14] and is known to aggregate due to the harsh treatment during PJI fractionation into PP [81]. Since PBBs normally contain N-rich sources such as peptides and amino acids, part of their growth-enhancing effect might be explained by the extra N supply they contribute to plants. However, part of their effect has also been attributed to their hormone-like activities [8]. The low molecular weight forms of organic N obtained from PBBs are easily taken up by plant roots, and then used by the plant as precursors in the biosynthesis of plant hormones that stimulate plant growth and development [22]. In our previous study [61], we compared the enhancing effect of HWG and PP on sugar beet with that of a nutrient solution with a comparable amount of N as in those two PBBs. Although the nutrient solution enhanced plant growth, it did not do so to the same extent as the PBBs, so the effect of the PBBs on sugar beet growth could not be explained solely by increased nutrient supply [61]. For a fuller understanding of the background and reasons for the growth-enhancing effects of PBBs such as HWG and PP, additional research needs to be carried out.

Furthermore, no studies have evaluated the long-term effect of HWG and PP as biostimulants for yield and sugar content in sugar beet. Such studies should be carried out to evaluate the full potential of the use of HWG and PP on sugar beet. However, early and strong plant development indicates a potential for high yield in a crop.

5. Opportunities and Future Directions for Use of PBBs

Having established the benefits of PBBs for crop growth and environmental safety, innovative solutions for their production, use, and application are needed [3,12,28]. One application of PBBs (as with other biostimulants) is use in seed treatment techniques such as seed coating, seed pelleting, and seed priming. Crops with small seeds can be primed, coated, or pelleted with PBBs, for enhanced establishment and productivity [92]. However, a more innovative way to use PBBs would be to develop superabsorbent polymers (SAPs) in a sustainable approach that could also help tackle the problems of abiotic stresses such as drought and soil nutrient issues [93]. SAPs, which can be either natural or synthetic, have the ability to swell in an aqueous solution by retaining water in their network and do not dissolve in water [86,92–96]. There are many potential areas of application for SAPs, due to their high water-absorbing capacity, rapid biodegradability, and low cost [86]. Synthetic

SAPs (usually from petroleum resources) are widely utilized due to their high absorption capacity, availability in a wide variety of raw materials, and long-lasting durability, but they are non-biodegradable [76,93]. Recent studies have shown that SAPs can be produced from agro-industrial protein side-streams using HWG and PP with a non-toxic dianhydride (EDTAD) [14]. This method is sustainable and eco-friendly, because it ensures the delivery of nutrients (peptides and amino acids from the protein), while water molecules are retained in the SAP network [77]. Therefore, the development of SAPs from agro-industrial wastes or side-streams is a promising future option. These products are eco-friendly, as they are developed using climate-smart technology and non-toxic components and can ultimately address the problems of soil nutrient and water deficits [70,77] (Figure 3).

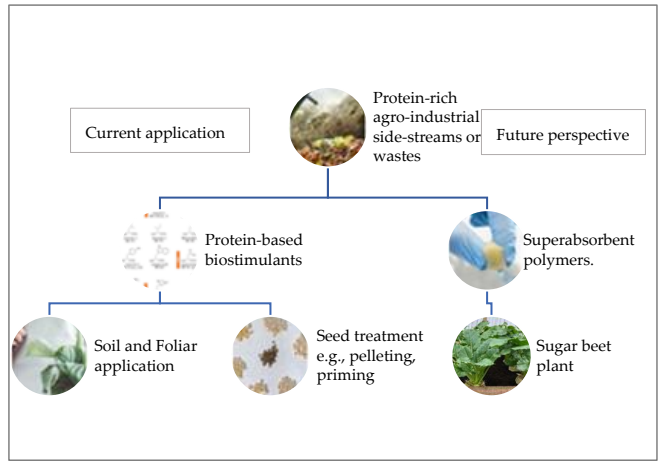

Figure 3. Chart illustrating current and possible future directions for the use of protein-based biostimulants (PBBs) developed from agro-industrial side streams.

6. Conclusions

The production of PBBs from agro-industrial wastes or side-streams is a sustainable way of addressing the problems of waste disposal and environmental pollution resulting from waste generation. PBBs can improve the agronomic and physiological performance of a wide variety of crops, as confirmed by metabolic and molecular data. They can also improve crop quantity and quality under different environmental conditions. Overall, PBBs have social, economic, and environmental benefits, by providing extra sources of income to agro-industries and other players along the value chain and improving soil structure. In socioeconomic terms, the costs of producing PBBs are covered by the higher crop yields obtained at harvest, although further cost–benefit analyses are required. The benefits of PBBs for the environment, apart from improvement of soil structure, include boosts to microbial communities and prevention of soil erosion. Tests on HWG and PP as possible PBBs for sugar beet crops have revealed greater plant height, plant canopy area, and biomass. PBBs are currently applied directly to soil or plant foliage, or through seed treatments such as priming, coating, and pelleting. A new future direction for the use of PBBs would be the development of SAPs to ensure the delivery of organic nitrogen (in the form of peptides), amino acids, and water to crop plants under normal and stressed conditions.

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Metabolic Processes and Biological Macromolecules Defined the Positive Effects of Protein-Rich Biostimulants on Sugar Beet Plant Development

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Abstract: Protein-based biostimulants (PBBs) have a positive effect on plant development, although the biological background for this effect is not well understood. Here, hydrolyzed wheat gluten (HWG) and potato protein film (PF) in two levels (1 and 2 g/kg soil) and in two different soils (low and high nutrient; LNC and HNC) were used as PBBs. The effect of these PBBs on agronomic traits, sugars, protein, and peptides, as well as metabolic processes, were evaluated on sugar beet in comparison with no treatment (control) and treatment with nutrient solution (NS). The results showed a significant growth enhancement of the plants using HWG and PF across the two soils. Sucrose and total sugar content in the roots were high in NS-treated plants and correlated to root growth in HNC soil. Traits related to protein composition, including nitrogen, peptide, and RuBisCO contents, were enhanced in PBB-treated plants (mostly for HWG and PF at 2 g/kg soil) by 100% and >250% in HNC and LNC, respectively, compared to control. The transcriptomic analysis revealed that genes associated with ribosomes and photosynthesis were upregulated in the leaf samples of plants treated with either HWG or PP compared to the control. Furthermore, genes associated with the biosynthesis of secondary metabolites were largely down-regulated in root samples of HWG or PF-treated plants. Thus, the PBBs enhanced protein-related traits in the plants through a higher transcription rate of genes related to protein- and photosynthesis, which resulted in increased plant growth, especially when added in certain amounts (2 g/kg soil). However, sucrose accumulation in the roots of sugar beet seemed to be related to the easy availability of nitrogen.

Keywords: agro-wastes; protein-based biostimulants; hydrolyzed wheat gluten; potato protein; sugar beet; growth; physiology and transcriptomic analysis

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1. Introduction

Biostimulants are described as bioactive substances that are either organic, inorganic, or microorganisms, which can improve crop performance when applied in small quantities [1–3]. Because biostimulants are able to enhance the growth and performance of crops [1,4], increased attention has been seen recently in utilizing them in agricultural and horticultural applications and productions. Reports on biostimulants have indicated their positive impact on crop performance in terms of significant increases in growth and metabolic processes, resulting in increased yield, nutrient- and water-use efficiencies, and

tolerance to abiotic stresses [2,4–7]. Of the different categories of biostimulants, protein-based biostimulants (PBBs), which are also known as protein hydrolysates and amino acids, have received increased interest lately [5]. The PBBs are normally highly available due to the abundance of their raw materials, and thereby the cost of assessing the raw materials and final product is often reasonable [8,9]. The raw materials utilized for the production of PBBs are usually protein-rich wastes, which are generated from agro-allied industries [10]. In some cases, such agro-wastes may also need to find other routes of use, to not end up being dumped into rivers or used as landfills, thereby contributing to environmental pollution [11]. Possible alternative uses of protein-rich residuals from agro-allied industries are in applications in material sciences [12,13] and bioenergy production [11], while they might also be developed into PBBs. Bio-based uses of protein-rich residues from agro-allied industries hold opportunities to result in eco-friendly and sustainable solutions [14], although their economic and environmental effects always need to be properly evaluated [15–18].

As described above, PBBs are often derived through the process of hydrolysis of protein-rich agro-wastes [8,19]. This hydrolysis process (chemical, thermal, enzymatic, or a combination of any of them) contributes to the breakdown of large protein molecules into smaller and more soluble entities [19,20]. The hydrolysis process eventually leads to a mixture of different types of molecules, including peptides and amino acids [8], which are then the main active ingredients in the PBB products [21,22]. Thus, when evaluating the effects of PBBs, it is important to understand effects based on an increased level of N available for the plants, derived from the peptides and amino acids, and of biostimulating effects of other origins [5].

Recent studies have, to an increasing degree, tried to understand the background of the biostimulating effects of PBBs on growth and physiological improvement in crops [23–25]. However, despite the fact that measurements of changes in metabolic processes are required to understand the background effects of PBBs, most studies till now have focused mainly on physiological changes [5]. Two PBBs that have been reported to have a biostimulating potential are hydrolyzed wheat gluten (HWG) and potato protein film (PF) [5]. These are both protein-rich streams from the wheat and potato starch industry, respectively [13]. Currently, as for most PBBs, there are no data on metabolic responses to support the physiological effect of HWG and PF on crop growth. Consequently, to improve the understanding of the effects of the use of PBBs and their biostimulating effect, their mode of action in terms of metabolic changes needs to be further evaluated and characterized.

Thus, the aim of the present study was to evaluate the effects of PBBs, i.e., HWG and PF, on the growth and physiological traits of sugar beet. Further, the aim was also to connect the changes in growth and physiological traits to changes in protein and sugar content and composition in the plants and metabolic responses through transcriptomic analysis.

2. Results

2.1. Effect of Treatments on Agronomic and Physiological Parameters

At low nutrient soil conditions (LNC), the samples treated with only nutrient solution (NS) were differentiated from the other samples by principal component analysis (PCA). The NS treatment is located on the negative axis of the first principal component (PC1; Figure 1A), indicating high sucrose and total sugar content in the roots (factors with a negative PCA value) and low values on the other parameters (factors with a positive value; Figure 1B). The high sugar and sucrose content in the NS samples at LNC and low values on the other parameters was also verified by mean values differentiated by Tukey's posthoc test (Table 1). No clear differentiation was observed based on the rest of the treatments (Figure 1), which was also verified by a large variation in early plant growth influenced by the different biostimulant treatments (Figure 1B, Table S1). However, the control and NS treatment generally resulted in the least plant growth for the three evaluated genotypes of sugar beet (Table S1).

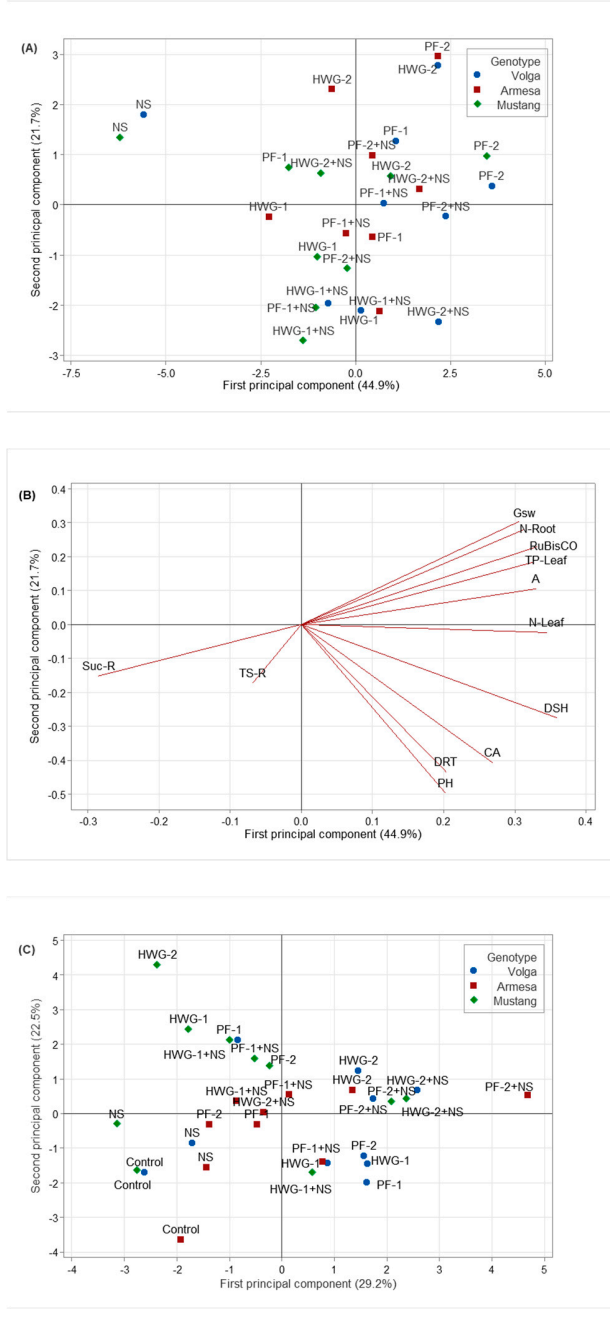

Figure 1. *Cont.*

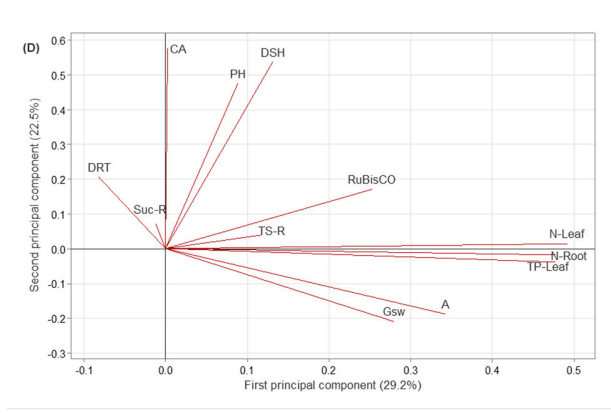

Figure 1. Principal component analysis of all agronomic and physiological parameters of three sugar beet genotypes under PBB and/or nutrient solution treatments. (**A**,**C**) Score and (**B**,**D**) loading plot from PCA of treatments under (**A**,**B**) low nutrient-rich soil conditions (LNC) and (**C**,**D**) high nutrient-rich soil conditions (HNC). PH: plant height, CA: canopy area, A: photosynthetic carbon assimilation rate, Gsw: stomatal conductance, DSH: dry shoot mass, DRT: dry root mass, TP: total peptide in leaf, NLeaf: nitrogen content in leaf, N-Root: nitrogen content in root, Suc-R: sucrose content in root, TS–R: total sugar content in root.

Additionally, at high nutrient soil conditions (HNC), control and NS samples were differentiated (with negative PC1 and PC2 values) from the biostimulant treated samples by PCA (Figure 1C), indicating low values on all the parameters analyzed (Figure 1D). Additionally, these results corresponded with an increased physiological plant development of plants treated with biostimulants (Table S1). Similarly, as for the LNC, the best biostimulant treatments varied in relation to sugar beet genotypes and plant character evaluated (Figure 1, Table S1).

2.2. Impact of PBB Treatments on Photosynthesis and Content of Nitrogen, Peptide, RuBisCO, and Sugar in Roots and Leaves

The photosynthesis capacity of sugar beet, measured as photosynthetic carbon assimilation, stomata conductance, and chlorophyll fluorescence, was generally low in control and NS samples (Table S1). High nitrogen content in leaves and roots was mostly found in HWG–2+NS, PF–2, and PF–2+NS samples for all three genotypes and under both LNC and HNC, although with some variation (Table 1). The nitrogen content in roots and leaves was generally low in control and NS samples (Table 1). The total peptide and RuBisCO content in the leaves were generally high in the HWG–2+NS, PF–2, and PF–2+NS samples and low in control and NS samples for the three genotypes and under both cultivation conditions, although with the exception of high total peptide content in Volga and Mustang under NS at HNC (Table 1). Differently from the N-related compounds, the sugar contents in the root, especially the sucrose content, were high in NS samples of all three genotypes under both growing conditions (Table 1).

Table 1. Physiological traits of sugar beet under PBB treatments in different soil nutrient conditions.

Genotype	Treatment	Low Nutrient Condition						High Nutrient Condition					
		N-Leaf	**N-Root**	**TP-Leaf**	**RuBisCO**	**Suc-R%**	**TS–R%**	**N-Leaf**	**N-Root**	**TP-Leaf**	**RuBisCO**	**Suc-R%**	**TS–R%**
Volga	Control	0.9h	1.0f	43.1e	9.5f	0.5d	1.0e	1.7d	0.8d	61.0g	13.7g	0.2g	3.3e
	HWG–1	1.8e	1.4d	44.8e	30.4e	3.0b	5.9a	3.4a	1.3b	57.7g	38.6d	2.0a	7.6b
	HWG–1+NS	1.4f	0.9f	57.8d	49.9d	3.2b	6.4a	2.4c	0.8d	149.9b	44.4c	0.4e	8.0b
	HWG–2	3.4a	2.7a	52.2e	75.3c	0.4d	3.8d	3.3a	1.2b	84.1f	36.0d	0.2g	9.8a
	HWG–2+NS	2.8b	1.4d	107.4a	85.0b	0.9c	6.4a	3.5a	1.4a	137.7c	52.2b	0.3f	3.9e
	NS	0.7h	1.1e	68.8c	8.0g	3.8a	5.3b	1.6d	1.0c	161.1a	23.5f	1.7b	8.2b
	PF–1	2.0d	2.0c	78.0b	52.4d	0.2e	5.1b	2.8b	1.3ab	126.5d	31.1e	0.6d	6.8c
	PF–1+NS	2.1d	1.9c	81.6b	76.6c	0.5d	5.8a	2.9b	1.3a	107.2e	53.6b	1.6c	5.3d
	PF–2	2.5c	2.5b	108.3a	136.1a	ND	ND	3.2ab	1.4a	137.0c	51.2b	1.8b	5.7d
	PF–2+NS	2.8b	2.9a	118.5a	74.1c	0.3e	4.5c	3.3a	1.3ab	139.6c	67.0a	1.8b	8.2b
Armesa	Control	1.2f	1.0g	33.2d	8.1g	2.2b	3.0d	1.6e	0.7f	52.2e	22.8h	4.6c	4.3c
	HWG–1	1.5e	1.0g	32.8c	11.9f	3.6a	7.1ab	2.8c	1.0d	58.0d	110.1c	4.3c	6.9ab
	HWG–1+NS	2.2d	1.3f	40.7c	12.5f	0.6c	7.7a	2.1d	0.9e	94.7b	121.9b	6.2ab	7.1a
	HWG–2	1.5e	1.0g	54.0b	88.3b	ND	ND	3.1b	1.0d	63.6c	72.2d	6.8a	7.6a
	HWG–2+NS	2.9b	2.2b	64.1a	86.1b	0.3e	4.9c	1.6e	1.0d	93.3b	68.7e	5.6b	6.3b
	NS	0.9g	0.7h	64.9a	3.3h	3.4a	5.3c	2.1d	0.9e	50.5e	70.4d	6.7a	7.3a
	PF–1	2.2d	1.4d	55.6b	43.5d	0.6c	6.5b	2.8c	1.1c	87.1c	69.9de	6.5a	7.3a
	PF–1+NS	2.1d	1.3e	56.5ab	28.9e	0.4d	6.6b	2.9bc	1.3b	51.6e	29.4g	5.9b	6.8ab
	PF–2	3.3a	3.0a	68.6a	133.7a	NS	ND	2.1d	1.0d	56.3d	66.1f	3.8d	6.5b
	PF–2+NS	2.4c	1.7c	57.2b	71.5c	0.6c	6.0b	4.7a	1.8a	184.4a	237.2a	6.1b	7.4a

Table 1. *Cont.*

Genotype	Treatment	Low Nutrient Condition						High Nutrient Condition					
		N-Leaf	N-Root	TP-Leaf	RuBisCO	Suc-R%	TS–R%	N-Leaf	N-Root	TP-Leaf	RuBisCO	Suc-R%	TS–R%
Mustang	Control	1.0d	0.8f	36.7h	4.3h	ND	ND	1.7f	0.9d	49.5f	36.6e	3.3e	6.0e
	HWG–1	3.2b	0.7g	41.8g	34.6e	2.7d	3.7d	2.1e	1.0d	48.3f	88.2c	6.2b	7.4c
	HWG–1+NS	2.1c	0.6h	54.9d	16.9g	2.7d	3.6d	2.8c	1.2b	60.7e	14.2g	6.0b	7.9b
	HWG–2	2.0c	2.3a	45.9f	96.0b	1.8e	4.7c	1.6fg	0.8e	87.5d	10.8g	4.4d	5.9e
	HWG–2+NS	2.1c	0.6h	72.7b	23.7f	ND	ND	3.8a	1.3a	42.6g	164.2b	4.0d	8.5b
	NS	0.8e	0.6i	49.5e	4.8h	3.9b	8.2a	1.5g	0.8e	142.9a	20.2f	4.2d	7.1c
	PF–1	2.2c	1.3d	52.0e	37.8d	4.9a	6.2b	1.2h	1.0cd	39.7h	168.0ab	5.0c	6.0e
	PF–1+NS	2.0c	0.9e	48.1f	18.9g	3.5c	4.7c	2.4d	1.1c	58.6d	165.0b	4.3d	7.0d
	PF–2	4.5a	1.9b	97.4a	144.8a	1.4f	4.1d	3.1b	1.0d	113.2b	52.8d	2.9f	7.0d
	PF–2+NS	2.7c	1.5c	66.2c	87.9c	3.5c	5.0c	3.5a	1.3a	105.0c	173.5a	9.5a	44.6a

N-Leaf: nitrogen content in leaf, N-Root: nitrogen content in root, TP-Leaf: total peptide in leaf, Suc-R: sucrose content in root, TS–R: total sugar content in root, RuBisCO: content of Ribulose-1,5-bisphosphate carboxylase/oxygenase, ND: not determined. Means are calculated from 3 replicates and separated using Turkey's posthoc test at $p < 0.05$. Means followed by the same letter along the column are not significantly different. HWG: hydrolyzed wheat gluten, PF: potato protein film, -1: 1 g/kg, -2: 2 g/kg, +NS, in combination with nutrient solution.

The Pearson correlation analysis revealed a higher degree of correlation between different parameters for LNC than for HNC (Table 2). In principle, a positive and significant correlation was found among all photosynthetic, agronomic, and nitrogen-related parameters for LNC (Table 2). However, sucrose and total sugar content correlated significantly and negatively with other measured parameters (Table 2). For HNC, significant and positive correlations were found among some agronomic parameters (PH, CA, DSH, and DRT), as well as among photosynthetic parameters (A, Gsw) and some of the nitrogen-related parameters (N-L, N-R, and TPL) (Table 2). In HNC, we also observed significant and positive Pearson correlations between Suc-R, DRT, and RuBisCO (Table 2).

Table 2. Correlation analysis of the effect of PBB treatment on sugar beet agronomic and physiological traits, with LNC below the diagonal and HNC above the diagonal.

		HNC												
		PH	**CA**	**A**	**Gsw**	**CF**	**DSH**	**DRT**	**N-L**	**N-R**	**RuBisCO**	**Suc-R**	**TS–R**	**TP–L**
LNC	PH	1.00	0.80 ***	−0.06	−0.13	0.19	0.67 ***	−0.15	0.09	0.12	0.08	−0.24	−0.14	0.14
	CA	0.87 ***	1.00	−0.15	−0.19	0.06	0.82 ***	0.30	−0.03	−0.07	0.18	0.03	−0.05	−0.07
	A	0.20	0.30	1.00	0.83 ***	−0.08	−0.01	−0.09	0.41 *	0.43 *	0.04	−0.03	0.01	0.44 *
	Gsw	−0.09	0.10	0.84 ***	1.00	−0.11	−0.09	0.06	0.31	0.27	0.12	0.10	−0.11	0.32
	CF	0.55 **	0.42 **	0.33	0.36 *	1.00	0.15	−0.39 *	−0.13	0.02	−0.10	−0.28	−0.06	−0.03
	DSH	0.87 ***	0.87 ***	0.56 **	0.37 *	0.52 **	1.00	0.31	0.24	0.15	0.21	0.06	0.08	0.14
	DRT	0.81 ***	0.77 ***	0.32	0.05	0.51 **	0.81 ***	1.00	−0.05	−0.29	0.17	0.46 *	0.06	−0.21
	N-L	0.62 ***	0.64 ***	0.53 **	0.50 ***	0.46 *	0.74 ***	0.45 *	1.00	0.84 ***	0.39 *	−0.02	0.25	0.91 ***
	N-R	0.22	0.31	0.55 **	0.67 ***	0.34	0.48 **	0.24	0.55 **	1.00	0.45*	0.00	0.17	0.79 ***
	RuBisCo	0.37 *	0.43*	0.47 **	0.61 ***	0.22	0.65 ***	0.28	0.72 ***	0.76 ***	1.00	0.47 **	0.33	0.26
	Suc-R	0.00	−0.22	−0.53 **	−0.54 **	−0.15	−0.19	−0.06	−0.31	−0.41 *	−0.36	1.00	0.41 *	−0.32
	TS–R	0.36 *	0.35	−0.17	−0.14	0.03	0.31	0.36 *	0.10	0.18	0.02	0.40	1.00	0.09
	TP–L	0.41 *	0.48 **	0.40 *	0.52 **	0.49 **	0.61 ***	0.40 *	0.66 ***	0.82 ***	0.77 ***	−0.41 *	0.07	1.00

PH: plant height, CA: canopy area, A: photosynthetic assimilation rate, Gsw: stomata conductance, CF: chlorophyll fluorescence, DSH: shoot dry mass, DRT: root dry mass, N-L: nitrogen content in leaf, N-R: nitrogen content in root, RuBisCO: content of Ribulose-1,5-bisphosphate carboxylase/oxygenase Suc-R: sucrose content in root, TS–R: total sugar content in root, TP–L: total peptide in leaf. Values are correlation coefficient R, *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

2.3. Impact of Amount of N on Agronomic and Physiological Performances

As the above-evaluated PBBs and NS differed in total N content, an extra experiment was performed, adding them in ratios so that the plants received an equal amount of N from them under HNC, to further reveal their biostimulating effects (Figure 2). As the purpose was to compare effects from different treatments holding the same N content, no PBBs+NS treatments were carried out. The score plot of the PCA differentiated the samples along PC1 based on the amount of N added (control samples with lower N aligned on the negative axis of PC1, while PBB and NS samples with an equal amount of N aligned on the positive axis of PC1) (Figure 2A). This indicated that an increase in nitrogen content as a result of PBB and NS treatments enhanced all agronomic and physiological parameters of sugar beet (Figure 2A,B, and Figure 3). Treatment types (NS versus biostimulants) were differentiated along PC2, explaining 20% of the variation (Figure 2A). Thus, the score plot revealed that the NS treatment (blue circle) favored root traits and the number of leaves (Figure 2B), while PBB treatments enhanced above-ground traits as well as physiological parameters (Figure 2A,B).

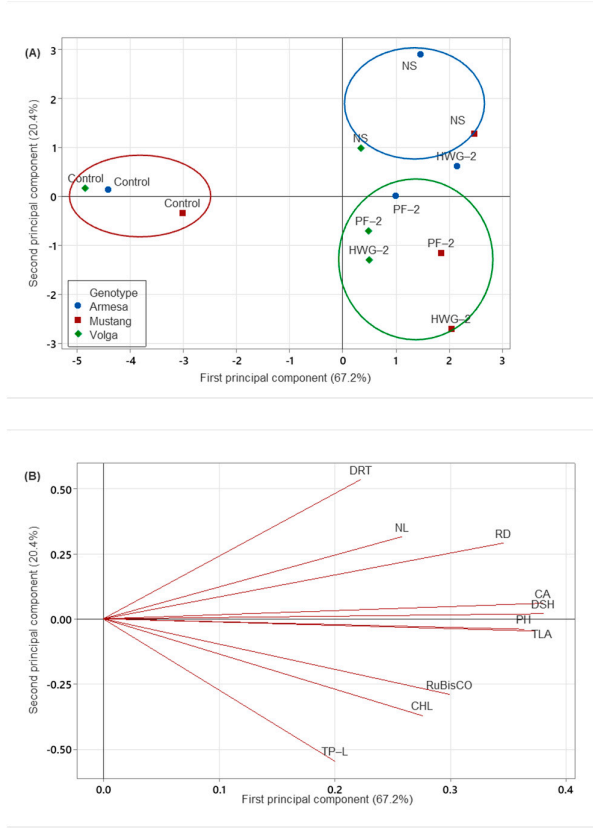

Figure 2. (**A**) Score plot and (**B**) loading plot from PCA of agronomic and physiological parameters of three sugar beet genotypes under equal nitrogen treatment from PBB and NS. CHL: chlorophyll concentration, PH: plant height, CA: canopy area, TLA: total leaf area, DSH: shoot dry mass, RD: root diameter, NL: number of leaves, DRT: dry root mass, TP–L: total peptide in leaf, RuBisCO: content of Ribulose-1,5-bisphosphate carboxylase/oxygenase. Treatments in blue (NS) and green (HWG–2 and PF–2) circles promoted growth parameters and physiological parameters respectively better than treatment in red (Control).

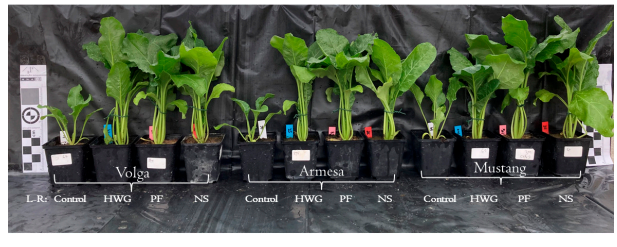

Figure 3. Plant height of three sugar beet genotypes under equal N from HWG, PF, and NS eight weeks after planting.

2.4. Differential Expression of Genes in Leaf and Root Samples of Sugar Beet Treated with PBBs

The transcriptomic analysis carried out based on the use of PBBs on sugar beet revealed large differential gene expressions. In total, 14,000–16,000 genes in both leaf and root samples were differently expressed (DE) compared to the control as a result of the PBB treatments (Table 3). The change in the expression of genes resulted in either an up- or a down-regulation of genes, and the present study clearly showed that a higher number of genes were down- than upregulated by the PBBs, especially in the root (Table 3). Furthermore, the change in expression of genes was generally higher in the roots as compared to in the leaves. Although the use of HWG resulted in a greater change in the expression of genes in the leaves than the use of PF, the opposite was found in the roots, where a greater change was obtained for PF than for HWG (Table 3, Figure 4).

Table 3. Total of 24,255 CDS sequences were processed and used for Differentially Expressed Gene (DEG) analysis with two thresholds set. One is only on FDR < 0.05, and another one is on both FDR < 0.05 and Log2FoldcChange (Log2FC) > 1.0. The total number of genes DE for these two cut-offs is shown below. One with a stringent threshold (FDR < 0.05 and log2FC > 1) and another one without a stringent threshold (FDR < 0.05 and no log2FC cut-off was set). These data are available in Supplementary Table S2.

Comparisons	Leaf		Root	
	HWG vs. Control	PF vs. Control	HWG vs. Control	PF vs. Control
Total DE genes	14,320	14,193	15,258	15,280
Number of genes (P5e-2_C0)	4525	2437	7448	8441
Number of genes (P5e-2_C1)	906	409	1756	2693

P5e-2_C0: number of genes down-regulated, P5e-2_C1: number of genes upregulated.

The down-regulation in the roots of genes associated with the biosynthesis of secondary metabolites was the most obvious change for both PBBs used as compared to the control, accounting for 50–60% of the down-regulated genes (Figure 4). Other genes that were down-regulated for both PBBs were some genes associated with specific secondary metabolites (tropane, piperidine, and pyridine) and genes associated with glutathione and galactose metabolisms.

Furthermore, when HWG was used, a down-regulation was found of genes associated with biosynthesis or metabolism of some amino acids (glycine, serine, threonine, valine, leucine, isoleucine, beta-alanine, aspartate, glutamate, cysteine, methionine, and zeatin), and of some fatty acid and respiration-related genes (Figure 4a). For PF, additional genes down-regulated in the roots included those involved in protein processing in the endoplasmic reticulum and plant-pathogen interaction genes (Figure 4b).

The most clearly upregulated genes were those associated with the ribosomes in both leaves and roots for the HWG-treated plants, while only in the leaves for the PF-treated plants (Figure 4). Additional upregulated genes for both HWG- and PF-treated plants were those for photosynthesis (Figure 4). Further, genes associated with cutin, suberin, and wax biosynthesis, tryptophan metabolism, and diterpenoid biosynthesis in the leaves and phenylpropanoid biosynthesis in the roots were upregulated for HWG-treated plants (Figure 4a). However, genes associated with the biosynthesis of aromatic essential amino acids (phenylalanine, tyrosine, and tryptophan), peroxisome as well as butanoate metabolism were upregulated in the leaves, and genes associated with DNA replication, mismatch repair, nucleotide excision repair, N-glycan biosynthesis, and base excision repair were upregulated in the roots of PF-treated plants (Figure 4b).

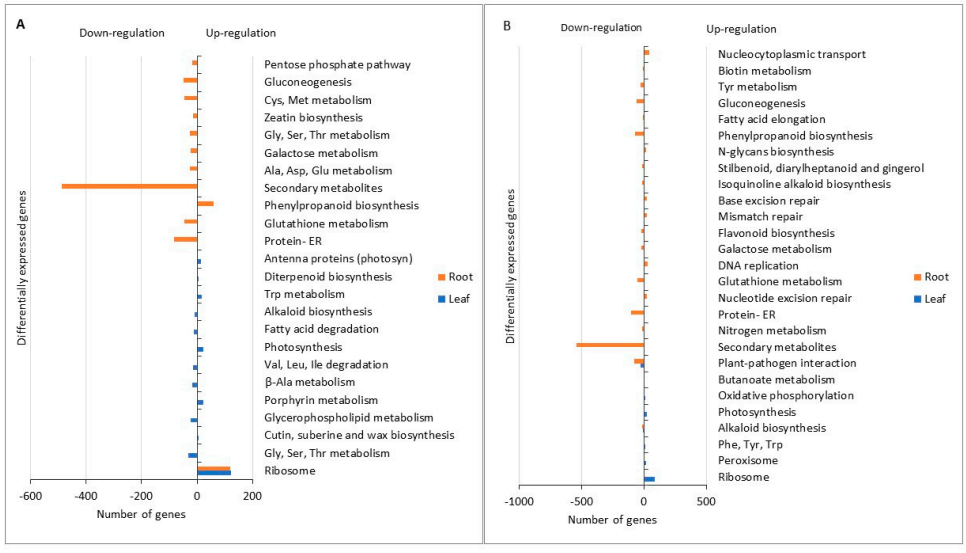

Figure 4. Metabolic processes determined by transcriptomic analysis showing differentially expressed genes from sugar beet leaf and root samples under (**A**) HWG and (**B**) PF treatment. Number of genes with negative values correspond to down-refulated genes, while number of genes with positive values correspond to up-regulated genesthat are relatd to the metabolic processes. Cys: Cysteine, Met: Methionine, Gly: Glycine, Ser: Serine, Thr: Threonine, Ala: Alanine, Asp: Aspartic acid, Glu: Gluatamic acid, Trp: Trptophan, Val: Valine, Leu: Leucine, Ile: Isoleucine, ER: Endoplasmic reticulum, Phe: Phenylalanine, Tyr: Tyrosine.

To validate the NGS results, the relative expression levels of five randomly selected genes were tested using a quantitative reverse transcription PCR (qRT-PCR). To that end, we selected three genes that are statistically significantly DE in all four treatments, *BvHSP70* (Gene ID 104883827), which encodes a chloroplast membrane-associated heat shock protein *BvHIPP24* (Gene ID 104905684), that codes for a heavy metal-associated isoprenylated plant protein, *BvGR2*(Gene ID 109135315), encoding glutamate receptor 2.6-like protein. Additionally, we validated the expression of, *BvIAA6* (Gene ID 104904637), which encode auxin-induced protein IAA6, and *BvSUSIBA2* (Gene ID 104890228), encoding a WRKY transcription factor that is involved in sugar signaling. The results indicated that the expression levels obtained from qRT-PCR analysis were largely consistent with the NGS data (Figure S1). However, the relative expression of *BvHIPP24* and *BvGR2* were upregulated in root samples of the plants treated with HWG according to the qRT-PCR as opposed to downregulated in the NGS data. Notably, the analysis indicated a positive correlation (correlation coefficient, r = 0.70) between the log2 fold change obtained from the NGS and qRT-PCR data. Interestingly, the correlation coefficient was above 0.90 for leaf samples treated with HWG and PF and root samples treated with PF, indicating a strong positive correlation between NGS and qRT-PCR data (Figure S1).

3. Discussion

The present study clearly showed that the used PBBs had a biostimulating effect on sugar beet. Thus, these PBBs contributed, when applied in specific amounts (2 g/kg soil), to up-and down-regulation of certain genes, resulting in an increased protein- and photosynthesis. These changes in gene activities resulted in an increase in the content of all nitrogen-related parameters in the leaves and thereby led to increased plant growth. How-

ever, in soils with HNC, nutrient solution (NS) with easily available nitrogen contributed specifically to increased root growth in the young sugar beet plants.

The present study showed that the enhancement of nitrogen-related parameters (nitrogen content, total peptide, and RuBisCO content) by the use of PBBs was a reflection of the activation of ribosome genes, as highlighted by the transcriptomic results. Nitrogen, peptide, and RuBisCO are N-rich molecules that are precursors for protein synthesis, which may have contributed to the increased ribosome activity, as the ribosome is an organelle for protein synthesis [26]. However, in roots treated with HWG or PF, genes associated with protein processing in the endoplasmic reticulum were down-regulated. This indicates, as also suggested by previous studies [27], that molecules such as peptides and amino acids are mobilized in the roots for further transport to the leaves, where protein synthesis takes place. Such a mechanism was also further verified here and in previous studies [28] by the high nitrogen and peptide content observed in leaf samples as compared to root samples treated with PBBs. Additionally, corresponding to previous studies [29], genes associated with photosynthesis were upregulated in the leaf samples of plants treated with either HWG or PF compared to the control. Such an up-regulation contributes to the enhancement of photosynthetic carbon assimilation and RuBisCO accumulation, as this enzyme is known to be highly involved in the process of photosynthesis [30]. Furthermore, genes associated with glycolysis and galactose metabolism, which are related to the respiration process, were down-regulated in the roots of samples treated with either HWG or PF. This down-regulation might be a result of the storage of sugar in sugar beet roots. It is well known that respiration is a catabolic process that results in the breakdown of sugars or other respiration substrates in order to release energy and carbon dioxide [31]. Thus, a down-regulation of the metabolic processes in the sugar beet roots may prevent respiration, thereby enhancing sugar storage in the root of the sugar beet plant.

The present study showed that RuBisCO at equal nitrogen additions, from the use of both NS and PBBs, were found to have a positive impact on sugar beet agronomy and physiology. However, the PBBs (both HWG and PF) enhanced all nitrogen-related parameters of sugar beet, including nitrogen content in leaf and root, and total polypeptide as well as RuBisCO content under both LNC and HNC as compared to control and NS, while NS was shown to favor root growth. RuBisCO in the leaves and the root dry mass were positively and significantly correlated to sucrose content in the roots at HNC. These results indicated differences in the mode of action of the NS and PBBs on sugar beet growth and physiology. The active ingredients in PBBs, similar to other protein hydrolysates, are mixtures of peptides and amino acids [2,5,24,32–34], unlike nitrate and other mineral elements present in NS. These active ingredients are usually low molecular weight compounds that are easily taken up by plant roots or foliage and which can act as precursors for phytohormones that are responsible for growth and development or nutrient sources used directly for growth [33]. This corresponds with the findings of the present study, indicating different pathways of plant growth and development in relation to used sources of nitrogen (NS versus PBBs).

In addition to the up-regulation of genes related to ribosomes and photosynthesis, genes for aromatic amino acids, e.g., tryptophan, were also upregulated. These aromatic amino acids (phenylalanine, tyrosine, and tryptophan) are known as precursors for auxins biosynthesis and other plant secondary metabolites, which enhance plant growth and tolerance to environmental stresses [35,36]. Furthermore, the HWG treatment resulted in an up-regulation of cutin, suberin, and wax biosynthesis as well as of diterpenoid biosynthesis in leaf samples. Cutin and waxes are known as water-resistant fatty acids derivatives, which are deposited on different parts of the plant, especially the leaves, and they provide minimum resistance to microbial penetration through leaf surfaces [37]. Previous studies have shown that diterpenoids are connected to the formation of gibberellin (GA), a phytohormone responsible for apical growth [29]. The HWG treatment also resulted in an up-regulation in the root of phenylpropanoid genes, which in previous studies have been linked to enhanced capacity to prevent microbial infection [38]. This is because

phenylpropanoids (also known as cinnamic acids) help to induce a response to fungal infections [39]. Furthermore, genes related to base excision repairs, nucleotide excision repair, mismatch repair, DNA replication, and nucleocytoplasmic transport were upregulated in the roots of the plants treated with PF, traits that previously had been linked to stress tolerance [40]. The differences in metabolic processes resulting from the differential gene expression by the use of HWG and PF confirmed that both PBBs are composed of different active ingredients (peptides and amino acids). Previous studies have indicated that differences in active ingredients are affected by the hydrolysis or other processes of making the PBBs [21].

The enhanced agronomic performance of plants treated with PBBs corresponds with the results of previous studies, primarily using protein hydrolysates but, in some cases, amino acids on a range of different plant types [14,20,21,33,34,41–43]. Additionally, small molecules such as polyamines have been reported to improve growth and stress tolerance ability in plants [44]. Previous studies have also reported positive physiological responses in terms of photosynthetic parameters in different crops from the use of PBBs [45,46], which our results verified. Additionally, the high sucrose content obtained in the present study in roots of sugar beet treated with HWG, PF, or NS, either individually or in combination, corresponded well with results from previous studies [43]. However, such a combination did not prove to be spectacular over solely applied PBBs. Furthermore, from the present study, no clear genotype differences were seen as to their performance in relation to the used PBB in regard to their agronomic and physiological parameters.

4. Materials and Methods

4.1. Plant Materials and Protein-Based Biostimulants (PBB)

Hybrid seeds of three sugar beet genotypes (Volga, Armesa, and Mustang) were generously provided by DLF Beet Seed AB, Landskrona, Sweden. These genotypes were selected because they represent the sugar beet gene pool in both the Nordic region and the EU. Two protein-based biostimulants (HWG and PF) were used in this study. Wheat gluten (WG) and potato protein (PP) are available as side streams from the industry [5]. In the present manuscript, we used the hydrolyzed WG (=HWG) as it is supposed to have better performance as a protein-rich biostimulant when the polymerized structure of the proteins is broken [5]. We preferred for this experiment not to produce the HWG by ourselves as it is easily purchased. HWG was purchased at A. Constanstino & Co. S.P.A., Favria, Turin, Italy. The PF was produced by ourselves in the lab from the PP received from the industry. We preferred to use PF and not PP based on previous results [5]. The PF was a film cast from potato protein powder, which was generously supplied by Lyckeby, Kristianstad, Sweden. PF was made by dispersing 50 g potato protein powder in 500 mL of milli-Q water over a 5 mm sieve. The suspension was placed on a magnetic stirrer for 10 min at 500 rpm at room temperature. The suspension was then dispensed into 100 mm × 15 mm Petri dishes at a volume of 50 mL per Petri dish. The dispensed suspension was placed in an oven for 48 h at 45 °C in order to form dry friable flakes (film) [47].

4.2. Soil Types and Biostimulants Treatments

Two trials were conducted in this study based on the soil types used. Soil type A is basically composed of sand, which was supplied by Bara Mineraler, Malmo, Sweden. This soil type (sandy) was chosen in order to be able to evaluate the effect of PBB in nutrient-deficient soil and for maximum root extraction for root biomass analysis. Whereas soil type B is a mixture of soil type A (sand) and peat-based soil in a ratio of 3:1. Peat-based soil was supplied by Emmal-Junga Torvmull AB, Sweden, and its physical and chemical components are presented in Table 4a. Soil type B was chosen in order to evaluate the effect of soil with improved physical and chemical characteristics in combination with PBB and/or NS on sugar beet growth and physiology. Both HWG and PF were mixed with the two soil types (A and B) in different concentrations (1 and 2 g/kg soil) either individually or in combination with nutrient solution (NS). NS used in this study was generously

provided by DLF Beet Seed AB, Landskrona, Sweden. The NS contained macronutrients and micronutrients in concentrations suitable for the greenhouse cultivation of sugar beet (Table 4b). The NS treatment used in the present study was 25 mL NS per plant at four weeks after planting, without the addition of any PBB. Ten replications of the treatments per variety were generated, and details are presented in Table 5. The choice of the concentrations (1 and 2 g/kg soil) was made based on the results from the previous study [5]. The untreated soil (no PBBs and/or no NS) was maintained as a control.

Table 4. (**a**). Physical and chemical components of the peat-based medium used in the high nutrient condition (HNC) soil medium. (**b**). Content of major elements present in nutrient solution (NS).

(a)		
Serial Number	**Description**	**Composition**
1	Light peat	50%
2	Dark peat	33%
3	Gravel	7%
4	exclay/LWA (2–6 mm)	5%
5	Clay	5%
6	pH	5.5–6.5
7	EC	2.0–4.0
	Additional component	
1	Crushed limestone	6 kg
2	Dolomite lime	2 kg
3	NPK 11-5-18 & Trace elements	1.5 kg
4	Extra trace element	0.1 kg
5	Optifer	0.1 kg
(b)		
Serial Number	**Element**	**Concentration (mg/L)**
1	N	271
2	P	56
3	K	331
4	Mg	70
5	Ca	169
6	S	61

Table 5. Description of the growing media used in the experiment.

Factors	**Levels**	**Remarks**
Genotype	I (Volga), II (Armesa) and III (Mustang)	
Treatment name	Hydrolyzed wheat gluten	13.1% nitrogen present HWG
	Potato protein film	13.1% nitrogen present in PF
Nutrient solution		NS contained major and minor nutrients, applied as a single solution and in one-time application (4 WAP). The solution contained 271 mg N per liter
Concentration (g/kg soil)	0, 1, 2	g of PBB per kg of soil
Treatments combinations	Control, HWG–1, HWG–2, HWG–1+NS, HWG–2+NS, NS, PF–1, PF–2, PF–1+NS, PF–2+NS	10 treatments per genotype

4.3. Design of Experiment and Environmental Conditions

The experiments were set up in the growth chamber of the Biotron at the Swedish University of Agricultural Sciences (SLU), Alnarp, in a controlled environment. The exper-

iments were laid out in a completely randomized design in ten replicates. The temperature (3.0/10.0 °C), relative humidity (60–70%), day length (13/11 h), and light intensities (0–1000 μmol m^{-2} s^{-1}) were set to mimic the weather condition during sugar beet planting season (March/April) in Southern Sweden. After four weeks in the Biotron, the experiment was moved to a greenhouse with a controlled relative humidity of 80–90% and temperature of 12–15 °C. The day length and light intensity in the greenhouse corresponded with the prevailing weather during the experiment, which was between March and August.

4.4. Effects of Equal Nitrogen Content from PBBs and NS on Agronomic and Physiological Parameters of Sugar Beet

In order to understand the mode of action of HWG and PF, an additional experiment was set up using equal amounts of nitrogen from HWG, PF, and NS supplied to the sugar beet genotypes. The nitrogen content (~13%) present in the HWG and PF was estimated using the Dumas method (Flash 2000 NC Analyzer, Thermo Scientific, Waltham, MA, USA, NX6.25) (more details under nitrogen content analysis). Thus, the addition of 2 g/kg HWG and PF at 2 g/kg as a PBB concentration for optimum growth of sugar beet [5] resulted in a supply of 260 mg N per kg of soil per plant. To contribute the same quantity of N by application of NS, ~960 mL of NS (composition described above) was supplied to the sugar beet plant with NS treatment.

Plants were raised under similar conditions as described above in both the Biotron and greenhouse for eight weeks. Similarly, agronomic and physiological data were collected at the end of the experiment.

4.5. Data Collection

4.5.1. Growth

Data on the growth of sugar beet were collected at eight weeks after planting (WAP). Growth parameters were measured as previously described [5] and included plant height (cm) and digital plant canopy area (% green pixel/cm^2). Plant height was measured using a transparent 50-cm meter rule from the topsoil to the tip of the tallest leaf. The plant canopy area was measured by taking aerial view photographs of plants from 60 cm above the plant using a Fuji Film Camera. The pictures were then analyzed using Easy-Leaf-Area (ELA), an open-source software for phenotyping [48]. The percent green pixel in relation to the area of coverage was taken to be the canopy area, but values were presented in squared centimeters (cm^2).

4.5.2. Gas Exchange Measurement

Gas exchange and chlorophyll fluorescence were measured on eight-week-old sugar beet seedlings under different PBB treatments. The measurements were taken on fully developed leaves in the morning (09:00–11:00) using a portable and open system equipped with infrared gas analyzers (model 6800; Li-Cor Inc., Lincoln, NE, USA). The leaf temperature during measurements was maintained at 25.0 ± 0.5 °C. Leaves were illuminated with a steady red and blue light source at a photosynthetic photon flux density (PPFD) of 1200 μmol m^{-2} s^{-1} [49]. The reference CO_2 concentrations in the cuvettes matched the treatment CO_2 concentrations to which sugar beet plants had been growing, i.e., 400 ± 2.5 μmol mol^{-1}. The vapor pressure deficit (VPD) was 1.1 ± 0.05 kPa, and the relative humidity was 55–65%. The gas exchange instrument was calibrated each day before the measurements and matched at least twice a day (between the curves). Data were recorded after sample acclimation in the cuvette for at least 15 min. Data were collected after the prevailing CO_2 had reached a steady state (2–3 min).

4.5.3. Biomass and Physiological Sample Collection

Plants were carefully uprooted and separated into shoots and roots at 8 WAP for analyses of biomass and physiological parameters (polypeptide and sugar analyses using HPLC as well as nitrogen content using the Dumas method). Shoot and root samples were

washed under gentle running tap water to remove soil particles. Excess water from the washed shoot and root samples was drained using a 3-fold paper towel. Three selected plants per treatment were put in separate brown paper envelopes (26 cm by 16.5 cm) and dried at 70 °C for 72 h in a ventilated drying cabinet for biomass analysis. Well-dried plant samples were weighed on a digital scale calibrated in milligrams. Thereafter, two plants, separated into shoot and root, were placed in separate plastic zipper bags and stored in a −80 °C freezer for analysis of physiological parameters.

4.5.4. Analyses of Biological Macromolecules

Sample Preparation and Protein Extraction; Total Polypeptide and RuBisCO Contents Analysis Using Size Exclusion (SE)-HPLC

Two plants per treatment, separated into shoot and root samples, frozen at −80 °C, and later used for RuBisCO, total polypeptide, sucrose, and total sugar content analyses using HPLC, as well as for analyses of nitrogen content using the Dumas method. The frozen (−80 °C) samples were freeze-dried for 72 h and then ground into powder using an MM 400 Retsch ball mill (Retsch Mill. Haan, Germany). Ground samples were put in 50 mL Falcon tubes and kept in a −4 °C freezer until further use.

The protein extraction protocol was similar to Gupta et al. [50], with some modifications. In order to extract protein, 16.5 mg of ground sample (leaf or root) was measured into 1.5 mL micro-centrifuge tubes in three replicates. Phosphate buffer (pH 6.9) prepared from a mixture of 0.05 M $NaH_2PO_4 \cdot H_2O$ (Solution A, MW: 137.99) and 0.05 M $NaH_2PO_4 \cdot 2H_2O$ (Solution B, MW: 177.99) solutions in ratio 1:1, was added to the sample in the tubes at the rate of 1.4 mL per sample. Sample mixtures were vortexed and then placed on a shaker for 5 min at 2000 rpm (IKA Vibrax VXR B, IKA Werke, Germany) for protein extraction. Samples were then arranged in the centrifuge and set at 10,000 rpm for 30 min for protein extraction. After centrifugation, the clear liquid phase above the samples was decanted into 2.0 mL HPLC vials and arranged in the HPLC autosampler.

Protein extracts in the HPLC vials were arranged in Waters e2695 HPLC machine with a Waters 2998 PDA detector (Waters Corporation, Milford, MA, USA), fitted with SEC s2000 column (Phenomenex, Torrance, CA, USA), which is suitable for analyzing polypeptides and small proteins (~3 kDa). The method was set to collect 20 µL per sample (at 25 °C), which runs for 37 min, and each treatment is made up of three replicates, while the column (SEC s2000) was maintained at 19 °C. A mobile phase of 0.05 M NaH_2PO_4, pH adjusted to 6.9, was applied at 0.5 mL/min. Absorption spectra (3D) were collected at 190 to 520 nm over 37 min, and for further analysis, absorption at 210 and 280 nm were collected. Phosphate buffer (solutions A and B, ratio 1:1, pH 6.9) was used as blank and was set at the end of running each replicate (containing ten treatments). The chromatograph from the SE-HPLC generated by Waters Software (Empower 2) was used to estimate the total peptide and RuBisCO contents present in each treatment by determining the area under the curve. Total peptide content per treatment was estimated by the sum of all the areas under the curves of the chromatogram, measured at 210 nm wavelength (Figure 5). However, RuBisCO content was estimated by calculating the area under the curve at a retention time (RT) of 10.0 min on the chromatogram at 280 nm wavelength.

Nitrogen Content Analysis Using the Dumas Method

Approximately 5 mg of ground leaf and root samples of sugar beet treated with PBBs were measured into thin aluminum capsules. The aluminum capsules were then folded and pressed to remove excess air that may be trapped in the capsule. The nitrogen content of PBB-treated sugar beet in leaf and root samples was determined using the Dumas method with a Flash 2000 N/C Analyser (Thermo Scientific, Waltham, MA, USA) [13]. The results of the nitrogen content of the samples were presented as averages from triplicates.

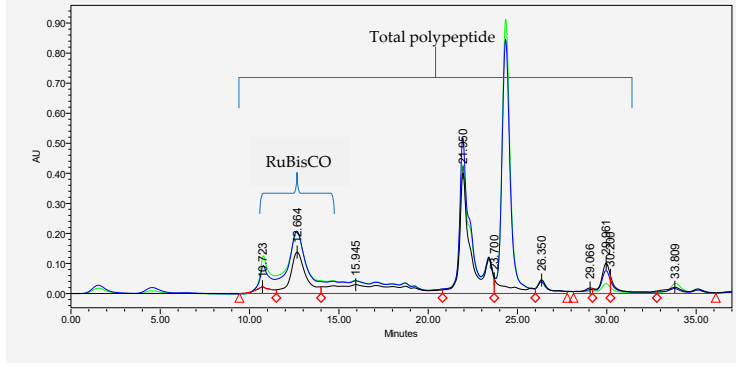

Figure 5. Example of SE-HPLC showing different peaks of protein analysis based on PBB treatment of sugar beet leaf sample. Blue line: HWG treatment; green line: PF treatment; black line: NS treatment; and red line: phosphate buffer. Red rhombus and triangle at the base of the figure are only to align the baseline to the origin (zero). Numbers above the peaks indicated the retention time for each peaks in minutes.

Sugar Content Analysis Using HPLC Method

Based on the result of a preliminary experiment, a 75 mg ground root sample was used for the analyses, and one-milliliter milli-Q water was added to each sample, followed by vortexing, shaking for 5 min, and centrifugation at 8000 rpm for 5 min. The supernatant was carefully decanted and diluted five times (100 µL in 500 µL milli-Q water) before running the samples in the HPLC system (Agilent 1100 OpenLab software ChemStation Edition v2.7, Santa Clara, California, USA). The HPLC system was connected to an Agilent 1260 Refractive Index Detector (RID), fitted with Asahipak NH2P 50 4E column, and eluted with 0.8 mL/min 5 mM H_2SO_4. Samples were injected into the system at the rate of 10 µL for 10 min and maintained at room temperature, while the RID was maintained at 35 °C. Standard sugar solution containing 2.5% each fructose (Janssen Chimica Geel Belgium) glucose and sucrose, as well as 0.5% raffinose from Sigma Chemical Co., St. Louis, MO, USA, were used to identify the peaks of the different sugars. The formula below was used to estimate the sucrose (%) and total sugar content (%) that is present in the root samples of sugar beet.

$$\text{Sucrose content}(\%) = \frac{\text{Area under curve by sample} \times \text{sucrose content in standard (2.5\%)} \times \text{dilution factor (5)}}{\text{Area under curve by sucrose standard}}$$

$$\text{Total sugar content}(\%) = \frac{\text{Area under curve by sample} \times \text{total sugar content in standard (8\%)} \times \text{dilution factor (5)}}{\text{Area under curve by total sugar standard}}$$

4.6. Data Analysis

All growth, biomass, and physiological parameters were subjected to analysis of variance (ANOVA) using a general linear model (GLM) of Minitab 19.2 in order to detect significant differences in the treatments. Thereafter, means were separated using the Tukey posthoc test, where differences were indicated with different lowercase alphabets. Principal component analysis (PCA) was carried out using all measured parameters (growth, biomass, and physiology) with Minitab 19.2. Correlation analysis was done using the "data analysis plug-in" of Microsoft Excel, and cells were formatted using the color scale tab of Microsoft Excel in order to evaluate the differences in the correlation of parameters.

4.7. Transcriptomics Analyses

4.7.1. RNA Sample Collection, Sequencing, and Data Analysis

Sugar beet genotype Armesa was used for the transcriptomic analysis because it largely represents the gene pool in the Nordic region. Armesa was sown in pots filled with peat-based soil containing sand and peat in a ratio of 1:3 (HNC) treated with 2 g/kg of HWG or PF, and untreated (no HWG and PF) pots were maintained as the control for eight weeks. At eight weeks after planting, leaf and root samples for transcriptomic analysis were collected as three biological replicates; each biological replicate was pooled from three individual plants. Leaf or root samples were collected and snap-frozen in liquid nitrogen and stored at −80 °C. RNA extraction was done following a similar method described by [51]. One hundred milligrams of tissue was homogenized in a motor and pestle in liquid nitrogen, followed by RNA isolation using an RNeasy Mini kit (Qiagen, North Rhine-Westphalia, Germany). Extracted RNA was then treated with a Turbo DNA-free kit (AM1907, Thermo Scientific, Waltham, MA, USA) to remove any genomic DNA contamination. The quality of RNA was assessed on Agilent Bioanalyzer. Paired-end mRNA reads were generated using Illumina high-throughput sequencing from the NGI facility. A quality control (QC) check was performed on independent samples with three biological replicates per sample using the FastQC v0.11.7 tool [52], and multiple sample visualization was evaluated using the MultiQC v1.6 tool [53]. An initial filtering step was performed on the removal of ribosomal RNAs (rRNAs) by aligning reads with silva and Rfam databases using the Sortmerna-v2.1b [54] tool, and all TruSeq3 adapters were trimmed with Trimmomatic-v0.36 [55] setting MINLEN:20 in bases and SLIDINGWINDOW:5:20 with other default parameters. The second round of QC checks was performed using the same tools mentioned above.

The whole genome of Beta vulgaris EL10_1.0 (https://phytozome-next.jgi.doe.gov/info/Bvulgaris_EL10_1_0; accessed on 15 May 2023) was used for reference alignment. The mRNA reads were aligned to the CDS coordinates using the splice aligner STAR-v2.7.5b [56] tool with --twopassMode Basic, --sjdbGTFfeatureExon CDS parameters, keeping other settings as default, the total number of reads processed can be seen in the Supplementary Table S3. Transcript abundance was estimated with Salmon v1.3.0 [57]. Raw read counts were used for Differential Expression (DE) analysis with DESeq2 [26,58], and an in-built cross-sample "Relative Log Expression" (RLE) [27] normalization was performed.

4.7.2. Pathway and GO Terms Enrichment Analysis

Kyoto Encyclopaedia of Genes and Genomes (KEGG) terms pathway enrichment analysis was performed using obtained gene coordinates from the closest variety of Beta vulgaris genome RefBeet-1.2.2 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000511025.2/; accessed on 15 May 2023). Each pairwise comparison of the DE gene set filtered with FDR cut-off < 0.05 was used for Gene Set Enrichment Analysis (GSEA). GSEA of KEGG were tested with the clusterProfiler version 3.18.1 [28] submodule gseKEGG with settings nPerm = 10,000, pvalueCutoff = 0.05; pairwise comparisons are shown in Supplementary Figure S2, and data tables are available in the Supplementary Table S4.

Singular Enrichment Analysis (SEA) of Gene Ontology (GO) categories was performed on AgriGO v2.0, the web-based tool using mapped coordinates obtained from the PLAZA3.0 database (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_pico_03/; accessed on 15 May 2023). The DE gene set was filtered with both FDR < 0.05 and log2FC > 1.0. GO significance level of FDR < 0.05 was set, and keeping the remaining settings as default, data tables are available in Supplementary Table S5.

4.7.3. qRT-PCR Analysis

qRT-PCR analysis was carried out as described previously [31]. Briefly, 500 ng of total RNA was used for reverse transcription using an iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). qRT-PCR reactions were performed using 2×DyNamo Flash SYBR Green Master mix kit (Thermo Scientific, Waltham, MA, USA) following manufacturer's

instructions with four microlitres of 10-fold diluted cDNA used as a template. Data analysis was performed using the BioRad CFX manager 3.1 with the Cq values of target genes normalized to that of BvGAPDH and BvEF1 alpha genes. The primer sequences are listed in Table S6. All reactions were performed with three biological replicates per treatment. Each biological replicate had three technical replicates for the qRT-PCR.

5. Conclusions

Hydrolyzed wheat gluten (HWG) and potato protein film (PF) are two novel protein-based biostimulants (PBBs) from agro-industrial side streams with biostimulating effects on sugar beet plant development. Both HWG and PF enhanced the early growth and development of sugar beet plants as well as the synthesis and/or accumulation of bio-macromolecules such as peptides, RuBisCO, sucrose, and total sugar content. Furthermore, the application of HWG and PF contributed to the up-regulation of genes associated with important metabolic processes such as protein synthesis, photosynthesis, and biosynthesis of metabolites and aromatic amino acids. All of these metabolic processes lead to enhanced growth and physiology of sugar beet, either directly through photosynthesis and protein synthesis or indirectly by the effect of amino acids (auxins and gibberellic acids) for plant growth and development and stress tolerance. Differences in up-regulation of genes, e.g., for DNA replication, mismatch repair, and nucleotide excision repair, in plants treated with HWG and PF indicated variation in the presence of active ingredients (amino acids and peptides) in the two PBBs, resulting in different effects on metabolic processes. The positive effect on the early growth of sugar beet plants and the biostimulating effect from the use of the HWG and PF indicate their possible use in crop production. As these PBBs and their raw materials are obtained from side streams of the agro-industry, they are expected to be environmentally friendly and sustainable, although this must be further verified.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24119720/s1.

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There is need to intensify efforts in finding alternative uses for agro-industrial side-streams, in order to prevent environmental pollution arising from their indiscriminate disposal. Conversion of these side-streams into biostimulants, is eco-friendly and sustainable. In this thesis, the biostimulant potential of hydrolysed wheat gluten, potato protein and Kitflokk™/Kitoflokk200™ were established based on their effect on growth and development of sugar beet as well as drought tolerance in wheat.

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