Amino acids as biostimulants to improve plant physiology, stress responses and crop yield

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I. Author’s declaration

I declare that the contents of this thesis are my own work, except where reference is made to other sources, and that I have not previously submitted this thesis for the award of a higher degree elsewhere.

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II. Abstract

The combined effects of a growing population and climate change require improvements in crop production to meet the global food demand. Together with genetic advances and breeding techniques, improving agricultural product inputs to crops presents an opportunity to minimise losses and increase plant yield, helping achieve the goal of food security. This thesis explores the effects of amino acid addition to plants and their potential usefulness as biostimulants, functioning in a way that affects plant physiology and biological processes regardless of nutrient content. Special attention is paid to pyroglutamic acid (PGA), a scarcely studied non-proteic amino acid that is used in commercial agricultural products.

The effects of a wide range of amino acids were screened *in vitro* in *Arabidopsis thaliana* plants, as well as in wheat in controlled environment, greenhouse, and field conditions. Results underline the differences in responses between different species and conditions. In *Arabidopsis*, high PGA concentration in solid media led to stress and toxicity symptoms. Seedling root growth varied between amino acids and led to different responses related to salt stress. Responses to amino acids were also altered in the *Arabidopsis* mutant *oxp1*, in which there is a disruption of the only known route of PGA conversion to glutamate (Glu). In greenhouse-grown wheat, root/shoot ratio and tillering appeared to change under the effect of PGA after two and four months of growth respectively, particularly under limited watering conditions, but neither PGA nor any other trialled amino acids altered yield parameters in non-stressed greenhouse plants grown to full development. In the field, yield-related physiological parameters differed after the application of different amino acids, as well as leaf protein content and nitrogen assimilation enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) during grain filling stage. A metagenomic analysis of soil bacterial populations revealed differences in the soils corresponding to different amino acid treatments, indicating that part of the effect of amino acid biostimulants may be driven by effects on rhizosphere microbial communities.

Overall, this thesis evidences the contrasts in the effects of amino acid application under different conditions, with clearer effects observed in plants under specific stresses or under more complex field conditions compared with controlled conditions. The data presented enhances the known characteristics of amino acids as biostimulants with potential to improve plant stress responses.

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V. Abbreviations

2-OG: 2-oxoglutarate

5-OPase: 5-oxoprolinase

Ala: alanine

AMOVA: analysis of molecular variance

ANOVA: analysis of variance

Arg: arginine

AS: asparagine synthetase

Asn: asparagine

Asp: aspartate

AtGLR: *Arabidopsis thaliana* glutamate receptor-like channel

bp: base pair(s)

BSA: bovine serum albumin

C(x+c): total carotenoids

C: carbon

Ca_2^+: calcium

Ca: chlorophyll a

Cb: chlorophyll b

cDNA: complementary DNA

CE: controlled environment

CFU: colony forming unit

CH_4N_2O: urea

Con: control

Cys: cysteine

DAMP: damage associated molecular pattern

DTT: dithiothreitol

DW: dry weight

ECM: the company Environmental Crop Management limited

EDTA: ethylenediaminetetraacetic acid

FW: fresh weight

GABA: γ-aminobutyric acid

GDH: glutamate dehydrogenase

GGC: γ-glutamyl cyclotransferase

GGT: γ-Glutamyl transpeptidase

Gln: glutamine

GLR: glutamate receptor-like channel

Glu: glutamate

Gly: glycine

GOGAT: glutamine oxoglutarate aminotransferase

GS: glutamine synthetase

GSH: glutathione

H: hydrogen

ha: hectare

His: histidine

HPLC: high-performance liquid chromatography

iGluR: ionotropic glutamate receptor

Ile: isoleucine

JA: jasmonic acid

LDA: linear discriminant analysis

LEfSe: linear discriminant analysis effect size

Leu: leucine

Lys: lysine

Met: methionine

MS: Murashige and Skoog (medium)

N: nitrogen

NAD: nicotinamide adenine dinucleotide (oxidised)

NADH: nicotinamide adenine dinucleotide (reduced)

NADP: nicotinamide adenine dinucleotide phosphate (oxidised)

NADPH: nicotinamide adenine dinucleotide phosphate (reduced)

NASC: Nottingham *Arabidopsis* Stock Centre

NH_4^+: ammonium

NH_4NO_3: ammonium nitrate

NO_3^-: nitrate

NPK: nitrogen-phosphorous-potassium

O: oxygen

oxp1: Oxoprolinase 1 defective *Arabidopsis thaliana* mutant

OXP1: Oxoprolinase 1 *Arabidopsis thaliana* gene

PAMP: pathogen associated molecular pattern

PBS: phosphate buffered saline

PCA: principal component analysis

PCoA: principal coordinate analysis

PCR: polymerase chain reaction

PGA: pyroglutamic acid

Phe: phenylalanine

PMSF: phenylmethylsulphonyl fluoride

ppm: parts per million

Pro: proline

PRR: pattern recognition receptor

PVPP: polyvinylpolypyrrolidone

qPCR: quantitative polymerase chain reaction

ROS: reactive oxygen species

RuBisCO: ribulose-1,5-biphosphate carboxylase-oxygenase

S: sulphur

SA: salicylic acid

Ser: serine

SPAD: single-photon avalanche diode

TCA: tricarboxylic acid

Thr: threonine

Trp: tryptophan

Tyr: tyrosine

UPGMA: Unweighted Pair-group Method with Arithmetic Means

USD: United States dollar(s)

Val: valine

WT: wild-type

α-KG: α-ketoglutarate

γ-EC: γ-glutamyl-cysteine

γ-GHM: γ-glutamyl mono-hydroxamate

Chapter 1. Introduction

1.1. Food security

Food demand increase is rising together with population, with the population being projected to reach as much as 9.7 billion people by 2050 and 11.2 billion by 2100 (UN, 2019). This increase affects different areas unequally (**Fig. 1.1A**), and current calorie demand will rise by as much as 50% from 2015 to 2050 (UN, 2015). However, the expansion of urbanisation, land erosion and salinisation, crop and land use for non-edible ends and climate change are limiting factors for an increase in food production (Parry *et al*., 2010), which in many cases is projected to pose a problem in the upcoming decades (**Fig. 1.1B**). Beyond population growth, undernutrition and micronutrient deficit of the current population is also an issue that emphasizes the need to increase the global food supply and availability. Despite the efforts in this area, between 720 and 811 million people have an insufficient daily calorie intake and over two billion people have micronutrient deficit (FAO, 2021A). Undernutrition rates have globally fallen during the last decades but have risen since the 2020 pandemic (FAO, 2021A).

Food production is being affected by human activity, changing the environmental conditions in which the global industry operates and facing the challenge of land, water and fishery limitations (Whitmee *et al*., 2015). Climate change can alter the conditions in which food is produced, with rising temperatures, increased greenhouse effect gas concentrations and more extreme climatic events such as severe rainfall (Harrison *et al*., 2016), altering food production and relationships among different factors in the ecosystem, which in agriculture include crops, pests, pathogens, weeds, pollination, water availability and ground level ozone concentrations.

Identifying the causes that threaten food security is key to address them and increase the food supply. From an agriculture point of view, finding ways to improve yields in globally used crops, as well as combating the loss from abiotic and biotic stresses can help bridge the gap between the global supply and demand of food.

Fig. 1.1. (A) World population increase in different areas of the world (SSA = Sub-Saharan Africa, LAC = Latin America and Caribbean, N America = North America, N Africa = Rest of Africa) **(B)** Past crop yield growth, projected future yield increase and yield increase needed to avoid an increase in harvested area (from the World Resources Institute, 2013).

1.2. Agriculture and climate change

Around 40% of the ice-free land in the planet is used as cropland and pasture, with a third of this being exclusively dedicated to agriculture (FAO, 2021B). Agriculture alone constitutes 83% of the worldwide production of consumed calories (FAO, 2021C), with irrigation being the largest human use of water and accounting for two thirds of water withdrawals for a total of 2,000 km^3 yearly (Brauman *et al*., 2016). Agricultural yields for each crop are determined by all factors affecting them and the dynamic balance between them, which include the soil, nutrients, water, sunlight, CO_2 and temperature, pests, pathogens and air pollution, all of which have already changed considerably with the effects of climatic change (Iizumi *et al*., 2017, Wang *et al*., 2018A).

Climate change is leading to global temperature rise (Gourdji *et al*., 2013) and a decrease in water availability (Lobell and Gourdji, 2012), posing a major risk to global crop yields. As temperatures rise so will crop exposure to damaging heat levels, with temperatures above 30^oC being considered prejudicial for rain-fed crops (Carlson, 1990; Schlenker and Roberts, 2009). Water availability reduction due to increased temperatures can also lead to water stress in crops, driving a further reduction of the yield (Muchow and Sinclair, 1991; Sinclair and Rufty, 2012).

CO_2 increase is a direct cause of anthropogenic climate change. Higher anthropogenic CO_2 concentration has direct positive effects in crop production as it increases photosynthetic rate and water efficiency (Long *et al*., 2006, Ebi and Ziska, 2018). This is true for both C3 and C4 species, although C3 crops such as soybean, wheat and rice will be more benefited in terms of growth and yield, as photosynthetic rate of C3 plants increases at higher CO_2, while C4 plants such as maize and sugarcane are close to CO_2 saturation for photosynthesis at current ambient concentration (Leakey *et al*., 2009).

However, negative effects of CO_2 increase are also notable, as higher CO_2 concentration alters nutritional composition of crops when transpiration is lowered and water efficiency is increased, reducing the protein content by alteration of the carbon-to-nitrogen ratios (Myers *et al*., 2014). Elevated CO_2 leads to lower mineral concentration, with up to 11% of decrease in zinc and iron in cereals and legumes at 550 ppm (Myers *et al*., 2014), and to a reduction of a wide range of minerals at higher concentrations of 690 ppm CO_2 (Loladze, 2014). These nutritional changes pose a risk of putting millions of people at new risks of zinc deficiency and exacerbating mineral deficiencies in a billion people (Myers *et al*., 2015).

Temperature increase also poses a risk to crop yields. General trends indicate a higher sensitivity to temperature increase for C3 plants compared to C4 ones (Challinor *et al*., 2014; Rosenzweig *et al*., 2014). Many crops like maize and wheat have yields that are 5% reduced compared to their theoretical production if temperature had not been increased since 1980 (Lobell *et al*., 2011). Other crops such as rice and soybean however have remained mainly unaffected by temperature increase (Lobell *et al*., 2011).

Climate change affects crops in different parts of the world unequally, with changes in temperature and rainfall having different effects on crop production in different locations and particularly at different latitudes (**Fig. 1.2**). Areas with already high temperatures and where crops are already at their physiological limit, such as tropics and arid regions, are much more likely to experience crop loss from rising temperatures (Gornall *et al*., 2010): considering temperature, water availability and CO_2 concentration, an increase of 4ºC globally could reduce low latitude maize yield by 15% and low latitude wheat yield by 25% (Rosenzweig *et al*., 2014). In contrast, crops grown at latitudes higher than 30º, where warmer temperatures are often convenient and where precipitation will overall increase (Donat *et al*., 2016) could experience an increase in yields as temperatures rise (Olesen *et al*., 2007, Challinor *et al*., 2014). The effects of climate change are leading to agricultural climate zones shifting to higher latitudes where yields can be higher (King *et al*., 2018), changing which crops can be grown in certain locations.

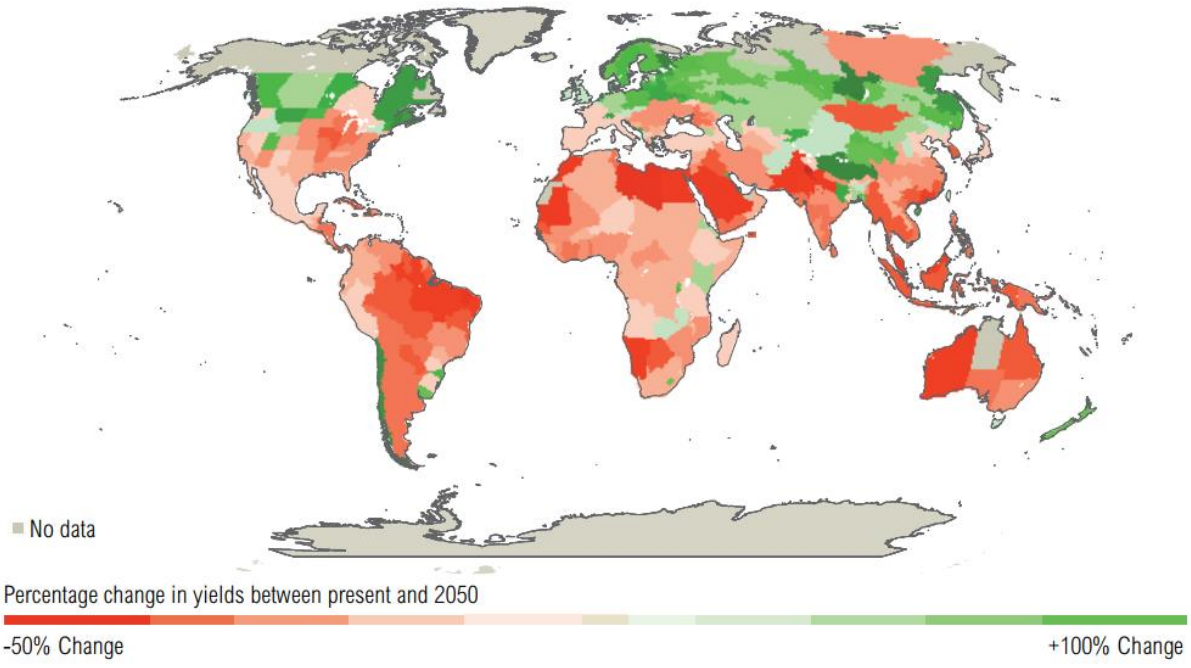


Fig. 1.2. Projected change of crop yields in different areas of the planet between 2013 and 2050 (from the World Resources Institute, 2013).

Pests such as insects, pathogens, bacteria, fungi and weeds are estimated to reduce crop yields by up to 40% yearly (Flood, 2010), and warmer temperatures can increase the winter survival of insect pests (Bale *et al*., 2002). Crop defence can also be compromised by a change in climate, exacerbating the effects of pests (Rosenzweig *et al*., 2001). However, extreme weather also poses a threat to pests and invasive plants, which can sometimes increase the competitivity of crops (Young, 2015). CO_2 concentration increase will also have effects in the relationship between plants and pests and pathogens, as plant defences are strengthened in higher CO_2 concentrations (Chauhan and Ramesh, 2015; Zvereva and Kzlov, 2006). Food protection practices to combat spoilage and pests also affect food yield (Hodges *et al*., 2010, Parfitt *et al*., 2010), and in events of drought or excess rainfall the exposure of crops to pathogenic bacteria, parasites, mycotoxins and viruses can increase (Rose and Wu, 2015). The relationship of crops with soil microorganisms is further described in **Section 1.3.1**.

The positive impact of insects in flowering species can also be affected by climate change as pollinator insect abundance is reduced and their regional distribution is changed (Memmott *et al*., 2007; Hegland *et al*., 2009; Abrol, 2012; IPBES, 2016). A change in the times of plant flowering and pollinator emergence can reduce the time overlap between these two processes (Myers *et al*., 2017). CO_2 concentration increase can change the nutritional values of pollinator food, with undetermined consequences for pollinator health. Animal pollinator reduction would threaten a considerable yield decrease in food crops that depend on them (Eilers *et al*. 2011; Chaplin-Kramer *et al*., 2014).

Human performance is also affected by changing climate conditions, particularly in less developed regions that rely less on mechanisation and where heat can limit human performance at specific times (Kjellstrom *et al*., 2016). Increased mechanisation can help mitigate the effect of human heat in labour, but economic requirements and labour consequences must also be considered. Food prices can also have a great influence on production and consumption, and market dynamics, often based in international relationships, can affect the supply and demand of specific food, posing a risk to food security (Schmidhuber and Tubiello, 2007).

All the stated consequences of climate change put an increasing amount of pressure into crop production, which at the same time needs to not only maintain but improve to address the population increase (Foley *et al*., 2011). Resources used in agriculture are limited, and the used of them often leads to contamination in the ecosystem: chemical fertilisers contribute to water eutrophication, release polluting gases to the atmosphere and affect soil chemistry and microbiology. (Machado and Serralheiro, 2017; Al-Ghussain, 2019). Ways of increasing crop yields while improving sustainability by reducing fertiliser and water inputs are being addressed, such as optimisation of fertilisers and pesticides (Lykogianni *et al*., 2021), genetic improvement of crops via breeding or genetic engineering (Gaba *et al*., 2021), improving the plant microbiome (Trivedi *et al*., 2021) and using compounds that impact plant biological processes to improve their performance. This last method will be the focus of this thesis, using amino acids as biostimulants to stimulate plant processes and improve crop performance.

1.3. Plant biology and ecosystem

Plants are surrounded and populated by microorganisms outside and inside them from the moment they are seeds. Microorganisms are known to be a major factor in helping plant ability to adapt to the environment and provide necessary functions to do so (Bulgarelli *et al*., 2013). The extent of the cooperative microbial symbionts in plants is so expansive that a plant is often regarded as an ecological unit known as holobiont that comprises the host plant and its surrounding microbiota (Zilber-Rosenberg and Rosenberg, 2008).

Outside of the plant holobiont, the aerial part of plants will be in contact with the atmosphere, from where plants will take CO_2, O_2 and solar energy for performing cellular respiration and photosynthesis. This is where they will also be most exposed to pests and pathogens as well as atmospheric conditions. On the other side of the ground, soil is the natural medium for plant growth in natural ecosystems, where roots will be developed and the majority of nutrients will be taken from. Plants will absorb water and chemicals from it, and the physical, chemical and biological characteristics of the soil will dictate plant growing conditions. Nutrient availability, such as H_2O, N, P K, S, Ca, Mg and micronutrients are necessary for plant life and growth, and can be taken up in several forms, both organic and inorganic. The form in which nutrients are present in the soil will also influence the ability of the plant to absorb and metabolise specific nutrients.

1.3.1. Plant microbial communities

As mentioned, microorganisms live around all parts of the plants and have considerable effects in their life. Endophytes (microorganisms inside the plant) spread mainly through the xylem to distinct compartments such as stem, leaves and fruits, creating different communities in distinct parts of the plant (Compant *et al*., 2010). Epiphytic microorganisms, the ones outside the plant in the aerial parts, have been reported to have their origin mostly in soil (Vorholt, 2012) and their populations are mainly influenced by environmental conditions rather than those in the plant.

The surrounding soil is the main source for microorganisms that are associated with the plants, and soil microbial community characteristics and environmental parameters are a more predominant factor for defining the root-associated microbiome than plant species or genotype (Bulgarelli *et al*., 2012; Schlaeppi *et al*., 2014; Bonito *et al*., 2014). However, plants also affect the soil microbial community around them (Marschner *et al*., 2004). Within the soil, the rhizosphere is the peripheral thin layer surrounding the roots that is in close relationship with

the plant. Plant exudates from the roots, including carbon (C)-rich organic molecules, oxygen (O) and antimicrobial compounds will directly impact the microbial community living in the area. At the same time, the microbial species living in the rhizosphere can help the plants, making this highly dynamic environment bear a distinct microbial population compared to the rest of the soil (Peiffer *et al*., 2013; Schreiter *et al*., 2014). Rhizosphere microorganisms play a role in plant growth and health as well as nutrient cycling, with interactions that can alter hormone and nutrient availability and suppress disease-causing microorganisms. The microbial communities in the rhizosphere differ in composition and relative abundance from the rest of the bulk soil, indicating the influence plant roots have in defining the microbial species surrounding their roots (Foster, 1986; Marilley and Aragno, 1999).

Because of the importance of the microbiome in plant life, different techniques to manipulate it have been researched. One of the most direct methods is the inoculation of a microbial strain or a consortium of microorganisms that can improve plant performance or stress response (Compant *et al*. 2019). Selecting or breeding plants to achieve certain microbiome characteristics is also possible as the specific microbiome of some species can change with evolution, breeding and domestication (Abhilash *et al*., 2012, Gopal and Gupta, 2016).

Agricultural input can also affect plant microbiome, potentially improving plant performance by adapting fertiliser use or adding certain compounds. One of the chapters in this thesis is dedicated to the study of plant microbiome changes after amino acid application in field wheat. Amino acids are of interest for both plants and bacteria, with the latter usually outcompeting plants in their assimilation (Sauheitl *et al*., 2009). Amino acids present in the soil can affect plant-microbe relationships (Moe, 2013), and amino acid application in the field can alter plant microbial population balance and even improve yield (Wang *et al*., 2019). Taking into account the wide array of functions and effects that amino acids can have in plants, as described in the following **Section 1.5**, it is reasonable to assume that amino acid application in fields is not only affecting plants directly but also the microbial populations associated to it. Studying the changes amino acids can provoke on the microbiome can help understand the use of amino acids in agriculture to improve crop characteristics.

1.4. Biostimulants

The concept of biologic materials affecting the metabolic and energetic processes of diverse organisms has been used since the 1940s (Filatov *et al*, 1944), and began being used for biological compounds that enhance enzymatic activity in plants around the same time (Filatov, 1951). The definition of the term biostimulants and its limitations has been altered throughout the decades (Yakhin *et al*., 2017), taking into account considerations such as suggesting that biostimulant products need to function in small doses and having reproducible benign benefits for agricultural practices. Zhang and Schmidt (1999) mention biostimulant effects as pre-stress conditioning, improving plant adaptation against these and producing better yields. Currently, biostimulant science in Europe is categorised according to the components in them and are subject to European Union legislation and regulation (EU, 2019). However, there is not a consensus about the exact definition of the word biostimulant (Yakhin *et al*., 2017). A generalised modern definition of plant biostimulants states that they are materials, compounds, organisms or products, other than fertilisers or pesticides, that can improve nutrition efficiency, health, stress tolerance, growth, development or crop yield and quality not because of their nutritional content, but because of their capacity to modify plant physiological and biochemical processes (Chojnacka *et al*., 2015; Du Jardin *et al*., 2015, Lovatt *et al*., 2015, Yakhin *et al*., 2016).

In practice, biostimulants are most commonly complex mixtures of components. Defining and categorising different complex biostimulants has typically been done according to the components of the biostimulant or their mode of action (Yakhin *et al*., 2017). Using a component-based classification does not fully explain the effects, which have often been referred to as “more than the sum of its parts”, “complex”, “emergent”, “unexpected” and related to systems biology (Johnson, 2006; Bertolli *et al*., 2014). Many biostimulants lack specificity about what components they include.

This specific mode of action refers to specific biochemical targets and the effects of the biostimulant compound on said targets, which are often not clearly defined nor explained. Frequently biostimulants have an unknown mode of action and just have a more general mechanism of action explained, a demonstrated general impact on specific biochemical processes, molecular pathways or physiological processes (Yakhin *et al*., 2017), which can demonstrate the efficacy without fully understanding the whole mode of action. However, there are cases of biostimulants in which not even a general mechanism of action is provided, raising

questions about the action of these products and leading to distrust, with some referring to the biostimulant industry as “snake oils” (Basak, 2008).

At present, the biostimulant market is growing as agricultural sectors are gaining awareness of the dangers of overusing fertilisers and need to provide protection to crops against biotic and abiotic stresses while optimising yield. In 2018, the biostimulant market in Europe accounted for 600 million USD and the global market for these products is estimated grow to 4.14 billion USD by 2025 (Madende and Hayes, 2020).

Proteins, peptides and their hydrolysates, as well as amino acids and mixtures of them are examples of biostimulant compounds that have been shown to elicit positive responses in plants at different levels. Microorganisms and their capacity to form mutualistic symbiotic relationships with plants may also stimulate the plants to improve their living conditions. These two and the relationship between them comprise the most relevant areas of biostimulant science for this thesis, with plant soil microbial communities having been discussed in **Section 1.3.1** and amino acid biostimulant effects discussed in the following **Section 1.5.2**.

Other biostimulant products used in plants are humic and fulvic acids, substances formed from biochemical transformation of plant, animal or microbial matter that can increase root growth (Rose *et al*., 2014) and reduce Pb toxicity (Shahid *et al*., 2012) because of their structural characteristics. Biopolymers such as chitosan have been used as biostimulants too, which can improve plant defence responses against infectious pathogens and physical damage (Pichyangkura and Chadchawan, 2015). Seaweed or algae extracts, on the other hand, can act as chelators by improving mineral utilisation, and can also enhance different life processes from germination to stress response and yield (Calvo *et al*., 2014), likely due to the presence of plant growth hormones, (Tarakhovskaya *et al*., 2007). Inorganic compounds on their own have also been reported to have biostimulant effects regardless of their chemical nature, with effects such as stress resistance via osmoregulation, thermal regulation, wall rigidification and reduced transpiration. The most researched elements with such characteristics are Al, Co, Na, Se, and Si (Pilon-Smits *et al*., 2009).

Biostimulants can have different effects in plant metabolism. They have been shown to improve assimilation of major elements such as C, nitrogen (N) and sulphur (S) (Jannin *et al*., 2013), increase photosynthetic activity and stress response (Caulet *et al*., 2014), alter senescence and enhance ion transport (Khan *et al*., 2009; Parađiković *et al*, 2011). However, due to the complex and often uncharacterised nature of biostimulants it is difficult to assume that an unknown amount of often uncharacterised molecules being taken up by plants via foliar or soil application can elicit reproducible positive responses into complex, dose dependent metabolic mechanisms (Yakhin *et al*., 2017). Thus, more precise information about the components of biostimulants and their application are required to accurately be able to determine the cause-and-effect relation of the components applied to plants.

Signalling, gene expression and hormone interaction effects of biostimulants are similarly complex and best understood when the components of the biostimulant are known. Full transcriptome and metabolome analysis are required to understand the full scope of genetic changes triggered by specific compounds. Humic, substances, organic materials, seaweeds, free amino acids, and plant extracts have stated an effect on plant hormonal status (Du Jardin, 2012; Yakhin *et al*., 2012), although this may be because of hormone-like components present in said biostimulants or because some components in them may induce the production of hormones by acting as precursors or activators (Parađiković *et al*., 2011).

1.5. Amino acids

Amino acids are molecules containing an amino and a carboxylic group, as well as a variable side chain, the R group, that gives individual amino acids their specific properties. The primary function of amino acids in biological systems is as the basic units of proteins, binding one to the next via peptide bonds to form full proteins for performing a vast variety of functions. For this reason alone, they are key in all living beings, but amino acids also perform a large variety of other functions carried out by specific amino acids and in specific conditions, which can also be essential and help organisms in specific conditions.

In plants, amino acids are primarily present in protein form. In plant amino acid synthesis, most of the the O and C have their origin in the air and the hydrogen (H) and N are taken from the soil water. Direct amino acid assimilation is possible in plants (Näsholm *et al*., 2009) but it is considered that microorganisms widely outcompete plants in this regard and the relevance of direct amino acid uptake by plants is controversial (Sauheitl *et al*., 2009; Warren, 2011), the mechanisms for this not being completely understood (Adamczyk, 2021).

Physiological activities have been shown to be altered by the presence of specific amino acids in plants, with effects such as stress resistance, effect on photosynthesis, chelating effect, action on stomas, phytohormone relation, pollination, fruit formation, soil flora equilibrium and others.

1.5.1. Nitrogen assimilation and amino acid synthesis

In N uptake by roots, the way N is fed to the plant, in the form of ammonium (NH_4^+), nitrate (NO_3^-), ammonium nitrate (NH_4NO_3) or urea (CH_4N_2O) affects several steps of N metabolism, N use efficiency and seed yield (Coleto *et al*., 2017; Furtado da Silva *et al*., 2020; Heuermann *et al*., 2021). Urea is the most used form of N fertiliser in crops and can be directly taken up by plants, but most of it is rapidly hydrolysed into NH_4^+ by soil ureases (Pinton *et al*., 2020).

Plant amino acids are synthetised from the precursors glutamate (Glu), glutamine (Gln), aspartate (Asp) and asparagine (Asn) (Xu *et al*, 2012). From these, Glu is the main route of N absorption and assimilation in plants. Glu assimilation mainly occurs by acquisition of NH_4^+ and NO_3^- from the roots. NO_3^- will be reduced to NH_4^+ via nitrate and nitrite reductases first in order to be assimilated by the plant (Lam *et al*, 1996). NH_4^+ will mainly be assimilated into the plant amino acid pool via the Gln synthetase-Gln oxoglutarate aminotransferase (GS-GOGAT) cycle, synthetising Gln, Glu, Asp and Asn, from which the rest of the plant amino acids will be synthetised (Xu *et al*., 2012). Glu will be transferred into different amino acids via

aminotransferases, and Asn synthetase (AS) will catalyse Gln formation from Asn and Glu, playing a crucial role in N metabolism (Lam *et al*, 1996). NH_4^+ is toxic for plants at higher concentrations, and NADH dependent Glu dehydrogenase (NADH-GDH) will be able to reduce NH_4^+ concentration in the plant by incorporating this molecule to create Glu by incorporating it to α-ketoglutarate (α-KG) (Masclaux-Daubresse *et al*, 2010). GDH is a reversible enzyme and can also function in the opposite direction by deaminating NH_4^+ from Glu to produce α-KG, replenishing the tricarboxylic acid (TCA) cycle, although only *in vivo* GDH activity has been demonstrated (Skopelitis *et al*., 2007). NADP(H) dependent GDH activity has also been seen in plants although it is less prevalent and less studied (Fontaine *et al*., 2012). Plant N biosynthesis pathways are summarised in **Fig. 1.3**.

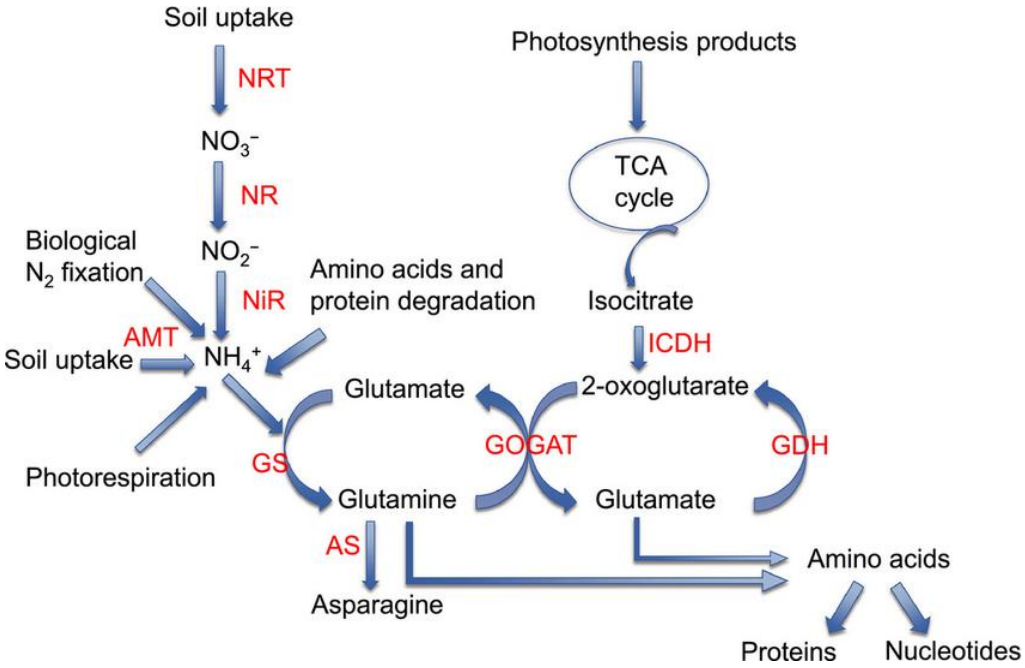


Fig. 1.3. Nitrogen-assimilation pathways in higher plants (from Lu *et. al.*, 2016). Inorganic nitrogen in the form of nitrate (NO_4^-) or ammonium (NH_4^+) becomes incorporated into amino acids and other organic molecules as depicted. The specific steps shown: nitrate transporters (NRT), nitrate reductase (NR), nitrite reductase (NiR), ammonium transporters (AMT), glutamine synthetase (GS), glutamate synthase (GOGAT), asparagine synthetase (AS), glutamate dehydrogenase (GDH), and isocitrate dehydrogenase (ICDH).

In plants, synthesis of main proteic amino acids is done via different metabolic branches, derived from glycolysis metabolites such as 3-phosphoglycerate (serine (Ser), glycine (Gly), cysteine (Cys)) and phosphoenolpyruvate (via shikimate pathway forming aromatic amino acids tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe)), from pyruvate (alanine (Ala), valine (Val), leucine (Leu)) or from TCA cycle molecules such as oxaloacetate (Asp, Asn, threonine (Thr), Isoleucine (Ile), methionine (Met), lysine (Lys)) and α-KG (Glu, proline (Pro), Gln, arginine (Arg), histidine (His)) (Yang *et al*., 2020). (**Fig. 1.4**).

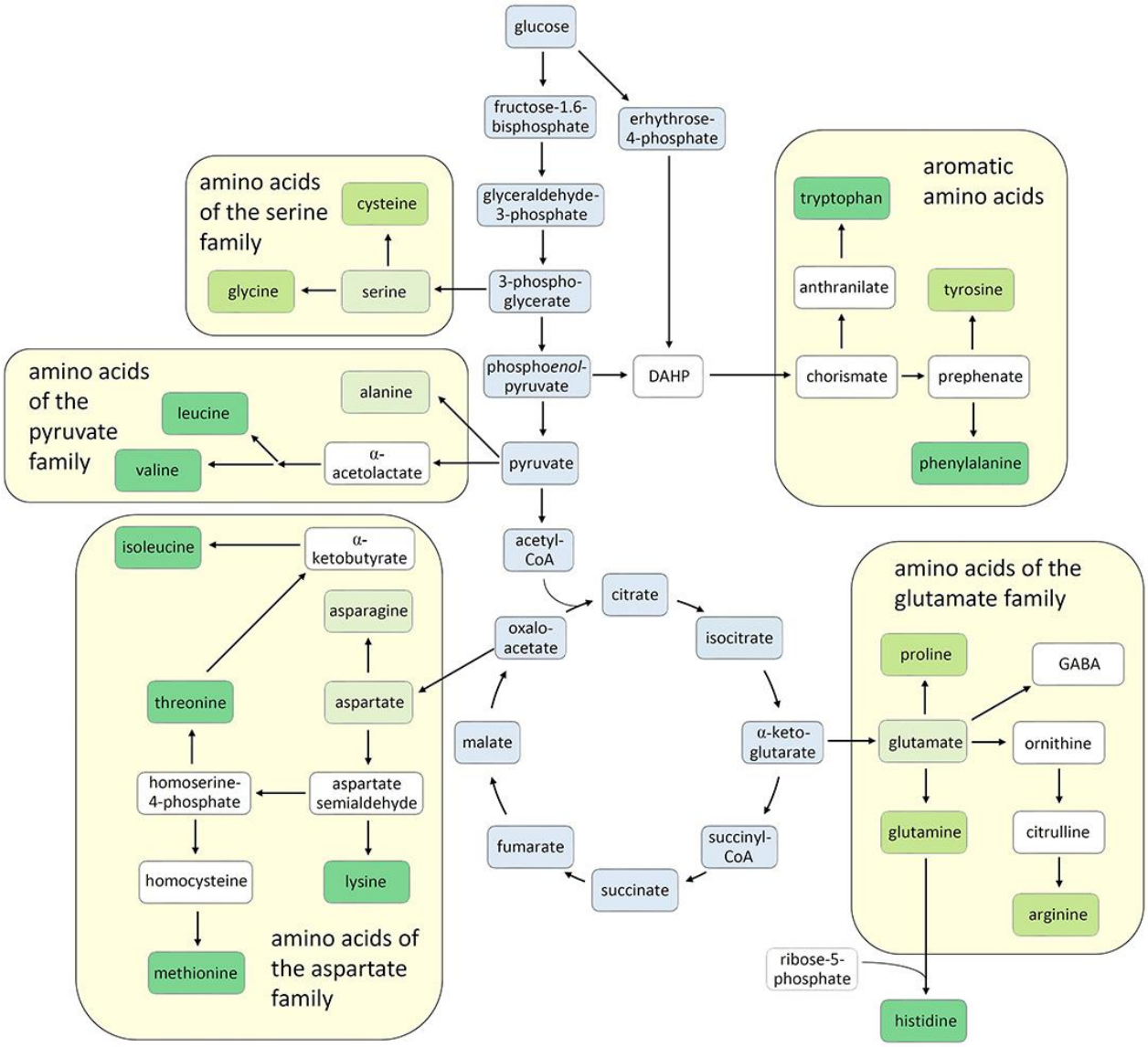


Fig. 1.4. Main amino acid biosynthesis pathways in plants (from Trovato *et al*., 2021). The carbon skeletons of amino acids are derived from different intermediates of the central carbon metabolism (boxed in blue). According to their respective precursors, the amino acids are grouped into five families derived from glutamate, serine, pyruvate, aspartate, or chorismate. The nine amino acids that cannot be synthesized in animals are shown in dark-green boxes, while those that can be synthesized but additionally need to be taken up with the diet are in brighter boxes. Proteinogenic amino acids that can be sufficiently synthesized in animals are in pale green boxes and non-proteinogenic amino acids and other important intermediates are boxed in white. DAHP = 3-deoxy-D-arabinoheptulosonate-7-phosphate.

1.5.2. Protein hydrolysates and amino acids as biostimulants

There have been a great number of products and claims regarding peptides or amino acids in terms of biostimulant action beyond their role as nutrient resource and protein formation, ranging from growth stimulation to improvements in stress response. These can be classified in two main categories: protein hydrolysates and individual amino acids (Calvo *et al*., 2014). Protein hydrolysates are usually a mixture of peptides obtained from animal or plant source and often contain other molecules as impurities. Because of their complex and often unspecified composition it can be difficult to identify which component is actually having an effect in plants. Individual amino acids, on the other hand, involve the addition of one or more proteic or non-proteic amino acids to plants. This entails a much more defined addition that can be controlled in terms of aggregated molecules and their concentrations, and evidence of the benefit of amino acid addition to plants is considerable (Sharma and Dietz, 2006; Forde and Lea, 2007; Liang *et al*., 2013).

Studies have shown mixed results regarding nutrient uptake and impact of yield when applying protein hydrolysates. Gajc-Wolska *et al*. (2012) found no statistically significant effect of the commercial product Aminoplant (Siapton) (Isagro, Milan, Italy) on endive, although Parrado *et al*. (2008) found increased plant height and flowers in Siapton fertilised tomato plants. Maini (2006) and Schiavon *et al*. (2008) showed that the same Siapton product enhanced activity of N metabolism enzymes such as NAD-GDH, nitrate reductase, nitrite reductase, GS, GOGAT and Asp aminotransferase, as well as TCA cycle enzymes malate dehydrogenase, isocitrate dehydrogenase and citrate synthase. Ertani *et al*. (2009) used hydrolysates of both plant (alfalfa) and meat (meat flour) origins and observed increased GS and nitrate reductase activities in both shoots and roots. Koukounararas *et al*. (2013) found increased levels of yield in tomato after applying the high amino acid containing commercial hydrolysate Amino16 (EVYP, Greek industry of hydrolysed protein LLP, Thessaloniki, Greece). There is evidence for a positive impact of protein hydrolysates to plant physiology via a coordinated C and N metabolism regulation (Calvo *et al*., 2014), but the nonspecific nature of complex protein hydrolysates (often with poor component descriptions in the literature) makes it difficult to narrow down specific action mechanisms responsible for changes.

In contrast to protein hydrolysates and complex mixtures, adding a single amino acid or a defined mixture of them allows pinpointing the effect of this addition much more precisely under the tested conditions. Either by exogenously applying the desired compounds or by forcing the plant to produce them, their effect can be better understood, and it can also help in

the specification of how the previously mentioned hydrolysate compounds are working. Various amino acids and peptides have shown to have a signalling function that regulates plant physiology (Forde and Lea, 2007; Ivanov, 2010) and exogenously applied amino acids have shown to be able to act as signalling molecules or influence hormone action (Tegeder, 2012).

Assessing the potential for the application of pyroglutamic acid (PGA) as a biostimulant was a key original aim of this thesis. Because of their close metabolic relationship, the amino acids Pro, Glu and PGA are considered to be of central importance in this thesis, and are therefore reviewed in detail in the following sections.

1.5.2.1. Proline

The most studied amino acid in terms of functions outside of protein metabolism is undoubtedly Pro. Pro is mainly synthetised from Glu through a number of reactions occurring in the chloroplast, and at the same time Glu can be converted into Pro through reactions occurring in the mitochondria. There are also other ways plant cells can obtain Pro, such as transamination from ornithine, with more relevance under stress (Xue *et al*., 2009).

Intracellular Pro levels are a result of a complex equilibrium of Pro metabolism as well as transport between cells and cellular compartments. Housekeeping Pro biosynthesis occurs mainly in the cytosol (Szekely *et al*., 2008) but when under osmotic stress conditions, production in the chloroplast increases (Strizhov *et al*., 1997, Szekely *et al*., 2008). Pro can be transported via specific transporters intercellularly (Rentsch *et al*., 1996) and between cellular compartments (Di Martino *et al*., 2006).

Pro accumulation can alter plant stress tolerance in different ways. It can function as a molecular chaperone to protect the integrity of some enzymes and enhance their activity during thermal (Rajendrakumar *et al*., 1994), heavy metal and osmotic (Sharma and Dubey, 2005) stresses. Exogenous Pro application via foliar spray has been shown to alleviate salt stress to an extent in *Brassica juncea*, although this was not enough to recover the plants at higher salt concentrations (Wani *et al*., 2016). Antioxidant properties has also been related to Pro, promoting reactive oxygen species (ROS) scavenging activity (Matysik *et al*., 2002) and a reduction of Pro in mutants shown to increase oxidative damage (Szekely *et al*., 2008). Increased Pro contents also protected the activity of enzymes associated with relieving stress (Hoque *et al*., 2008). Factors such as water stress, changes in light, pathogen detection, changes in photoperiod and salt stress can alter Pro metabolism leading to increased Pro biosynthesis

and accumulation of this amino acid. Relieving plants from specific stresses such as rehydration can increase Pro catabolism towards Glu (Szabados and Savouré, 2010).

Studies have also suggested a role of Pro as a regulatory molecule as well, having signalling functions. Pro content supply has been related to adequate plant development (Szekely *et al*., 2008), with enhanced Pro content leading to early flowering in *Arabidopsis* and a lower content leading to late flowering (Mattioli *et al*., 2008). In *Arabidopsis*, it has also been observed that a third of rehydration inducible genes can also be induced by Pro (Oono *et al*., 2003). Pro can also play a role in apoptosis, by enhancing other expression factors related to ROS and pathogen or viral infections (Fabro *et al*., 2004).

1.5.2.2. Glutamate

Glu plays a major and central role in plant amino acid metabolism. It can be synthetised via Glu dehydrogenase (GDH) to assimilate NH_4^+ with α-KG (also known as 2-oxoglutarate (2-OG)), in a reversible reaction that can also release NH_4^+ in the other direction under specific conditions. It is also part of the inorganic N assimilating GS-GOGAT cycle, where it is formed by GOGAT (which is also known as Glu synthase) using Gln and 2-OG, and where Glu is used for synthetising Gln by incorporating NH_4^+ using the GS enzyme. The amino group in Glu can serve as basis for several amino acids via different aminotransferases and its C skeleton and amino group are used in the synthesis of Pro, as well as also providing the same amino group for Arg and γ-aminobutyric acid (GABA) synthesis (Ginguay *et al*., 2017). Glu is also a precursor for chlorophyll synthesis in developing leaves (Yaronskaya *et al*., 2006).

Different N supply to the plant has shown to alter Glu levels in different ways, while also affecting the levels of other related amino acids metabolically linked to it. An increase in N input to plants has shown to slightly increase Glu concentration in leaves, while a greater concentration increase occurs in Arg, Gln, Asp and Ser, with lesser effects on roots (Geiger *et al*., 1999). Comparing NO_3^- starved tobacco (0.2 mM), to plants with high concentration of NO_3^- (12 mM) shows that the latter increases concentrations of Glu much less than it does with Ala, Asn, Asp, Gln, Gly and Ser, which reached 10 to 20-fold increases while Glu did not increase over 2-fold (Fritz *et al*., 2006). In *Arabidopsis* seedlings, NO_3^- starved plants decrease Glu and Gln concentrations while 2-OG levels increased, which was reversed after adding NO_3^-. NH_4^+ nutrition has shown to increase in-tissue amino acids levels up to 30-fold in tobacco, while Glu levels remain steady (Terce-Laforgue *et al*., 2004). Increased NH_4^+ assimilation helps to mitigate NH_4^+ toxicity at higher concentrations (Coleto *et al*., 2017). To maintain homeostasis, Glu metabolism is regulated by N nutrition and its quantity and form

availability, and higher N levels in the medium will increase N uptake by the plant, which will not mainly be accumulated as Glu but converted into other amino acids that have much more variable rates than Glu under different nutrition and stress conditions. The relatively constant Glu levels in plant have been suggested to be related to the large number of enzymes that form Glu or use it as a substrate, which depend on the availability of 2-OG to form Glu from Gln via aminotransferases or GS and on the supply of other 2-oxo acids to form other amino acids from Glu via transamination (Forde and Lea, 2007). GS-GOGAT cycle and GDH are the main enzymatic pathways that regulate Glu concentration, together with controlling N assimilation (Masclaux-Daubresse *et al*., 2006).

1.5.2.3. Pyroglutamic acid

PGA, also known as 5-oxoproline, is the cyclic form of Glu, a little-studied intermediary in the glutathione (GSH) degradation pathway. It can be found in tissues as a free compound and is not present in proteins, except for the N-terminal residue of some (Van der Werf and Meister, 1975). It can also be produced from the spontaneous cyclisation of the highly unstable activated Glu in some incomplete reactions (Kumar and Bachhawat, 2012). However, the aforementioned GSH degradation pathway, shown in **Fig. 1.5**, is the main source of PGA in plant tissues and PGA synthesis from GSH will occur in the cell cytoplasm by action of γ-glutamyl cyclotransferase (GGC). In mammals, GSH degradation is mainly done via γ-Glutamyl transpeptidases (GGT), but in plants this is a secondary pathway that takes place in the extracellular space and provides Glu and Cys-Gly conjugates to the GSH cycle (Ohkama-Ohtsu *et al*., 2008).

PGA will be degraded to Glu by action of 5-oxoprolinase (5-OPase), in an ATP-dependent reaction that is, to our knowledge, the only major *in vivo* demonstrated way of degrading PGA in plants. The disruption of the 5-OPase encoding OXP1 gene in *Arabidopsis* has shown that this mutant accumulates PGA while reducing the amount of Glu present in tissue (Ohkama-Ohtsu *et al*., 2008), confirming GGC is the mayor GSH degradation pathway in plants. Glu can be recycled via γ-glutamyl-Cys (γ-EC) synthetase and GSH synthetases to produce GSH. Notably, all steps of GSH metabolism are not experimentally confirmed, such as GSH conversion to PGA via GGC and alternative GSH degradation pathways that bypass PGA (Ohkama-Ohtsu *et al*., 2008).

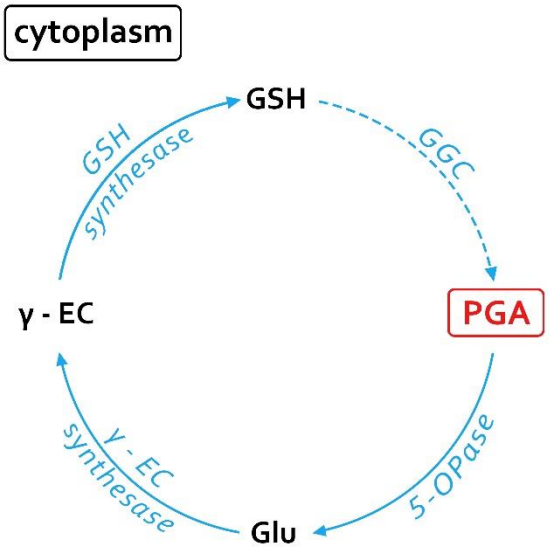


Fig. 1.5. Pyroglutamic acid metabolism as part of the GSH degradation pathway. Solid lines are experimentally confirmed pathways and dashed lines represent proposed pathways. PGA = pyroglutamic acid, GSH = glutathione, Glu = glutamate, 5-OPase = 5-oxoprolinase, γ-EC = γ-glutamyl-cysteine, GGC = γ-glutamyl cyclotransferase.

For a long time, PGA has been regarded solely as an intermediary in the GSH degradation cycle and its application has been ignored (Mazelis and Creveling, 1978; Kumar and Bachhawat, 2012). In recent years, however, this molecule has been more widely studied for its potential usefulness, such as demonstrating its potential to reduce plant sensitivity to the antibiotic sulfamethoxazole by blocking the metabolisation of PGA to Glu using 5-OPase-defective *Arabidopsis* mutants. (Schreiber *et al*., 2012). It can provide crop protection against fungi, improving barley response to *Fusaria* by affecting the biosynthesis of phenolic acids and flavonoids (Bilska *et al*., 2018) and conferring protection against the dominant fungal pathogen *Zymoseptoria tritici* in wheat (Mejri *et al*., 2019). Low concentration of PGA can also stimulate specific secondary metabolic routes including phenylpropanoids (Bilska *et al*., 2018). All these suggest a role of PGA in stress response and for plant protection. Furthermore, in the cases where PGA was externally added, the low concentration of this addition implies that the changes brought by PGA addition may be through signalling rather than simply by metabolic assimilation.

In terms of improving crops for agriculture, a recent study by Jiménez-Arias *et al*. (2019) used PGA to promote drought tolerance in lettuce, with plants under drought-stress having enhanced photosynthetic rate and maintaining osmotic and water balance. The same authors have further used PGA in a study this year to present data that also highlights the potential of this amino acid to prevent losses from drought-stress in maize (Jiménez-Arias *et al*., 2022).

Although scientific literature on PGA is scarce, it has been directly related to a number of patents for agricultural use (Unkefer *et al*., 2004, Alvarez Builla Gomez *et al*., 2015) and it is used in agriculture claiming improved N uptake and assimilation (Fulvix PGA, Unium Bioscience, Doncaster, UK). This raises questions about the use of it in commercial agricultural products and emphasises the need to understand its effect in crops.

1.5.3. Amino acid-related GLR signalling in plants

As described above, amino acids play a role in several plant physiological processes beyond protein formation. Particularly, they are involved in stress-response functions. For example, alternative metabolic routes using amino acids as substrates can be activated when anabolic reactions are supressed using a recently elucidated Cys signalling pathway (Heinemann and Hildebrandt, 2021). However, the use of amino acids as signalling molecules and the study of associated processes are still an emerging topic and fundamental discoveries are still to be made.

One of the mechanisms associated with amino acid-mediated signalling in plants are Glu receptor-like channels (GLRs), homologs to mammal ionotropic Glu receptors (iGluRs), which are non-selective cation channels gated by ligands that play a role in the nervous system (Mayer, 2016). Plants GLRs, despite having Glu in their name, have shown a wide array of amino acid ligands with as many as 12 of them, and with Glu not always having the highest affinity (Qi *et al*., 2006; Forde and Roberts, 2014). These GLRs are considered Ca^{2+} channels (Vincill *et al*., 2012; Tapken *et al*., 2013) and have been related to many physiological effects including stomata movement (Cho *et al*., 2009), changes in root development (Vincill *et al*., 2013) and both abiotic and biotic stress responses such as jasmonic acid (JA)-related Ca^{2+} mechanisms (Kang *et al*., 2006), wound-signal transduction (Mosauvi *et al*., 2013) and pathogen and damage associated molecular pattern (PAMP/DAMP) recognition (Kwaaital *et al*., 2011; Moeder *et al*., 2019).

Although there is no fully confirmed mode of action for plant GLRs as part of plant signalling processes, studies to date suggest that GLRs can perceive changes in apoplastic amino acid concentrations resulting from cell damage (damage-associated molecular patterns (DAMPs)) or from PAMP mediated exocytosis. In this way, GLRs work in parallel with other receptors and pattern-recognition receptors (PRRs) to activate JA-dependent defences in case of herbivore attack or salicylic acid (SA)-mediated defences in case of pathogen attack (Forde and Roberts, 2014) (**Fig. 1.6**).

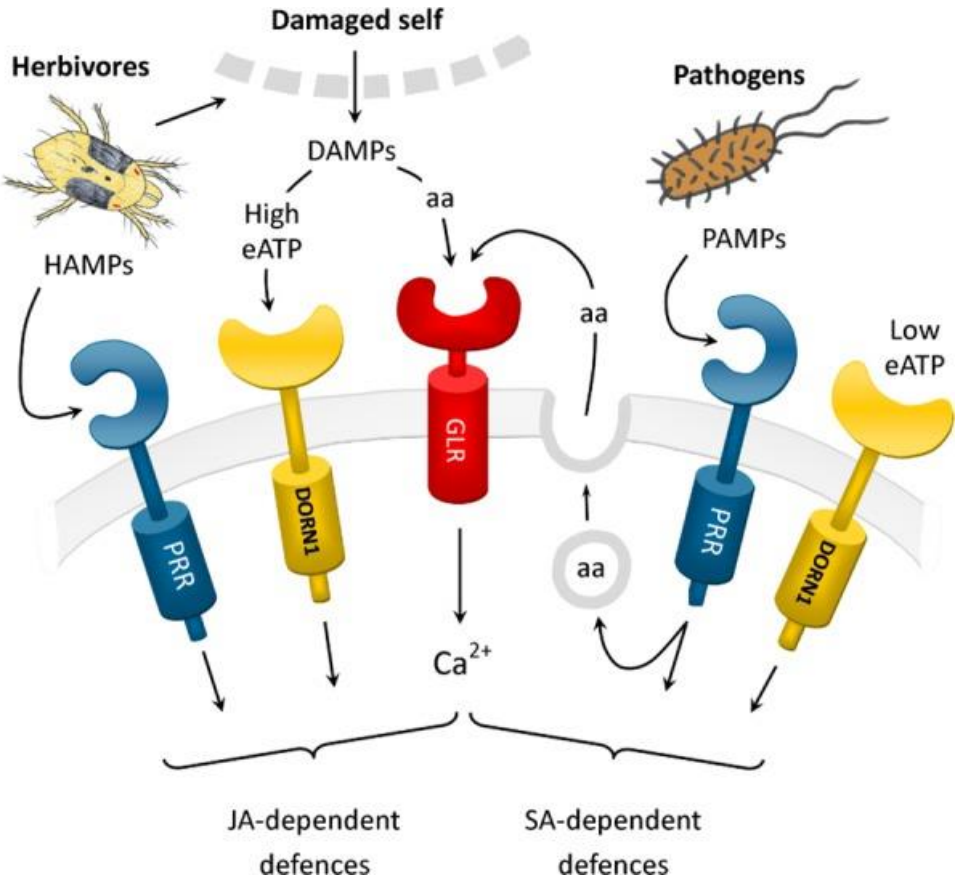


Fig. 1.6. Speculative model for the role of glutamate-like receptors in the regulation of plant defence responses (from Forde and Roberts, 2014), acting in parallel with other receptors such as plant purinergic receptor DORN1 and pattern-recognition receptors (PRR) to activate jasmonic acid (JA) and salicylic acid (SA)-mediated defences.

1.6. Aims

Interest in biostimulants is on the rise, representing an opportunity to improve crop performance and reduce reliance on traditional fertilisers. The central idea behind this thesis is the study of amino acids and their functions as biostimulants to identify novel uses for them that can contribute to their application in crop science and agriculture. This is divided into two general aims that branch into more specific objectives.

General aim 1: To study PGA, an amino acid that has garnered the interest of the agricultural industry despite its metabolism and effects on plants not being completely clear, discerning if it can be useful for crop improvement. This main aim is broken down in the following specific objectives:

- To determine and measure biostimulant effects of pyroglutamic acid on plant growth and performance.
- To establish if the effect of pyroglutamic acid is the same in the model plant *Arabidopsis thaliana* and in wheat.
- To investigate whether its effects are dependent on the absence or presence of stress and in controlled and uncontrolled environments.
- To identify whether the effects of pyroglutamic acid on plant physiology are a product of downstream metabolic conversion of pyroglutamic acid to glutamate or they are different and independent of conversion.

General aim 2: To compare the biostimulant effects of several amino acids under the same conditions, contrasting with most studies on the topic, which focus on a small number of amino acids and are not consistent with experimental conditions. This aim has the following specific objectives:

- To screen a variety of amino acids applied in low concentration, identifying and comparing biostimulant effects of each.
- To determine if the biostimulant effects of the studied amino acids are the same under all conditions or are triggered by specific circumstances.
- To identify changes in the rhizosphere of field-grown wheat after the application of amino acids, addressing the microbiome as a target of interest for biostimulants.

Chapter 2. Amino acid biostimulant application has differential impacts depending on species and stress conditions

2.1. Introduction

Crop improvement practices in agriculture are as much a commercial goal as a global necessity, with the current and future prospects of rising food demand and decrease in food security (FAO, 2021A). Research and development in plant science often faces challenges when translating molecular biology into controlled environment studies and these into field trials (Nelissen *et al*., 2014), resulting in difficulties applying known mechanisms of interest into commercial crops or when adding compounds such as biostimulants that can be effective, but their detailed mode of action is not completely understood (Yakhin *et al*., 2017).

Compared to the technical difficulties and legislative limitations of biotech crops (Halford, 2019), improving the nutrients and compounds available in the field is more straightforward to translate into crops. Crops generally feed from soil nutrients, receive water from rain and additional sources and can be supplied with pesticides for protecting yield from biotic stressors (AHDB, 2021). Spray application of major nutrient sources can be done using NPK (nitrogen-phosphorous-potassium) fertilisers and their need and efficacy are well documented (Yousaf *et al*., 2017; Arifin, 2019), but other additions, such as biostimulants that act not as nutrients but as triggers for specific natural processes or effects are on the rise as a multibillion-dollar market aiming to decrease fertiliser usage and improve yields (Madende and Hayes, 2020).

Amino acids are of utmost importance in plant life due to their role in protein synthesis and N metabolism. Additionally, other roles for different amino acids have been proposed: Pro accumulation under stress conditions and its protective functions have been widely reported and reviewed in *Arabidopsis thaliana* and crops (Forlani *et al*., 2019; Hanif *et al*., 2021; Meena *et al*., 2019; Mattioli *et al*., 2020), giving special relevance to this amino acid concerning stress defence. Other amino acids have also been reported to have desirable specific effects, such as GABA, which is related to several developmental and metabolic processes and improves stress response (Gramazio *et al*., 2020), and aromatic amino acids (*e.g*., Phe, Trp, Tyr) which have been associated with secondary metabolism synthesis and tolerance to both biotic and abiotic stresses (Oliva *et al*., 2021).

PGA or 5-oxoproline is the cyclic form of glutamic acid. This non-protein amino acid is formed during the GSH degradation cycle (Ohkama-Ohtsu *et al*., 2008), and later converted to Glu via

5-OPase (Mazelis and Creveling, 1978). This is the only known metabolic use of PGA in plants (Mazelis and Pratt, 1976). This molecule has generally been regarded as an intermediary metabolite (Kumar and Bachhawat, 2012) in both animals and plants, meaning that other potential effects have been largely ignored. As PGA metabolism directly leads to Glu formation, its role of metabolic intermediary is unequivocally relevant: Glu is known to have signalling functions (Forde and Lea, 2007) related to GLRs that are able to bind Glu as well as other amino acids and GSH to provide sensitivity towards internal or external stimuli (Price *et al*., 2012; Weiland *et al*., 2016). Plant GLR functions are not clearly defined but there is evidence of an inter-cellular signalling pathway related to their function as Ca^{2+} channels gated by amino acids (Forde, 2014). GLR genes and their alteration have shown to play a role in the immune responses of the plant and the defence against pathogens (Forde and Roberts, 2014).

However, a few recent studies have been using PGA as a plant biostimulant of interest in crop development and protection, *e.g*. helping cope with abiotic plant stress (Jiménez-Arias *et al*., 2019), increasing protection from *Zymoseptoria tritici* fungus (with positive results using *in vitro* antifungal assays and more limited success in greenhouse wheat using soil inoculation) (Mejri *et al*., 2019), and stimulation of secondary metabolism including trichothecenes and phenylpropanoids (Bilska *et al*., 2018). These studies all use PGA in low concentrations that do not change overall amino acid or N contents in tissue. All these studies also focus on the overall changes in crop growth and stress protection, with very little to define any mechanism responsible at a metabolic, biochemical or cellular level. Metabolic presence of 5-OPase (Rennenberg *et al*., 1981) and its relevance in Glu levels (Ohkama-Ohtsu *et al*., 2008) has been demonstrated in plants but very few studies have put a focus on discussing PGA effects in metabolism (Paulose *et al*., 2013) and the processes it may have a role in (Bilska *et al*., 2018).

Agricultural crop market products have been seen including PGA as a yield enhancer and protector (Fulvix PGA, Unium Bioscience, Doncaster, UK). However, as mentioned above, the effects of PGA in plants and its specific modes of action are not fully understood, representing an interesting opportunity to understand a metabolite that is potentially useful in plant and agricultural science. As other amino acids also show changes in different circumstances, the effects of PGA addition can be addressed as a step to identify biostimulant actions of this amino acid. This chapter addresses the behaviour of *Arabidopsis* and wheat after PGA addition, as well as the effects of altering PGA metabolism by means of a 5-OPase defective mutant. The experiments also expand to screenings of a number of other amino acids added in low concentrations in both mentioned species to identify direct biostimulant effects.

2.2. Materials and methods

2.2.1. Wheat growth assays

For growth assays, winter wheat (*Triticum aestivum)* plants of the commercial variety KWS Lily (Einbeck, Germany, www.kws.com) were used. Plants were grown for two and four months in controlled environment (CE) rooms, with the two-month growth assay being repeated twice as independent experiments and the four-month growth assay being a single experiment. First, germination was induced by putting the seeds in Petri dishes with wet folded filter paper for three days, and then moving them to the CE room. Seedlings were planted in 11 cm 1 L pots with Levington Advance Pot & Bedding M3 compost (ICL, Ipswich, UK) and grown for two months with 16 h days at 23ºC and 8 h nights at 18ºC with 50% relative humidity and with a light intensity of 120-150 µmol m^{-2} s^{-1} during the day. Well-watered plants were watered two to three times a week as needed to maintain moisture on the soil, while the plants with limited watering were watered half as often. Spray treatments of water, NPK mixture (Nutrifast Accolade, Nutrel, Lincoln, UK) or NPK + PGA were applied once after three weeks for plants that would grow for two months. For NPK, 2.3 mL were diluted in 1 L water to dilute it in the same way the product is recommended to be applied in the field (3 L/ha), and for NPK + PGA treatment 25 µL of 50 g/L (0.39 M) PGA was added to the first undiluted NPK solution and the same dilution was applied afterwards. These concentrations of NPK and PGA were in line with the ones used by the partner company Environmental Crop Management Ltd. (ECM) in their previous trials and in the commercial use of similar products. Plants were randomised in the growth space and randomly moved around each week. For plants grown for four months, the same growth procedure was used with two spray application after three and seven weeks, also using the same spray application procedure and concentrations.

Shoot fresh weight (FW) was calculated by cutting the above ground plant parts and weighing them. In two-month-old plants root FW was calculated immediately after cleaning the soil from the root with water and drying the root with paper towels. Total FW was calculated as a sum of both root and shoot weights and the shoot/root weight ratio was calculated by dividing shoot weight by root weight. In four-month-old plants, tillers were counted by hand and hydration % (water weight %) was calculated taking disk samples from flag leaves, drying them for 48 h in a 60ºC oven and calculating the % of lost weight.

2.2.2. Wheat yield assays

For wheat taken to full yield, spring wheat (*Triticum aestivum* cv Paragon) and Petersfield compost (Petersfield, Leicester, UK) were used. Plants grew in semi-controlled environment in a greenhouse in Lancaster Environment Centre (LA1 4YQ, Lancaster, UK), with a 16 h photoperiod at 25ºC and 8 h dark period at 18ºC and a photosynthetic photon flux density of approximately 300 μmol m^{-2} s^{-1} at the top of the canopy. Plants were individually potted and located in pots in a randomised way along the greenhouse, changing the layout every two to three weeks. Plants were planted 2 cm deep in 11 cm diameter 2 L pots and were loosely covered with dark plastic for three days in fully wet pots for germination. The pots were then uncovered and watered as needed. The amino acid treatments were applied via spray in the same concentrations used in the experiments of **Section 2.2.1** and at the same time points as the plants grown for four months in said section.

For measuring parameters, the endpoint to the experiment was defined when the plants were fully ripe, having a completely golden-brown colour and the grain was hard. Tillers for each plant were counted by hand and plant height was measured with a metre ruler. Subsequently the full above ground plant mass was cut from the soil and weighed. The number of spikes was counted, and three representative spikes were chosen per plant for manually counting the average number of spikelets. The spikes were put through a wheat threshing machine (Haldrup LT-15, Haldrup, Ilsofen, Germany) to separate the seeds from the chaff and the number of seeds were counted taking 3 g of seeds per spike with the SeedCounter app on Android using a smartphone (Komyshev *et al*., 2017) and extrapolating this to the fresh grain yield. All of the seeds were later put in bags and dried at 60ºC for 48 h for measuring the dry seed yield.

2.2.3. Arabidopsis assays

2.2.3.1. Rosette growth assay

Col-0 *Arabidopsis thaliana* seeds were used for growing in half strength (2.15 g/L) Murashige and Skoog (MS) basal media (Duchefa Biochemie, Haarlem, Netherlands) (Murashige and Skoog, 1962) with 1% sucrose, 0.8% agar and a pH adjusted to 5.8. Sucrose was used in the seedling growth media as it increases growth rates under conditions when photosynthesis is limited by poor transpiration and gas exchange (like parafilm-closed plates) (Lei *et al*., 2011). The addition of sucrose, although common practice, can function as a signal in plants, impacting phenotypes. However, the 1% concentration used here is relatively low and has even been used as a baseline for trialling the effect of sucrose itself (Roycewicz and Malamy, 2012).

Before plating them, seeds were sterilised with a 5 min rinse with 70% ethanol + 0.1% Triton-X and two 1 min rinses with 95% ethanol. 12 seeds per plate were plated in each of the three plates per PGA concentration, with concentrations of 0 (control), 1, 10 and 100 μM and 1 and 10 mM, previously filter-sterilised with 0.2 μM filters and added to the media at the time of plating the seeds. Differences were observed after 21 days of growth.

2.2.3.2. Root growth assays

Col-0 *Arabidopsis thaliana* plants were used as WT in root growth assays. Oxoprolinase 1 (*OXP1;* AT5G37830) defective *Arabidopsis thaliana oxp1* mutants were obtained from Nottingham *Arabidopsis* Stock Centre (NASC) (Nottingham, UK). These *oxp1* mutants had a T-DNA insertion in the second exon of the *OXP1* gene (designated *oxp1-1* in Ohkama-Ohtsu *et al*., 2008) in the Col-0 background (SALK line N578745; Alonso *et al*., 2003).

Half-strength MS basal media at the same conditions described in **Section 2.1.3.1.** was autoclaved and 100 mM NaCl was subsequently added through filter sterilisation using a 0.2 μm filter for salt treatments. 9 cm diameter sterile plates were filled with 30 mL medium inside the flow hood and previously filter sterilised (0.2 μm filter) L-amino acids were added in concentrations of 100, 200, 300, 400 and 500 μM. The used amino acids were PGA, Glu, Pro, Gly, Ala and Arg. The amino acids were added as pure L isoforms, except for Glu, which was added as L-Glu potassium salt monohydrate to make it soluble in water.

Arabidopsis seeds sterilised as described above were plated by placing 8 seeds per plate in a row at one edge of the plate, using a total of three plates (24 seeds) per treatment. Plates with seeds were closed with parafilm and left to stratify for 48 h at 4ºC covered in foil. Plants were grown in vertical stands with an inclination of 10-15º for the roots to grow into the agar, with a light intensity of 120-150 μmol m^{-2} s^{-1}, a temperature of 22-23ºC, a relative humidity of 50% and a 16/8 h photoperiod.

Root growth was measured after 11 days in the vertical plates by photographing the plates and measuring the roots with Fiji package of ImajeJ2 software (Schindelin *et al*., 2012).

2.2.4. qPCR confirmation of mutant *Arabidopsis thaliana*

WT and *oxp1 Arabidopsis thaliana* were grown for three weeks, flash-frozen in liquid nitrogen and ground to powder with using a mortar and pestle while frozen. RNA extraction was performed using the Spectrum plant total RNA kit (Merck Life Science UK, Gillingham, UK) following the manufacturer instructions, using approximately 100 mg of tissue per sample and adjusting the lysis mixture to account for the weight differences in each sample.

Total RNA was treated with DNaseI (Thermo Fisher Scientific UK, Altrincham, UK) and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Renfrew, UK) and oligo(dT)20 according to the manufacturer instructions. Real-time PCR analysis was carried out using the AriaMx real-time PCR (qPCR) system (Agilent technologies UK, Stockport, UK) using q-EvaGreen (Qarta bio, Carson, CA, USA) as an amplification and detection mix. The following primers were used to amplify *OXP1*:

OXP1 **F** (5'- CGTTGACGTACCACCCATATCA -3')

OXP1 **R** (5'- GGGAACTACTGTGGCAACGAAT -3')

Ubiquitin was used as a control gene with the following primers:

UBQ **F** (5'- GCCAAGATCCAGGACAAGGA -3')

UBQ **R** (5'- GCTGCTTTCCGGCGAAA -3')

An initial denaturalisation of 15 min at 95ºC was made, followed by 40 cycles of 15 s at 95ºC for denaturalisation, 20 s at 62ºC for annealing (Tm was estimated at 66ºC for the used primers and the annealing temperature was adjusted to the manufacturer recommended Tm – 4ºC) and 20 s at 72ºC for extension. The qPCR analysis was used to determine the allele status of the mutant plants, be it homozygous (no amplification), heterozygous (half the relative gene expression) or non-mutant (same relative expression as WT plants). All mutant plants showed no amplification, confirming the homozygous nature of the mutation for all analysed plants without the need of calculating ΔCt for calculating changes in expression vs WT plant plants, which all amplified the *OXP1* gene.

2.2.5. Statistical analysis

All statistical analysis was performed with IBM SPSS Statistics 20 software package for Windows.

For two- and four-month-old wheat plants in **Section 2.3.1.1**, a two-way ANOVA analysis was used to analyse the effects of treatment and water status. Additionally, Tukey post-hoc tests ($\alpha = 0.05$) were performed for the parameters that showed a significant effect in the results, as well as a pair-wise analysis ($\alpha = 0.025$) between each treatment for the parameters that showed a statistical interaction effect between both analysed variables as well as for water status effect alone.

For fully-grown wheat in **Section 2.3.1.2**, a one-way ANOVA analysis was performed for each of the experiments to determine significant amino acid treatments as well as a Tukey post-hoc test for possible significant groups between treatments ($\alpha = 0.05$).

For *Arabidopsis* root growth assays in **Section 2.3.2**, results are shown as relative root growth instead of absolute growth because the experiment was done over the course of a prolonged period of time due to the closure of the growth facilities between the months of March and September 2020, which affected the ability to obtain controls that were equivalent between all amino acid treatments. Statistical tests were also performed from these relative results, including a three-way ANOVA analysis for the parameters of plant genotype, amino acid concentration and salt status, as well as the combination of these, for each of the analysed amino acids.

2.3. Results

The experiments in this chapter focussed on addressing the objectives of determining biostimulant effects of PGA in terms of growth and performance, both in *Arabidopsis thaliana* and wheat, and in ideal and stress conditions, to determine how it may be advantageous to use this amino acid as an addition to crops, and if its action is dependent or independent of the metabolic conversion of PGA to Glu. Additionally, the effect of a number of amino acids were observed in *Arabidopsis* and wheat in controlled and semi-controlled conditions, with the specific objective of screening the potential positive impact of some of them in plant growth that can lead to further crop related studies.

2.3.1. Impact of amino acid addition on NPK fertilisers on controlled environment-grown wheat

Agriculturally significant crops like wheat depend on the addition of fertilisers and additional compounds to optimise their yield output. Generally, spray mist application of fertilisers such as NPK mixtures are applied, from which they will obtain nutrients that add to the ones available in the soil. Although their mode of action is not always fully understood, biostimulant addition to macro- and micronutrient fertiliser mixtures is a fairly common practice to combat stress and improve yield, using molecules such as biopolymers, algae extracts, protein hydrolysates and amino acid mixtures for this matter (Du Jardin, 2012), . The potential benefits of PGA when added to an NPK fertiliser via foliar spray were assessed in this work.

2.3.1.1. Impact of PGA addition in wheat growth

The PGA addition experiments presented in this section, using wheat, were designed so that, although carried out in controlled environment conditions, resembled the way amino acids are typically applied in the field as much as possible. For this reason, mist-spray application was used at different growth stages and with different soil water availability as described in **Section 2.2**.

Two-month-old plants showed no significant effect of treatment, water status or interaction of both for shoot FW, root FW or total FW values despite a reduction on the means in the control treatment when water was limited as opposed to the rest of treatments (**Figs. 2.1A, 2.1B, 2.1C** and **Table 2.1**). Shoot to root ratio however showed a treatment effect, identified in both the ANOVA (**Table 2.1**) and the post-hoc analysis (**Fig. 2.1D**), with NPK treatment showing a statistically higher shoot to root ratio than the control. Water status on the other hand did not change the shoot to root ratio.

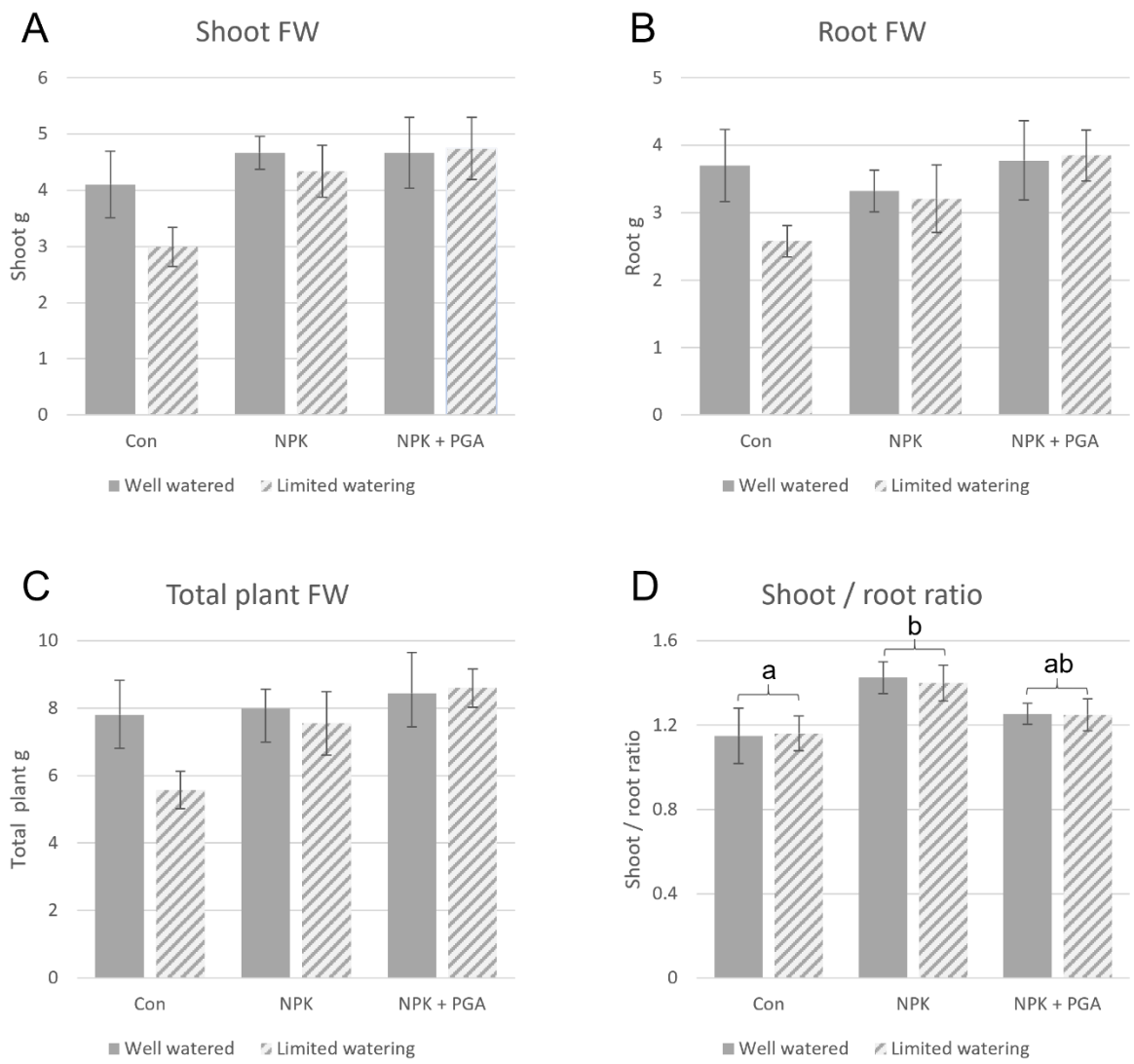


Fig. 2.1. Quantification of wheat growth after two months since germination under abundant and limited watering conditions and after the spray addition of a control treatment (Con) (using water), a commercial NPK mixture (NPK) and a commercial NPK mixture and PGA (NPK + PKG). The following parameters were measured: shoot fresh weight (FW) **(A)**, root FW **(B)**, total plant FW **(C)** and shoot/root ratio **(D)**. Columns represent mean biomass in grams ± standard error in shoot, root and total mass and the ratio ± standard error in the shoot/root ratio (n = 10). Post-hoc analysis comparing treatment is shown with letters representing statistically similar groups in shoot/root ratio, the only parameter that generated different groups (α = 0.05). Full two-way ANOVA analysis is shown in **Table 2.1**.

Table. 2.1. Two-way ANOVA analysis of the 2-month-old wheat for each of the parameters represented in Fig. 2.1: shoot FW, root FW, total plant FW and shoot to root FW ratio.

	Treatment	Water status	Treatment*Water status
Shoot FW	0.063	0.273	0.486
Root FW	0.288	0.294	0.363
Total FW	0.139	0.258	0.392
Shoot/Root FW ratio	**0.040**	0.939	0.981

After studying the effects of PGA addition in two-month-old wheat, we studied how the same plants would react after four months of growth and with the same treatments (applied twice in this case). Measuring differences in older, bigger plants allowed us to see clearer effects of plant development after applying the treatments. However, root volumes were much larger after the extra growth period, making it practically impossible to separate from the soil. Due to this, growth measurements were focused on above-ground parameters.

As expected, plants grown under limited water availability had reduced shoot FW (**Table 2.2** and **Fig. 2.2A**) The number of tillers showed differences by water status and a significant interaction between treatment and water status (**Table 2.2**). It is evident from **Fig. 2.2B** that each treatment had a different behaviour when the water status was changed, with the control treatment having very similar number of tillers for both water statuses but the other two treatments showing an increased number of tillers in plants with limited watering. Additional post-hoc analysis for tiller results showed that water status significantly affects tillering in the NPK + PKG treatment ($p < 0.001$), with NPK treatment also having notably different tillering, although non-significant ($p = 0.065$). When analysing the water content in leaves (hydration % in **Fig. 2.2C**) we saw the treatment impacted the results (**Table 2.2**). Paradoxically, this did not generate different groups in the subsequent post-hoc analysis by treatment despite being close ($p = 0.051$ between control and NPK + PGA treatments), due to the differences in statistical power of the full ANOVA and the post-hoc Tukey tests.

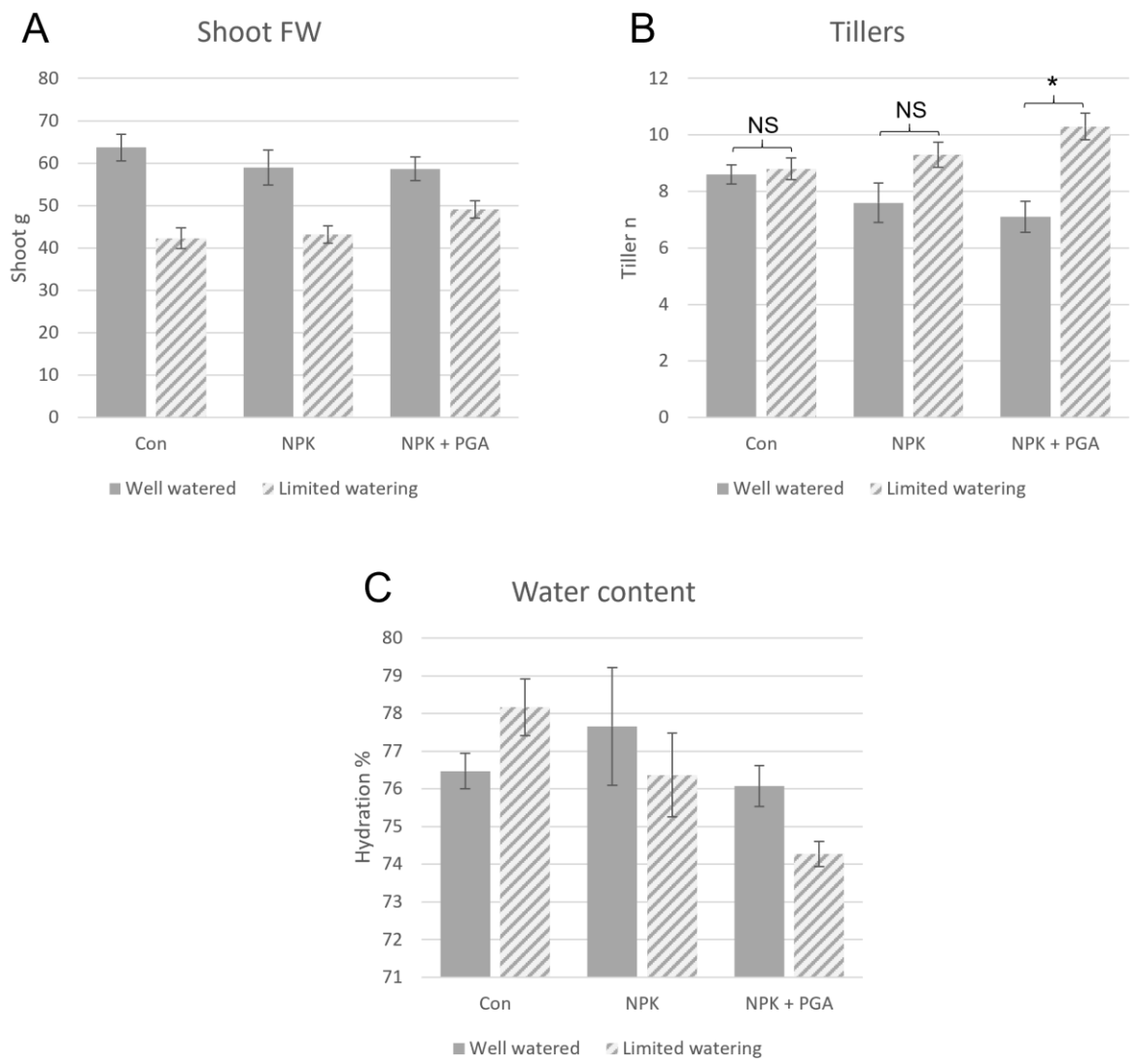


Fig. 2.2. Quantification of wheat growth after four months since germination under abundant and limited watering conditions and after the spray addition of a control treatment (Con) (using water), a commercial NPK mixture (NPK) and a commercial NPK mixture and PGA (NPK + PKG). The following parameters were measured: shoot fresh weight **(A)**, tillers **(B)**, and % of water weight in leaves (as a % of lost weight after 48h of oven drying) **(C)**. Columns represent means for shoot grams (A), number of tilers per plant (B) and water weight % (C) ± standard error (n = 10). Pair-wise analysis is shown to evaluate if water status affects each treatment in tillers, the only parameter with a significant interaction between treatments and water status (asterisk indicates significant effect of water status for the treatment, NS = non-significant, α = 0.05). Full two-way ANOVA analysis is shown in **Table 2.2**.

Table. 2.2. Two-way ANOVA analysis of the 4-month-old wheat for each of the parameters represented in Fig. 2.2: shoot fresh weight, number of tillers per plant and hydration %.

	Treatment	Water status	Treatment*Water status
Shoot FW	0.597	**<0.001**	0.109
Tillers	0.993	**<0.001**	**0.009**
Hydration %	**0.033**	0.514	0.099

2.3.1.2. Impact of amino acid application in yield parameters of fully developed wheat

PGA application in two- and four-month-old wheat showed effects on growth and development. In order to evaluate the possible effect on wheat yield and related parameters, we performed additional experiments taking wheat to full development to address these metrics. Additional amino acid treatments were also included in these assays to screen for amino acids that can lead to positive effects in yield, potentially identifying some that can be suggested as candidates for crop improvement practices.

The following growth assay was used with the aim of determining if wheat grown in controlled conditions develops a different yield after the application of amino acid-including fertilisers. This was carried out in a greenhouse, sacrificing some of the control of fully controlled-environment rooms but still enabling controlling the main growing conditions while allowing more space for plants developing in individual pots.

Fig. 2.3 shows a screening of wheat grown under the spray application of eight distinct amino acids in order to determine differences in key yield parameters. However, the ANOVA for these treatments (**Table 2.3**) showed no significant effect on any of these parameters, with no group differences between any treatments. Some trends are visible in some of the parameters with PGA, Pro and Asn treatments having higher averages than the rest of the treatments and the control NPK treatment in yield (**Fig. 2.3A**), dry weight (DW) (**2.3B**), number of spikes (**2.3C**) and number of seeds (**2.3D**), but none met the statistically differential threshold. The number of spikelets (**Fig. 2.3E**) showed values that are very similar to the NPK control for all amino acid treatments.

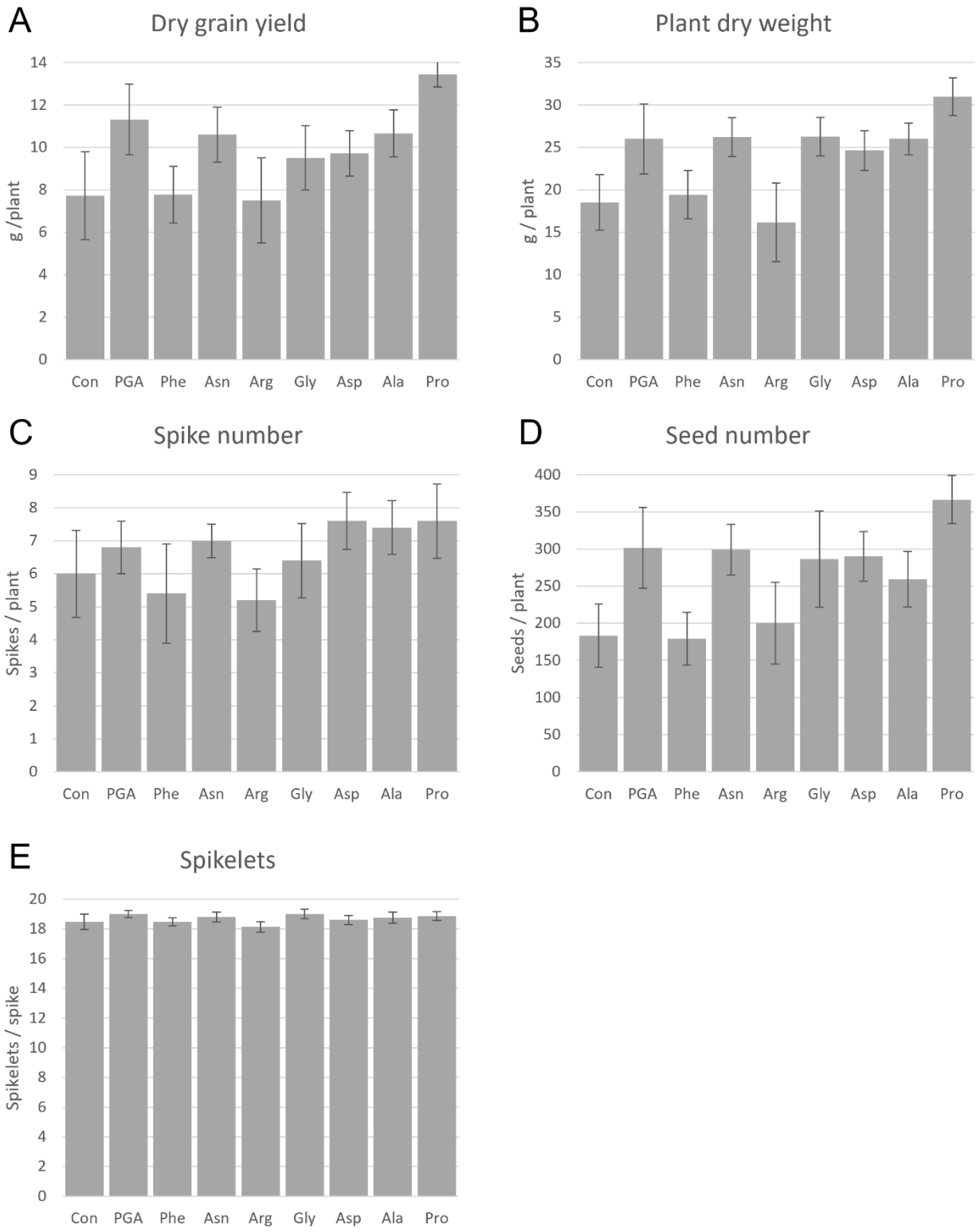


Fig. 2.3. Wheat growth parameters at full maturity: dry grain yield **(A)**, dry plant weight (DW) **(B)**, number of spikes per plant **(C)**, number of seeds per plant **(D)** and number of spikelets per plant **(E)**. Columns show mean values represented by the units on the Y axis of each graph ± standard error for each trialled amino acid in the X axis. There are no statistical differences ($\alpha = 0.05$) for intra-graph columns ($n = 5$). Full one-way ANOVA analysis is shown in **Table 2.3**.

Table. 2.3. One-way ANOVA analysis of the fully matured wheat for each of the parameters represented in Fig. 2.3.

Parameter	Treatment
Grain yield	0.061
Plant mass	0.129
Spike number	0.131
Seed number	0.164
Spikelets per spike	0.921

The results in PGA are particularly interesting as, despite being non-significant, they had a considerable higher yield mean compared to the control and were second highest after Pro, suggesting a stimulation of yield after application as has been observed for commercial products including PGA for wheat. These results will be complemented and expanded in the following **Chapter 3** with field experiments that also show a similar trend for this amino acid.

While results in **Fig. 2.3** are useful as a screening of a high number of individual amino acid additions in NPK fertilisers, the high number of treatments came with space constraints that allowed for a small number of biological replicates. A new batch of wheat plants were grown to yield in order to repeat the experiment, this time using a lower number of treatments but increasing the number of biological replicates. A new control that did not include NPK fertiliser (spraying water instead) was also added to observe its performance versus the NPK fertilised treatment and the amino acid treatments. This allowed to obtain more robust results in regards to the differences amino acid application creates in greenhouse wheat, as well as to follow up on the trends seen in **Fig. 2.3**. For this purpose, two of the treatments with highest yields were chosen for this new batch of plants (PGA and Pro), as well as one of the amino acids that did not have increased averages compared to the NPK-only treatment (Phe).

All treatments, including the water control and the NPK without added amino acids, showed similar results for all measured parameters: plant height (**Fig. 2.4A**), number of tillers per plant (**2.4B**), seed yield (**2.4C**), spike weight (**2.4D**) and number of spikes per plant (**2.4E**). The one-way ANOVA analysis (**Table 2.4**) confirmed that there were no significant differences between treatments.

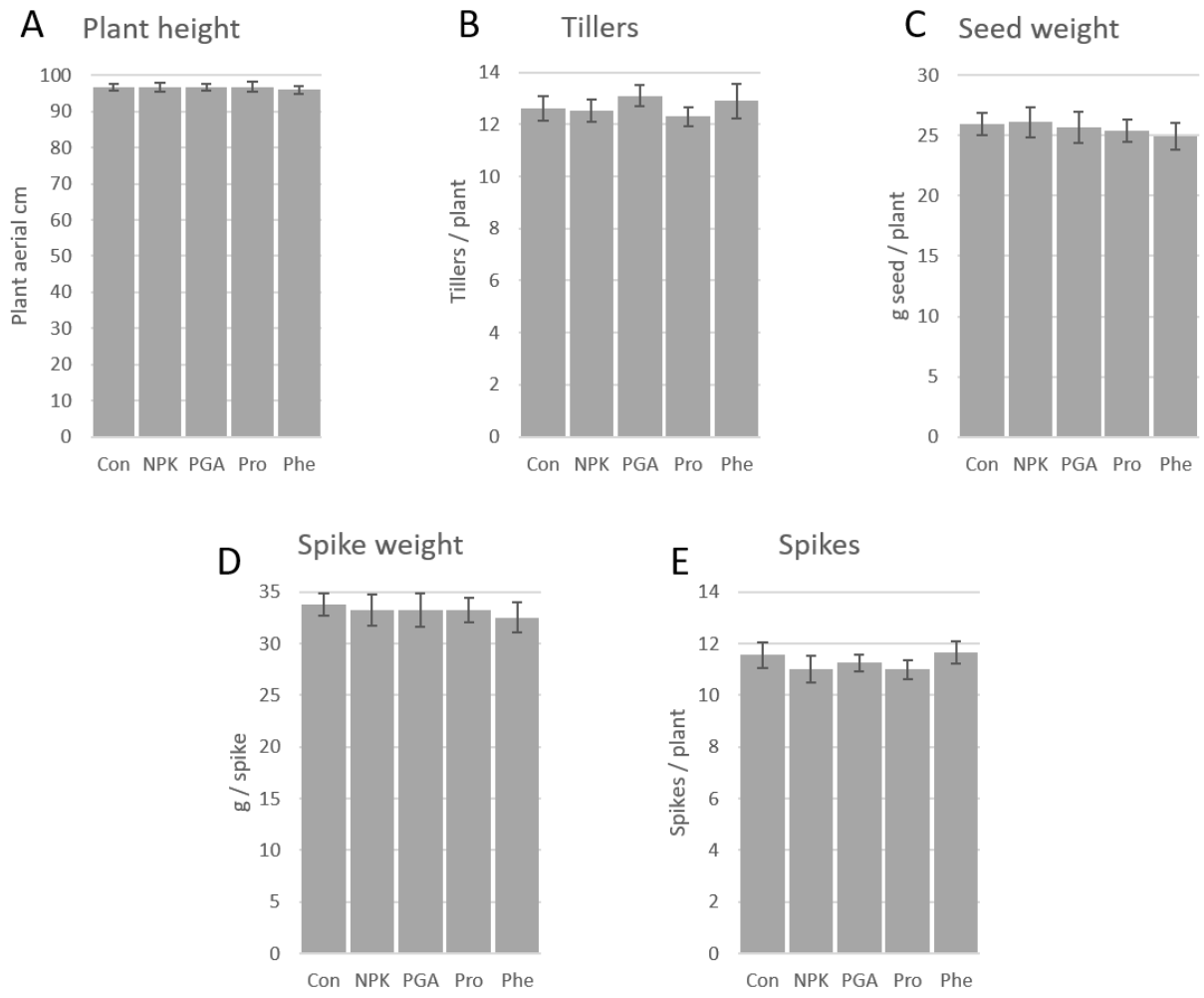


Fig. 2.4. Wheat growth parameters at full maturity: plant height **(A)**, number of tillers per plant **(B)**, seed weight per plant (yield) **(C)**, fresh weight of each spike **(D)** and number of spikes per plant **(E)**. Columns show mean values represented by the units on the Y axis of each graph ± standard error for each trialled amino acid. There are no statistical differences (α = 0.05) for intra-graph columns (n = 10). Full ANOVA analysis is shown in **Table 2.4**.

Table. 2.4. One-way ANOVA analysis of the fully matured wheat for each of the parameters represented in Fig. 2.4: plant height, number of tillers, grain yield, spike weight and spike number.

Parameter	Treatment
Plant height	0.987
Tillers	0.788
Grain yield	0.952
Spike weight	0.982
Spike number	0.741

2.3.2. Impact of individual amino acid addition in *in vitro Arabidopsis* seedlings

The experiments presented in this work so far show that in a crop of agricultural interest such as wheat and in controlled environment experiments, the addition of the scarcely studied PGA has some impact on growth. Yield parameters, however, are not affected by PGA or any other amino acid under the studied conditions. This contrasts with what has been seen in the next **Chapter 3** when applying amino acids similarly under field conditions, which shows more evident yield differences among amino acids.

As an alternative experimental system to investigate the effects of PGA and other amino acids on plant growth and development, both in the presence and absence of abiotic stress, we turned to the measurement of root growth in *Arabidopsis* grown on agar *in vitro*. The use of *Arabidopsis* root growth assays as a system to study the effects of external molecules and specifically amino acids has been previously seen and proven effective (Walch-Liu *et al*., 2006; Forde, 2014). Although different from the experiments conducted with wheat, above, the *Arabidopsis* root growth assay provides a much more high-throughput system for making accurate quantitative measures of the effects of many different amino acids across a range of concentrations and in different genetic backgrounds. Being a function of genetics and the environment, measurement of root growth allows comparisons of the effects of different compounds during early development (Dinh *et al*., 2015; Burrell *et al*., 2017). Root growth was measured in seedlings grown on vertical agar plates containing different amino acids over a range of concentrations. To introduce an abiotic stress, salt was included as a variable in the experimental design, since this has well-documented effects on root growth in *Arabidopsis*.

A preliminary experiment was conducted to observe *Arabidopsis* seedling rosette growth at a range of PGA concentrations (**Fig. 2.5**) in order to evaluate visible changes in plant growth during early development. Seeds failed to germinate on media containing 10 mM PGA, whilst at 1 mM PGA, seedlings exhibited altered morphology, indicating phytotoxicity. Lower concentrations can show some signs of yellowing, but this was not consistent between plates (particularly *in situ* compared to photographs), and rosette morphology was not altered. Based on these results, subsequent root growth comparison screenings were conducted using a maximum PGA concentration of 0.5 mM, where there may be root growth alteration, but the plant was clearly able to germinate and grow.

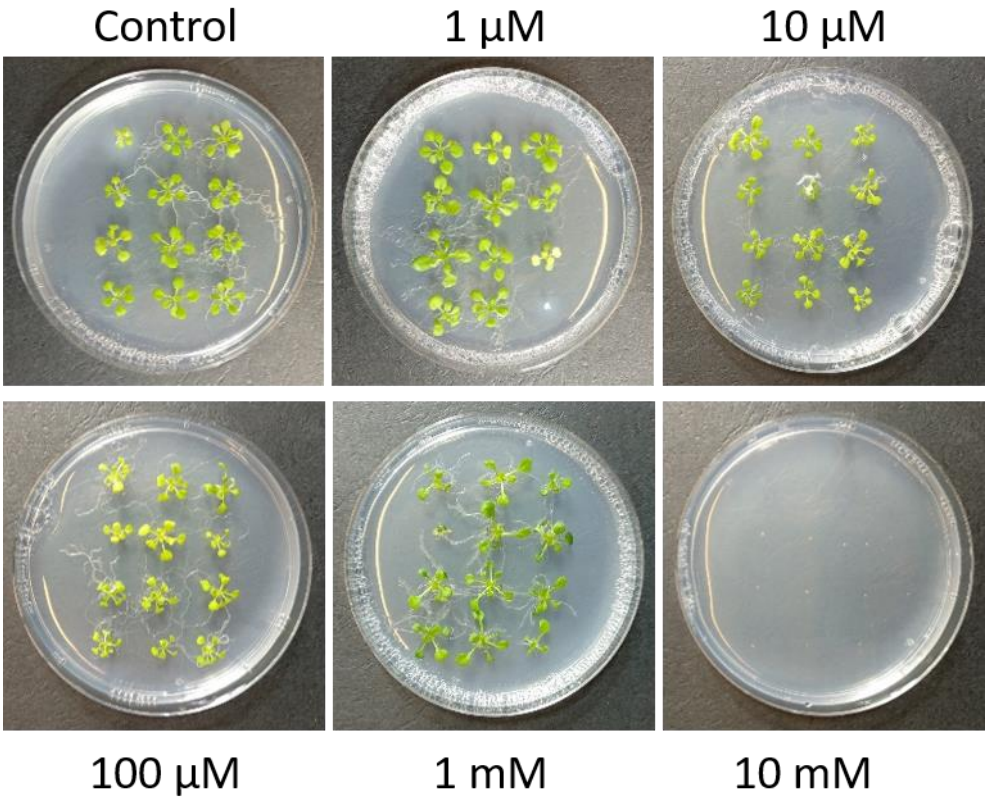


Fig. 2.5. *In vitro Arabidopsis thaliana* rosette growth in PGA including growth media at the concentrations of 0, 1, 10 and 100 µM as well as 1 and 10 mM. One representative plate out of three grown for each concentration is shown.

To focus on the mechanisms of action of PGA, the 5-OPase defective mutant *oxp1* was included in the root growth screens. 5-OPase is the only known pathway for PGA degradation, making it the only known gate to avoid PGA overaccumulation in the plant. Since *oxp1* cannot convert PGA to Glu, externally applied PGA should remain as PGA, and therefore any biological effects observed can be attributed to PGA rather than Glu or its downstream metabolites.

A RT-qPCR was performed with WT and *oxp1* mutant plants, confirming the disruption of the *OXP1* gene mRNA transcript with the absence of oxp1 amplification. The genetic sequence for *OXP1* was not amplified when using cDNA isolated from the *oxp1* mutants, whilst expression was detected in WT plants (**Table 2.5**). The homozygosity of the mutation was also confirmed by the absence of amplification instead of reduced amplification relative to WT.

Table 2.5. Ct values for the qPCR performed to validate the *oxp1* mutant *Arabidopsis thaliana* plants. No primers were added for the negative control. Each sample represents a pool of three plants.

Variety	Gene	Biological replicate	Ct
WT	Negative control	1	No Ct
		2	No Ct
		3	No Ct
	Ubiquitin	1	18.23
		2	18.20
		3	18.19
	OXP1	1	29.73
		2	28.06
oxp1	Negative control	1	No Ct
		2	No Ct
		3	No Ct
	Ubiquitin	1	18.17
		2	18.32
		3	18.02
	OXP1	1	No Ct
		2	No Ct

Following these preliminary tests, root growth responses were measured in the WT Col-0 and *oxp1 Arabidopsis* over a range of concentrations of PGA, Glu, Pro, Gly, Ala and Arg, either in the absence or presence of 100 mM NaCl (**Table 2.6, Figs. 2.6-2.9)**. This concentration of NaCl was chosen as it constitutes a moderate stress in which *Arabidopsis* is able to germinate *in vitro* and grow while presenting reduced root growth (Julkowska *et al*., 2014).

Table 2.6 Three-way ANOVA analysis of the vertical *in vitro* root growth in *Arabidopsis thaliana* for the factors of genotype (WT or *oxp1*), amino acid concentration (up to 500 µM) and salt status (0 or 100 mM) as represented in Figs. 2.6, 2.7, 2.8 and 2.9.

Applied amino acid	Genotype	Concentration	Salt status	Genotype*Concentration	Genotype*Salt status	Concentration*Salt status	Genotype*Concentration*Salt status
PGA	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Glutamate	**<0.001**	**0.004**	**<0.001**	0.271	**<0.001**	**0.002**	0.115
Proline	**<0.001**	0.716	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.074
Glycine	**<0.001**	0.533	**<0.001**	0.145	**0.033**	**<0.001**	0.637
Alanine	0.505	**<0.001**	**<0.001**	**0.004**	0.930	**<0.001**	**0.002**
Arginine	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.257	**<0.001**	**<0.001**

The ANOVA analysis (**Table 2.6**) suggests that genotype, concentration and salt status affected root growth responses to most amino acids, and there were also significant interactions between these three variables in most cases. This ANOVA table is the main result for the root growth assays, showing which factors and combinations of them present significant differences for the measured parameter, and that is why it is presented before the quantitative results in **Figs. 2.6-2.9**. This is also the reason why no letters are displayed in said figures, as this would require a complex concurrent comparison of all plots that would difficult understanding the differences and their causes. Moreover, the concurrent plotting of all graphs is complicated by the fact that they are standardised measurements and not absolute values.

Overall, concentration had a significant effect on root growth for PGA, Glu, Ala and Arg, but not for Pro and Gly. Examination of the plots in **Figs. 2.6-2.9** shows clear dose responses for PGA and Arg (growth was inhibited with increasing amino acid concentration) and Glu (dose-dependent promotion of growth) in the various conditions analysed. In WT plants, PGA addition led to root growth reduction from 100 μM (**Fig. 2.6A**), while Arg did so from 300 μM (**2.6F**). Glu (**2.6B**), Pro (**2.6C**), Gly (**2.6D**) and Ala (**2.6E**) on the contrary showed little difference compared to the control, even increasing growth in some concentrations. Ala promoted growth at lower concentrations but was inhibitory at higher concentrations.

As expected, salt reduced root growth and there were interactions between salt and amino acid concentration in all amino acid treatments (**Table 2.6**). When salt was added to WT plants, PGA (**Fig. 2.7A**) and Arg (**2.7F**) addition led to less root growth, in concentrations above 200 μM and 100 μM respectively. Ala (**2.7E**), which showed no growth decrease without salt addition, showed signs of decrease from 300 μM when under salt stress. Glu (**2.7B**), Pro (**2.7C**) and Gly (**2.7D**) on the other hand saw no reduction at any of the measured concentrations, even showing slight root length increases at higher concentrations.

oxp1 mutant *Arabidopsis* plants were also assayed in the same way as WT plants to determine if the disruption of *oxp1* led to PGA related toxicity or any other differences comparing to their WT counterparts. All previously used amino acids were also assayed using *oxp1* plants to explore if the alteration PGA pathway can lead to effects in the wider amino acid metabolism. In non-salt stressed *oxp1* plants (**Fig. 2.8**), PGA (**2.8A**) led to a slight increase in root growth when adding 100 μM, and plants with concentrations greater that experienced decreases similar to the WT plants. Glu (**2.8B**) and Pro (**2.8C**) led to increased root growth compared to the control in most of the applied concentrations, while Gly (**2.8D**) root growth was slightly less in higher concentrations and Ala (**2.8E**) concentrations led to no impact in growth. Arg (**2.8E**) addition reduced root growth, but much less than in WT plants.

In *oxp1* plants with added NaCl, PGA addition (**Fig. 2.9A**) led to a greater root growth reduction than any of the conditions seen before, with over 70% growth reduction at 500 μM. Glu (**2.9B**) and Pro (**2.9C**) however saw an evident root growth increase, higher in relative terms than those of the WT or the non-salt *oxp1*. Gly (**2.9D**) addition did not lead to an obvious variation of root length, while Ala (**2.9E**) addition did over 300 μM, as well as Arg (**2.9F**) addition from 100 μM. The significant interaction between genotype and salt status for PGA, Glu, Pro and Gly suggest that these amino acids can alter the root growth inhibition of salt differently in WT and *oxp1* plants, whilst Ala and Arg cannot.

Interestingly, the main effects analysis indicated that the *oxp1* mutation affected responses to all amino acids except Ala and interacted with dose response and/or salt responses for all amino acids (**Table 2.6**), suggesting effects in a wider range of amino acids when PGA/Glu metabolism is altered. Dose responses differed by genotype for PGA (inhibitory effects of PGA are exaggerated in *oxp1*), Pro (no effect in WT, but growth increases with dose in *oxp1*), and for Ala and Arg, where dose responses were attenuated in *oxp1*.

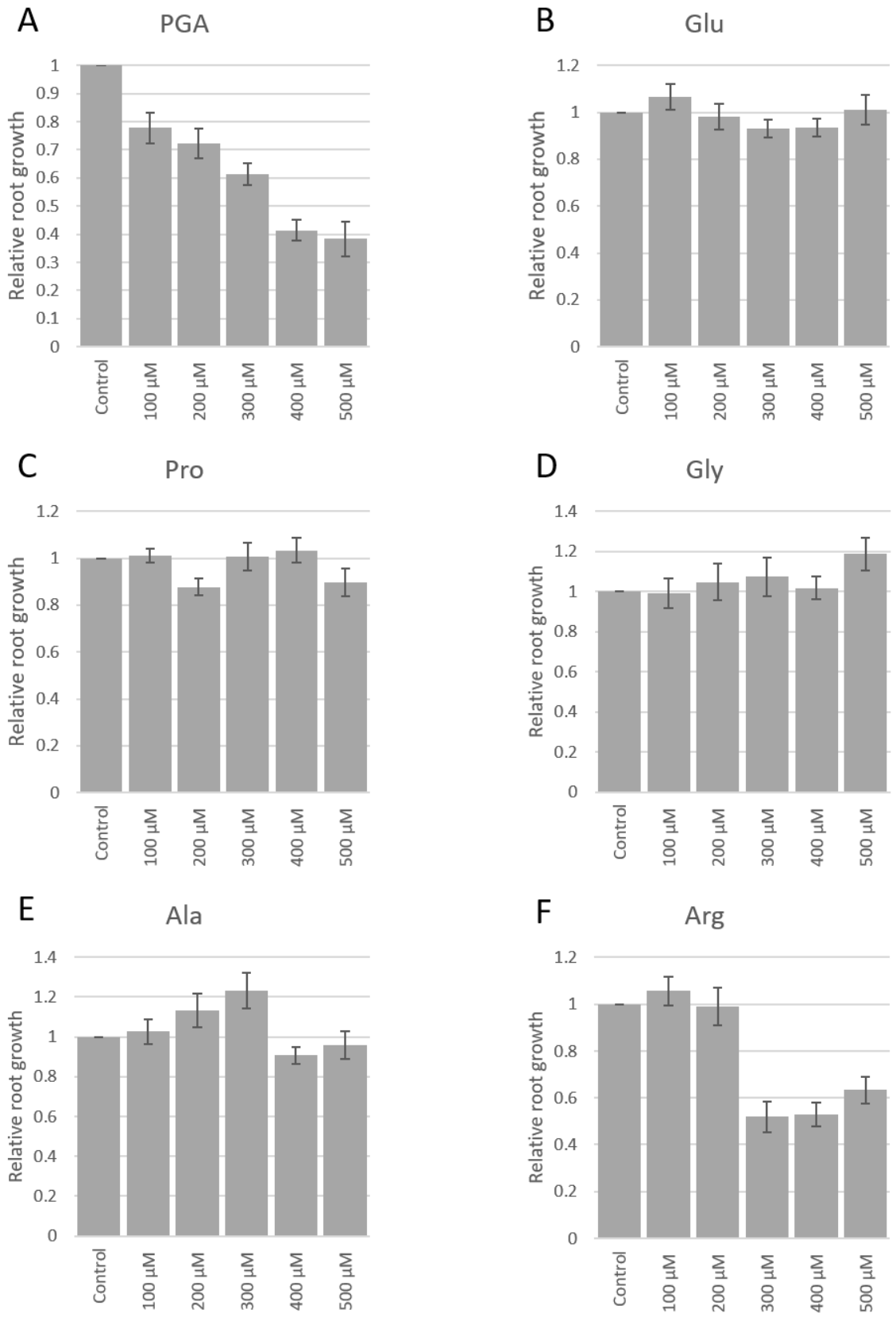


Fig. 2.6. *In vitro* vertical root growth of WT *Arabidopsis thaliana* under the application of different concentrations of pyroglutamic acid **(A)**, glutamate **(B)**, proline **(C)**, glycine **(D)**, alanine **(E)** and arginine **(F)**. Control columns for each plot correspond to no added amino acid. Columns represent means of growth relative to the control ± standard error (n = 27).

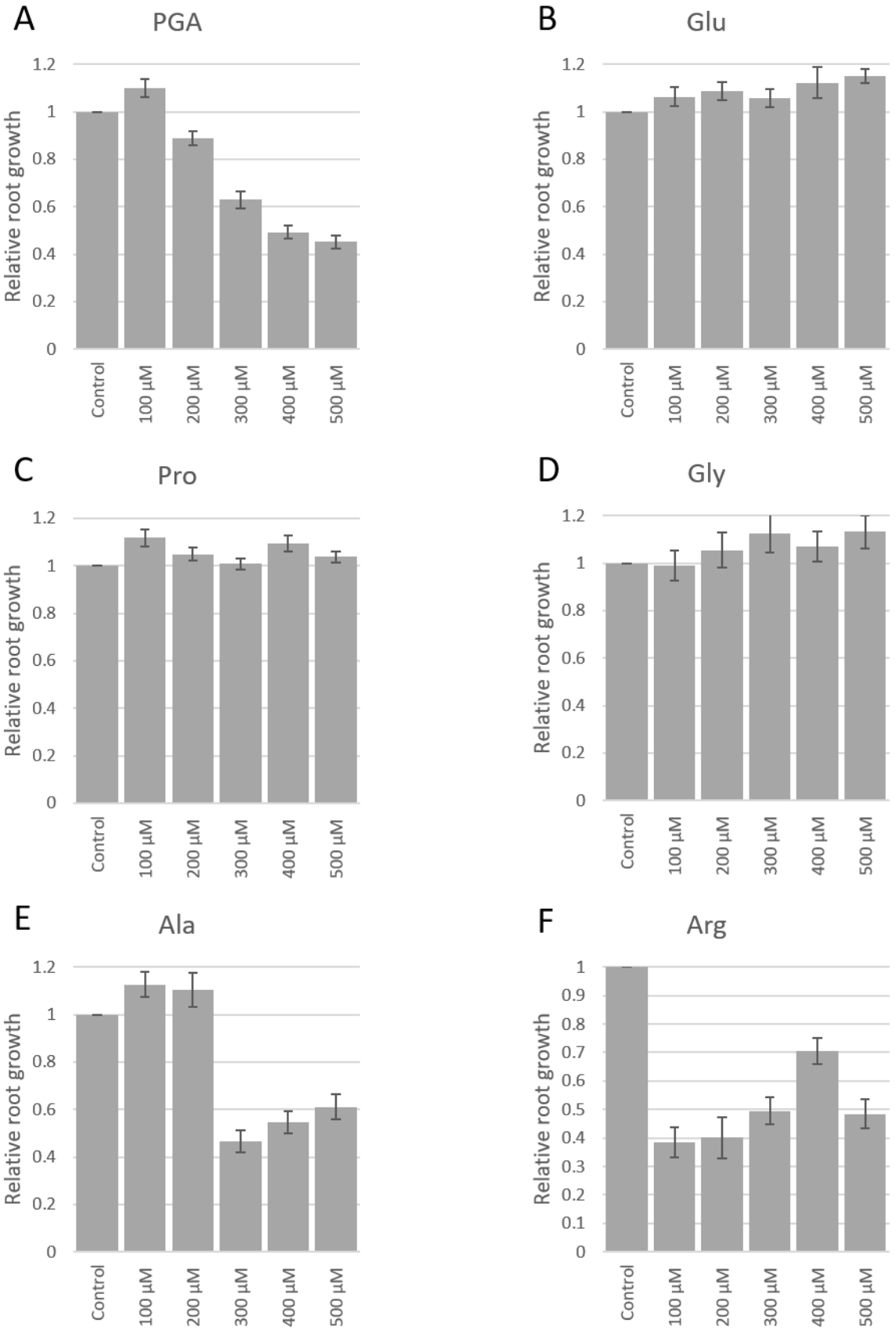


Fig. 2.7. *In vitro* vertical root growth of WT *Arabidopsis thaliana* under the application of different concentrations of pyroglutamic acid **(A)**, glutamate **(B)**, proline **(C)**, glycine **(D)**, alanine **(E)** and arginine **(F)** and 100 mM salt. Control columns for each plot correspond to no added amino acid but 100 mM salt being added. Columns represent means of growth relative to the control ± standard error (n = 27).

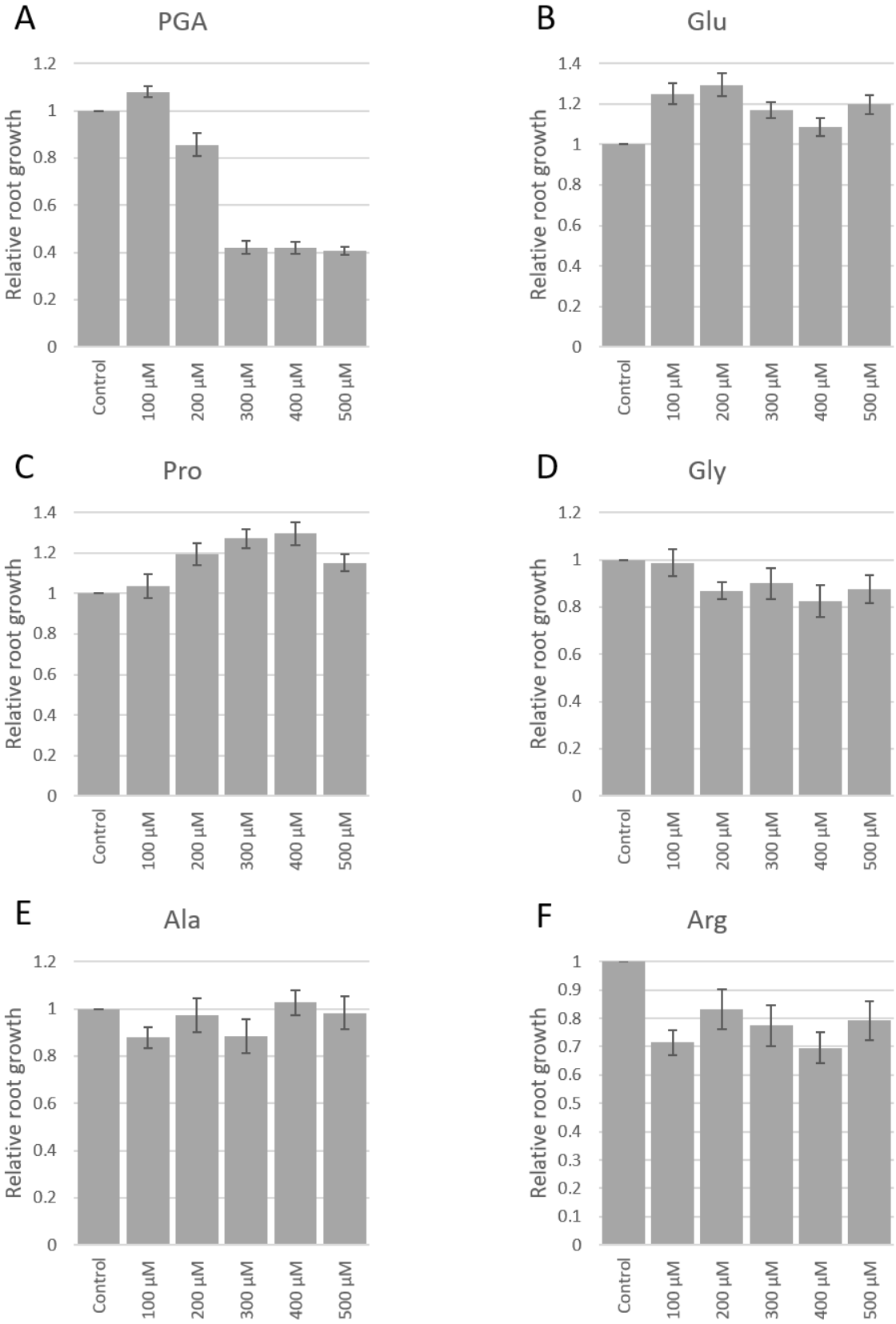


Fig. 2.8. *In vitro* vertical root growth of *oxp1* mutants of *Arabidopsis thaliana* under the application of different concentrations of pyroglutamic acid **(A)**, glutamate **(B)**, proline **(C)**, glycine **(D)**, alanine **(E)** and arginine **(F)**. Control columns for each plot correspond to no added amino acid. Columns represent means of growth relative to the control ± standard error (n = 27).

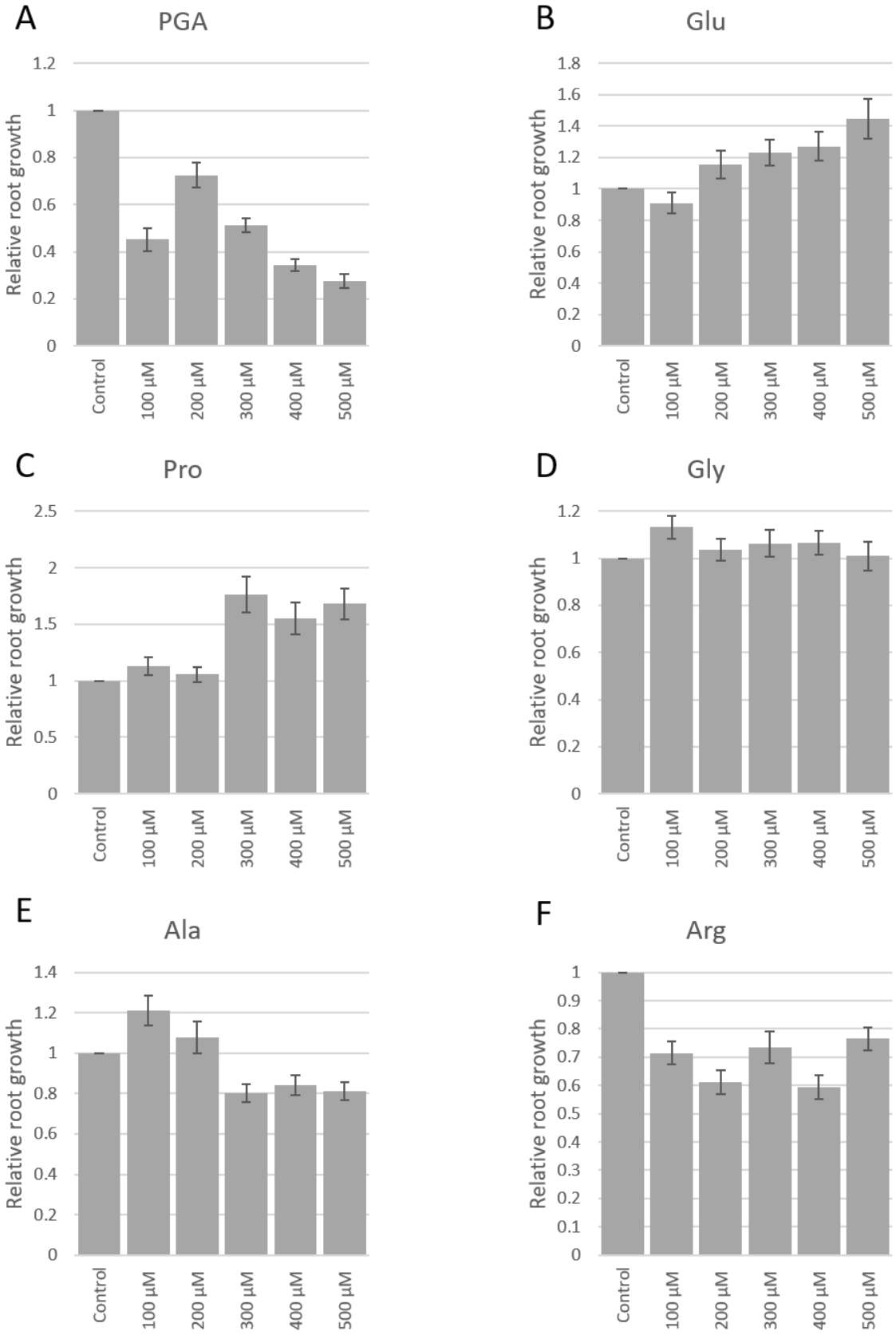


Fig. 2.9. *In vitro* vertical root growth of *oxp1* mutants of *Arabidopsis thaliana* under the application of different concentrations of pyroglutamic acid **(A)**, glutamate **(B)**, proline **(C)**, glycine **(D)**, alanine **(E)** and arginine **(F)** and 100 mM salt. Control columns for each plot correspond to no added amino acid but 100 mM salt being added. Columns represent means of growth relative to the control ± standard error (n = 27).

2.4. Discussion

Amino acids perform a number of roles in the plant life cycle, from essential and widely known ones such as protein building blocks and N cycling to more specific ones that may be triggered only under certain circumstances (Rai *et al*., 2002), or only by specific amino acids (Roberts, 2007; Vranova *et al*., 2011; Hayat *et al*., 2012). Studying the effects of amino acids using either an internal increase in concentration or external addition to plants has been useful to discover biostimulant effects that go beyond adding nutrients (Guo *et al*., 2021). Many of the particular effects of specific amino acids remain to be completely described (Trovato *et al*., 2021). The dataset from this chapter aims to evaluate the effects of externally adding the often-overlooked PGA into plants in controlled environment conditions and studying the relationship these effects have with PGA conversion to Glu, as well as evaluating a set of additional amino acids to determine if they impact plant growth.

Due to it largely being seen as an intermediary molecule in GSH cycle (Rennenberg *et al*., 1981) and with a single known degradation pathway to Glu via 5-OPase (Mazelis and Kreveling, 1978; Kumar and Bachhawat, 2012), few works study the usefulness of this molecule on its own, which hint at improved yield (Jiménez-Arias *et al*., 2019) as well as resistance to toxins and microbial disease (Fernandes *et al*., 2017; Bilska *et al*., 2018; Mejri *et al*., 2019). Despite the presence of PGA in some commercial crop products, the scarce literature available about its use in crops leaves a gap for further understanding its effects and mode of action.

The results presented in this chapter show some of the effects PGA has under controlled conditions, whether *in vitro* using the model plant *Arabidopsis thaliana* or in wheat in controlled growth rooms and glasshouse conditions. Interestingly, results showed clear PGA related stress and toxicity symptoms in *in vitro Arabidopsis* at the concentrations of 1 and 10 mM (**Fig. 2.5**). Other studies that evaluate the effect of PGA do so using high-volume but low-concentration application in field crops via spray, irrigation or drenching (Bilska *et al*., 2018, Jiménez-Arias *et al*., 2019). To our knowledge, this work represents one of the first evidences of stress and toxicity caused by excessive PGA in higher plants and particularly in *Arabidopsis*. Other studies using a range of amino acids in root growth assays have identified similar primary root growth inhibiting effects in *Arabidopsis* seedlings when applying exogenous Glu (at concentration of as little as 50 μM) and distinct effects with other amino acids (Walch-Liu *et al*., 2006; Forde, 2014). These studies however use three- and four-day old seedlings, and here we observed no inhibition of root growth after a longer growth time (**Fig. 2.6B**) suggesting that

growth will stabilise after a number of days, consistently with other studies that have added as high as 20 mM external Glu concentration at later growth stage (Goto *et al*., 2020).

Despite the fact that PGA is included in biostimulant products, *in vitro* rosette assays (**Fig. 2.5**) evidenced toxicity at mM order concentrations and lower concentrations in root growth assays showed that increasing concentrations of PGA in both WT and *oxp1 Arabidopsis* led to a decline in root growth (**Figs. 2.6A, 2.8A**), which was even more pronounced with salt stress (**Figs. 2.7A, 2.9A**). Glu treated plants (**Figs. 2.6B, 2.8B**) on the other hand did not see any reduction and in salt-stressed plants (**Figs. 2.7B, 2.9B**) and they even showed an increase in root growth over the control. Added PGA in WT plants was expected to be converted to Glu, as opposed to *oxp1* plants, maybe alleviating PGA related stress.

A possible explanation for plant responses to PGA is that they are essentially responses to Glu, which can act as a receptor in GLRs by gating Ca^{2+} channels, involving amino acids in signalling roles (Forde, 2014; Bjornson *et al*., 2021). This will happen if PGA is converted to Glu by 5-OPase. To investigate this possibility, *oxp1* Arabidopsis mutants were obtained and confirmed (**Table 2.5**) to provide a background in which externally applied PGA could not be converted to Glu. The effects of PGA were very similar for WT and *oxp1* plants, suggesting that 5-OPase activity in WT plants is limited and cannot effectively alleviate PGA toxicity above 100 μM. Importantly, the clear differences in responses to PGA and Glu mean that the growth responses observed following application of PGA cannot be explained simply as a consequence of its conversion to Glu. The differences between the effects of PGA and Glu therefore underline that both amino acids have different biological effects in *Arabidopsis*, with PGA having functions beyond being an intermediary in the GSH degradation route to Glu synthesis.

In the wheat studies, however, PGA showed signs of improved growth in developing wheat at two months (**Fig. 2.1**) and four months (**Fig. 2.2**), particularly under water limitation. Tillering at four months showed an interesting increase in PGA-including treatment under water stress, although higher tillering under water stress will not always mean more spikes that can produce yield (Hazra *et al*., 2014). In the glasshouse wheat yield study, the average number of tillers was slightly above the control after PGA spray application, but the grain yield remained unchanged (**Figs. 2.3, 2.4**). The data from water-limited four-month-old plants (**Fig. 2.2**) is consistent with other studies done with PGA in different species, such as the lettuce used by Jiménez-Arias *et al*. (2019), where a PGA drenching treatment increased yield in water-stressed

plants, also showing increased values in above-ground plant mass. The authors of that paper describe the possible changes occurring in plants under water limitation: the photosynthetic rate and the stomatal conductance are positively related, and stomatal control promotes rapid growth under water stress conditions, with PGA-treated plants showing a higher water use efficiency under drought conditions. Osmolyte accumulation under water stress such as Pro (which can be converted from PGA via Glu) is also a response mechanism to this specific stress to prevent cells from damage by using molecules with osmo-protectant characteristics. It is possible that PGA directly impacts drought-resistance in wheat using those responses in a situation where water is limited, without having any effect on plants that are not under water limitation. It is also possible that the lack of impact of PGA in yield is because plants were grown in a controlled, favourable environment, as opposed to more challenging field conditions where stress adaptability may be challenged and the effect of additional molecules that can have a positive impact may be more evident.

Furthermore, the impact of plant age in the effect of amino acids and specifically PGA needs to be considered, as Jiménez-Arias *et al*., (2019) saw a fresh weight increase in lettuce after PGA application via soil-drenching, where parameters such as photosynthetic rate, stomatal conductance, water use efficiency and total carbohydrate count were altered from the first week after treatment. Although in a very different plant, this underlines that there can be an impact in plant physiology shortly after PGA is applied, and although the parameters presented here do not change in 2-month-old wheat plants there is a possibility of changes at a short age that are not present in this species, in the experimental conditions used here or in the parameters this study has focused on.

In the glasshouse experiments presented here (**Figs**. **2.3**, **2.4** and **Tables 2.3**, **2.4**), the concentration of N added by PGA was negligible compared to that provided by the carrier NPK solution and the nutrients present in the soil. Therefore, it can be assumed that the effect of PGA in two- and four-month-old water limited wheat plants (**Figs. 2.1**, **2.2** and **Tables 2.1**, **2.2**) was not due to the addition of N or of PGA assimilation, suggesting a biostimulant action of PGA that may be triggering some mechanism leading to a positive impact in water limited wheat growth. It is possible this is related to GLRs (Glu receptor-like channels), cation channels that have been related to physiological effects in plants (Cho *et al*., 2009; Vincill *et al*., 2013), and can bind a variety of amino acid ligands in addition to Glu (Weiland *et al*., 2016). It could be possible that PGA, as many other amino acids, can bind to GLRs leading to activation of Ca^{2+} channels involved in molecular signalling, either as GLR agonists, or antagonists of Glu or

other ligands. In the future, it would be interesting to test this hypothesis by measuring responses to PGA in *Arabidopsis* GLR mutants.

Pro, on the other hand, is an amino acid with proven effects as a growth enhancer (Kavi Kishor *et al*., 2005; Teh *et al*., 2016) that can improve stress resistance in plants (Ali *et al*., 2007; Hayat *et al*., 2012; Mansour and Ali., 2017). In the root length assays from this work, WT plants reacted similarly to Pro with and without salt stress (**Figs. 2.6C, 2.7C**). Although Pro in these concentrations did not significantly impact growth on its own (**Table 2.6**), there was some effect of concentration in salt response, which was interestingly more accentuated in *oxp1* plants (**Fig. 2.8C, 2.9C**). It is possible that with GSH degradation to Glu being interrupted by a defective 5-OPase, Glu sourced Pro levels will be lower and a bigger proportion of Pro will be from the external source, which has been seen to have specific effects (Ali *et al*., 2007; Deivanai *et al*., 2011; Teh *et al*., 2016). This work does not help identify specific intracellular changes after external Pro addition, but does suggest a reaction to adding low external Pro concentrations in relation to salt stress. Historically Pro accumulation and salt stress resistance have been tightly related. Most studies focusing on the exogenously applied Pro-related salt resistance are done with higher concentrations of Pro in the 10 to 100 mM range (Jain *et al*., 2001; Roy *et al*., 2014; Wani *et al*., 2016; Ali *et al*., 2017), while a lesser number of studies have shown that concentrations as low as 500 μM (Cuin and Shabala, 2005) can impact salt resistance by reducing NaCl related K^+ efflux from the cell. This could explain the lack of clear positive effects of Pro in the growth of WT plants even under salt stress in our work. It would be interesting to further study effects of Pro addition at low concentrations and specifically compare the effect of external-sourced Pro to internal sources, particularly Glu-sourced as 5-OPase disruption directly impacts one of the main Glu generating metabolic routes.

The greenhouse assays did not show any stimulation after Pro application either, again underlining that using a low concentration of this amino acid and under ideal conditions there is not necessarily a positive effect of adding an amino acid such as Pro that is generally considered positive for plant growth. This lack of impact in controlled and ideal condition greenhouse experiments extended to all other foliarly sprayed amino acids, from the already discussed PGA and Glu to the rest such as Phe, Asn, Arg, Asp and Ala (**Figs. 2.3**, **2.4** and **Tables 2.3**, **2.4**). Although this may seem to contradict the utility of amino acids as biostimulants, it perhaps just underlines that amino acids, starting from the well-known Pro, have effects that are specific to certain conditions, and in the wheat experiments the aim was to specifically study the effect of amino acid addition at low concentration and without external

stress factors. As positive effects in plants by amino acids are often related to stress, when accumulation of a certain amino acid occurs, a lack of response in plants grown under ideal conditions is to be expected and adds to the hypothesis that potential biostimulant actions by amino acids may require stress conditions. There are however authors who observed growth improvement using foliarly applied amino acids, like Gly and Gln, at low concentrations (Noroozlo *et al*., 2019) in a similar timeframe as used in these experiments, although using a very different crop such as lettuce. In our work, several amino acids, including Gly, showed growth improvement trends in wheat (**Fig. 2.4**), although we were not able to achieve statistical significance (**Table 2.4**), again underlining the importance of conditions in which amino acid application may make a difference.

The remainder of amino acids used in *Arabidopsis* assays (Gly, Ala, Arg) do show some impact in seedling growth. When added to WT plants, Gly led to a slight increase in root growth at certain concentrations compared to the control (**Fig. 2.6D**), even when salt was added (**Fig. 2.7D**). In contrast, in *oxp1* plants Gly only increased root growth in the presence of salt (**Fig. 2.9D**), while the non-salt-stressed plants had a slight reduction in root growth (**Fig. 2.8D**). This difference suggests that the response to Gly is altered when PGA/Glu homeostasis is disrupted. Gly addition to the medium in other plants such as coriander has shown to increase plant growth at lower concentrations, while concentrations above 40 mg/L decrease growth (Mohammadipour and Souri, 2019). Our results show similar upward trends (except for non-salt *oxp1*), with the ANOVA indicating that concentration increases lead to different effects when under salt stress (**Table 2.6**).

Higher Ala and Arg addition on the other hand decreased root growth in WT plants (**Fig. 2.6E, 2.6F**), with effects being even more evident when adding salt (**Fig. 2.7D, 2.7F**). 5-OPase defective mutant plants however only exhibited a growth reduction at higher concentrations and with salt (**2.9E, 2.9F**), showing a growth similar to the control without NaCl (**2.8E, 2.8F**). Free Ala (Misra *et al*., 2006) and Arg (Ramadan *et al*., 2019) levels in plant tissue have shown to increase their levels under salt stress, but the lower root growth on WT with no salt stress is unexpected and does not correlate with other studies as far as we are aware. Furthermore, the growth reduction in salt stressed mutants for these two amino acids brings more questions about the far-reaching effects of altered Glu metabolism via disruption of PGA conversion to Glu that are not answered by these assays.

Whilst these experiments provide a useful platform to study the specific role of PGA and the interaction between PGA/Glu metabolism and responses to other amino acids, to extend current knowledge of their biostimulant activity in crops it is necessary to address their effects under field conditions in order to understand how abiotic and biotic stress conditions can be helped by amino acids. Those conditions are addressed in the following **Chapters 3** and **4**, showing how different environments can lead to distinct effects of the same amino acids.

2.5. Conclusions

The results in this chapter present interesting insights on the effect of PGA in plants, as well as of other amino acids. We show that PGA, an amino acid that has been scarcely studied and is mainly considered an intermediary in plant metabolism, induces stress in *Arabidopsis thaliana* seedlings at concentrations above 1 mM and completely inhibits germination at a concentration of 10 mM. *In vitro* root growth is also reduced at concentrations above 300 μM, and genetically blocking the conversion from PGA to Glu gives similar growth reduction. This growth reduction in the presence of PGA is additive with the inhibitory effects of salt. We propose a role of PGA in plants beyond an intermediary of GSH metabolisation to Glu, as the effects of Glu on *in vitro* root growth were completely different to the effects of PGA, with increased growth under salt stress which was even more striking in the 5-OPase defective *oxp1* mutant where PGA conversion to Glu does not occur. Low concentrations of other amino acids such as Pro and Gly also increased root growth, particularly under salt stress, and this effect is accentuated in 5-OPase defective plants. In Ala and Arg containing media root growth was decreased in WT plants, but *oxp1* mutants experienced no decrease when not stressed and less decrease when salt-stressed. Hence, PGA metabolism has impacts extending also to responses to other amino acids.

In controlled environment-grown wheat, growth at two months showed some differences after spray PGA application and when watering was limited, with a change in shoot/root ratio. At four months, there was an effect of PGA on the water content of shoots, as well as a different effect of PGA on tillers with or without limited watering. Fully grown wheat plants however showed no significant changes in yield parameters after the spray application of PGA or any of the other seven amino acids tested. We underline the importance of environment conditions for amino acids to function not solely as nutrients but to have biostimulant actions that may be revealed only under more challenging conditions.

Chapter 3. Effect of individual amino acid application in the growth, yield and N metabolism of field-grown wheat

3.1. Introduction

Agricultural demand increase (Tilman *et al*., 2011; Ray *et al*., 2013) and climate change mean food security will be challenged in the upcoming years (Ray *et al*., 2015; Tilman and Clark 2015), with climatic events such as drought or excessive rain periods (Beniston *et al*., 2007) being more common and the relationship of crops with the soil, atmosphere, pests, and diseases being altered (Bebber *et al*., 2013; Dhankher and Foyer, 2018). There is an evident need for improving crop yields and make them more resilient to the rapidly changing conditions they are faced with (Long *et al*., 2015; Paul *et al*., 2017). To achieve this, it is essential for both crops and agricultural techniques to improve, providing a viable adaptation to the conditions in their specific lands. Breeding techniques for crop improvement have slowed over the last decades and especially in the most recent ones (Long *et al*., 2010) and methods involving *in vitro* cultivation or genetic engineering, although useful for enhancing yield, are limited due to legal regulation depending on the country and biological origin of the modification (Laaninen, 2019).

Improving agricultural input is also key for obtaining higher yield on available crops. The use of fertilisers and herbicides enables the proper growth of crops but is also an energy and resource intensive practice that entails a source of land and water pollution, such as soil acidification and mineral toxicity, water eutrophication and atmospheric contamination by nitrous oxide (Khan *et al*., 2018). This underlines the importance of optimising fertiliser use and finding ways to stimulate plant growth and yield with the least possible application. Including components that may stimulate plant natural processes to benefit plant development, nutrient use efficiency and stress protection is also helpful for this matter (Calvo *et al*., 2014).

Field trials remain the most accurate way of studying how a specific crop will behave under real agricultural conditions and can provide more accurate information of how specific treatments will behave if applied in commercial agriculture (Sommer *et al*., 2013; Rozbicki *et al*., 2015). The use of model plants such as *Arabidopsis thaliana* is widespread for all research aspects of plant science and shares a lot of common characteristics with crops, but translating knowledge from a model plant to a crop and to field conditions is often difficult (Nelissen *et al*., 2014). Greenhouse studies enable the use of specific crops of agricultural interest without the need of model species, directly studying the effect on crop species under specific controlled

conditions. However, greenhouse conditions can still be very different from field conditions, where there are a lot of changing and uncontrolled variables that have a large effect on the crop, as studies have shown that specific stresses in greenhouse and field experiments might trigger similar reactions but the effect on yield might be different, specifically in wheat (Evans *et al*., 1999).

The field trial in this study has used wheat as the crop of choice for being a cereal that is the most important crop in the UK with a production of 16 million tonnes per year (DEFRA, 2020). By using this crop, the present work aims to analyse the potential benefit of using novel biostimulant additions under authentic UK field conditions.

Amino acids are mainly synthesised from soil N assimilation (as previously described in **Fig. 1.3** from **Chapter 1**), where NO_3^- will mainly be reduced to NH_4^+ in the shoots after being transported via xylem. NH_4^+ will then be incorporated to the GS-GOGAT pathway to create amino acids. GDH, another NH_4^+ assimilating enzyme, is a bidirectional enzyme that can aminate 2-OG to create Glu or deaminate Glu in the other direction. GDH performs both directions *in vitro*, although it functions primarily in its deaminating direction *in vivo*, generating 2-OG as a C recovery and remobilisation enzyme in times of need for the plant (Fontaine *et al*., 2012). In *in vitro* studies, GDH has been related to stress-coping under different conditions through its assimilating direction, such as under increased salinity (Skopelitis *et al*., 2006; Hessini *et al*., 2013) and drought (Zhou *et al*., 2015). Plants can also absorb amino acids directly from the soil, although this is limited by their availability in the rhizosphere and the amino acid transporters in the cell membranes that are in contact with it (Jamtgard *et al*., 2010).

Beyond protein formation, amino acids have roles in plant physiology as metabolites and signal molecules, including acting as precursors of other metabolites, providing stress defence capabilities, stimulating developmental processes and hormone metabolism, and acting as chelating agents (Rai, 2002; Szabados *et al*., 2010; Popko *et al*., 2018). Applying external products of biological origin can create a number of these effects beyond a simple increase in their concentrations: this is the very definition of biostimulants (Yakhin *et al*., 2017). A number of amino acid products are currently being used or considered as biostimulants in commercial agricultural practices as an attempt to improve crops. Amino acids have also been related to signalling, as GLRs are integral membrane proteins with ligand-gated ion channel activity that have Glu as well as other amino acids as ligands and may function as sensors that trigger various biological processes (Forde and Roberts, 2014; Weiland *et al*., 2016).

Amino acid biostimulants may be administered in the form of protein hydrolysates or individual amino acids (Calvo *et al*. 2014). Amino acids can be assimilated via roots or foliage (Colla *et al*., 2015; Calvo *et al*., 2014) and translocated into the plant. Foliar application of amino acids has been related to effects of interest in crops like alleviation of stress (Jiménez-Arias *et al*., 2019) and an increase in photosynthetic pigments, biomass and carbohydrates (Sadak *et al*., 2014). While protein hydrolysates have been used with more frequency, their complex composition makes it more difficult to decipher which amino acid or components are leading to an effect in plant physiology, growth and yield. Despite the difficulties protein hydrolysates present due to their complexity, the effect of individual amino acids in crops has not been widely studied. There has been evidence that spray applications of some amino acids like Gly, Cys and Phe can impact the activity of enzymes related to oxidative stress and N metabolism when applied in µM concentrations, acting as signal molecules (Teixeira *et al*., 2017, Teixeira *et al*., 2018).

In this study, a number of amino acid treatments were applied in field-grown spring wheat at low concentrations and parameters related to plant physical characteristics, growth, yield, chlorophyll and N assimilation were observed in order to analyse changes that may be derived from said application. This provides an insight of the effect of applying amino acids in wheat and how these amino acids can be used as biostimulants for improving crops.

3.2. Materials and methods

3.2.1. Plant material, experimental design and growth conditions

Winter wheat (*Triticum aestivum* L.) of the KWS Siskin variety (KWS SAAT SE & Co. KGaA, Einbeck, Germany) was used for these field trials. This variety is known for its high breadmaking yield, as well as for its physical grain quality and for offering a wider sowing window (KWS.com).

Taking into account the field characteristics, a control treatment and eight different amino acids treatments were established, all amino acids being in their L isoform. These treatments were:

- Proline (Pro)
- Arginine (Arg)
- Asparagine (Asp)
- Glycine (Gly)
- Phenylalanine (Phe)
- Pyroglutamic acid (PGA)
- Alanine (Ala)
- Aspartic acid (Asp)

Each of the amino acids was applied in a concentration of 50 g/L to a standard NPK solution (Nutrifast Accolade, Nutrel, Lincoln, UK). This solution was then diluted 200 times for the spray application of the treatments to the growing winter wheat at Zadoks growth stage 32 during stem elongation stage, applying 3 L/ha. This application of NPK solutions with added amino acids was made by the farmer of the land in collaboration with the partner company ECM, and the concentrations above were selected by ECM as they were the standard used in their previous trials and products.

The distribution of treatments in the field was done longitudinally, with an area of 1 ha (400 m long and 25 m wide) for each treatment, distributed as shown in **Fig. 3.1A**. Six of the treatments were located next to each other while the other two were divided from the rest by an untreated area. The distribution of treatments in the field, where treatments are one next to another without a randomised design of the distribution, was the only available way to conduct the experiment as a field trial in an ECM-managed field. All samples for each treatment were taken in different points of the longitudinal 400 m but around the centre of the 25 m wide distance to avoid interference between different treatments. Control (Con) treatment samples were measured and taken from the area shown on the right of Asp.

The field used for this experiment was located in Little Eccleston, PR3 0YR, UK (53º50'49.4" N 2º53'58.4"W) (**Fig. 3.1B**). The soil was previously characterised as adequate for wheat growth, with appropriate levels of all relevant chemical components. Only rainwater was used for the crops, without additional irrigation systems apart from the spraying. The monthly maximum and minimum temperatures for the duration of the trial are shown in **Table 3.1**.

Fig. 3.1. **(A)** Distribution of treatments in the field (not to scale) **(B)** Satellite image of the field where the trial took place.

Table 3.1. Maximum and minimum monthly temperatures during the duration of the field trial, taken from the Hazelrigg weather station.

	Sep-18	Oct-18	Nov-18	Dec-18	Jan-19	Feb-19	Mar-19	Apr-19	May-19	Jun-19	Jul-19	Aug-19
Maximum temperature	21.4	21.8	13.8	11.5	9.6	18.8	12	22	21.5	25.1	31.4	28.1
Minimum temperature	3.6	-1.1	0.9	-0.1	-5.1	-5.1	1.4	-0.6	1.5	5.8	7.7	10

3.2.2. Measurement of growth parameters

Plant growth parameters were measured in flowering stage (Zadoks stages 65-69). Height of the plants was measured by hand using a metre rule (n = 50). The length of leaf tip yellowing was also measured the same way (n = 50). In lower leaves, the amount of dry leaf material was estimated by eye using an arbitrary scale of 0 to 10 for comparative purposes (n = 50).

Spikes per square metre were calculated by taking advantage of the plant distribution in the field, which was in rows. For each of the ten sites per treatment measured, the number of spikes per one metre long of plant line were counted, as well the number of plant rows per metre width. The number of spikes per m^2 in the site would be the product of those two factors.

Spikes were sampled three days before harvest, with five spikes taken in each of the sampling sites with 10 sites per treatment (n = 50). The tallest spike of each sampled plant was cut off and transported to the lab, where it was measured, weighed and threshed. Once threshed, seeds were also counted, dried for 72 h at 65ºC and weighed to calculate their dry weight.

All measurements described in this section were taken spacing samples along the whole 400 m length of each treatment area, while taking samples from the middle of the 25 m width.

3.2.3. Sampling and storage of flag leaves

Flag leaves (being the last leaf of each plant to emerge, signalling the transition from crop growth to grain production and being the most important leaf for grain potential) were cut off at grain filling stage (Zadoks growth stage 77-83) from 10 sites along each treatment, with 30-50 m between sites to use the whole 400 m length and from the middle of the 25 m width. For each site, a pool of three flag leaves was taken from plants next to each other. These were immediately wrapped in foil bags and submerged in liquid nitrogen. The foil bags were taken out of the liquid nitrogen for transport in dry ice to keep the plants frozen and subsequently stored at -80ºC.

3.2.4. Chlorophyll estimation and determination

Single-photon avalanche diode (SPAD) measurements were taken at flowering stage (Zadoks 65-69), using an Apogee chlorophyll concentration meter (Apogee Instruments Inc, Logan, USA) and measuring the centre of four flag leaves in each of the sites, taking 10 sites per treatment for a total of 40 measurements in each.

For chlorophyll determination, flag leaf tissue from grain filling stage (Zadoks 77-83) was taken from the -80ºC freezer and ground up with mortar and pestle while maintaining the tissue frozen

with liquid nitrogen. Approximately 20 mg of tissue per sample were placed in a microtube and 1 ml 80% acetone was added. This was kept cold and in the darkness for 30 minutes, vortexed and centrifuged for 15 min at 10,000 rpm at 4ºC. The supernatant was removed and all the steps from adding acetone were repeated until the pellet no longer contained any green colour. 200 µL of each sample were placed in 96 well plates and spectrophotometrically measured for chlorophyll A (Ca), chlorophyll B (Cb) and carotenoid (C(x+c)) contents following the equations used by Lichtenthaler and Buschmann (2001):

$$\mathrm{Ca}\left(\frac{\mu g}{mL}\right) = (\ 13.36 \times A664 - 5.19 \times A649\ ) \times \frac{\mathrm{Dilution}}{1000} \times \mathrm{FW} \qquad (\mathbf{A})$$

$$\mathrm{Cb}\left(\frac{\mu g}{mL}\right) = (\ 27.43 \times A645 - 8.12 \times A662\ ) \times \frac{\mathrm{Dilution}}{1000} \times \mathrm{FW} \qquad (\mathbf{B})$$

$$\mathrm{C(x+c)}\left(\frac{\mu g}{mL}\right) = \frac{(1000 \times A470 - 2.13 \times Ca - 97.49 \times Cb)}{209} \times \frac{\mathrm{Dilution}}{1000} \times \mathrm{FW} \qquad (\mathbf{C})$$

Equation 3.1. Formulas used for calculating chlorophyll a (Ca) (**A**), chlorophyll b (Cb) (**B**) and Carotenoid (C(x+c)) (**C**) contents in flag leaves.

3.2.5. Protein quantification and nitrogen assimilation enzyme activity assays

Extraction for protein content quantification and enzymatic activity assays was performed based on the method described by Gibon *et al*. (2004) with some modifications: approximately 50 mg of frozen flag leaf tissue from grain filling stage (Zadoks 77-83) was ground to powder using a mortar and pestle. The extraction was performed with a buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol (DTT), 0.1% Triton X-100, 10% glycerol, 0.05% bovine serum albumin (BSA), 0.5% polyvinylpolypyrrolidone (PVPP), 50 mM HEPES (pH 7.5) and a cocktail of protease inhibitors: 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM ε-aminocaproic acid, 10 µM leupeptin, 1 mM benzamidine. For said extraction, a 1:20 dilution (1 mg tissue / 20 mL buffer) was mixed in 1.5 mL microtubes and centrifuged at 14,000 *g* at 4°C for 20 minutes, recovering the supernatant and keeping it on ice.

Protein content quantification was done following the Bradford method (Bradford, 1976): 1 µL sample extract were mixed with 9 µL distilled H_2O and 190 µL Bradford reagent (Sigma-Aldrich Co., St Louis, MO, USA) in triplicates in 96 well plates and kept inside a spectrophotometer (LUMIstar omega, BMG Labtech, Ortenberg, Germany) at 30°C for 10 minutes for the reaction to complete, following absorbance measurement at 595 nm wavelength with four biological replicates per treatment (n = 4). Standard was measured with 1-10 µL BSA 1 %, adding water up to 10 µL and then adding 190 µL Bradford reagent.

GS activity was measured monitoring the formation of γ-glutamyl mono-hydroxamate (γ-GHM) in a reaction buffer containing 50 mM Tris-HCl (pH 7.6), 20 mM $MgSO_4$, 80 mM sodium glutamate, 6 mM hydroxylamine, 4 mM Na_2-EDTA and 8 mM ATP. To do so, 50 µL of extract and 100 µL reaction buffer were mixed in 1.5 mL microtubes and incubated 30 minutes at 30ºC. The reaction was stopped with 150 µL of stop buffer (60 mM $FeCl_3$, 240 mM TCA and 2 M HCl). Samples were centrifuged at 14,000 *g* for 10 minutes; the supernatant was recovered and absorbance of γ-GHM was read at 540 nm at 30ºC over 20 minutes in 96 well plates with three technical replicates per sample with n = 5. Results were compared with a calibration curve done with γ-GHM as standard in stop buffer at 1 to 4 mM concentrations.

GDH activity determination was carried out in both aminating and deaminating directions at 30ºC. The reaction buffer for the aminating reaction contained 100 mM Tricine-KOH (pH 8), 1 mM $CaCl_2$, 13 mM α-KG, 50 mM $(NH_4)_2SO_4$ and 0.25 mM NADH or NADPH). For the deaminating reaction, the buffer contained 100 mM Tricine-KOH (pH 9), 1 mM $CaCl_2$, 30 mM glutamic acid and 0.25 mM NAD or NADP. The kinetic activity was monitored spectrophotometrically adding 185 µL reaction buffer to 15 µL of sample in 96 well microplates (n = 4) and quantifying consumption of NADH and NADPH for the aminating direction and the synthesis of them for the deaminating directions, at 340 nm during 20 minutes. Results were compared against a standard NADH calibration curve that measured increase in absorbance per increase in concentration.

3.2.6. Statistical analysis

Statistical analysis for all data in **Figs. 3.2-3.11** was performed with IBM SPSS Statistics 20 software package for Windows. One way ANOVA analysis was used for each of the measured parameters to compare treatments among each other, with Tukey post-hoc test to establish statistically significant differences at $p < 0.05$.

For the data in **Fig. 3.12** the Pearson product-moment correlation coefficients for each pair of parameters was calculated using Excel software for windows using the means for each treatment in a given parameter and calculating the $p < 0.05$ threshold by using Student's left-tailed t-distribution, performing a two-tailed test. The heatmap in **Fig. 3.13** was created using the ClustVis browser tool (Metsalu and Vilo, 2015) removing clustering parameters.

3.3. Results

3.3.1. Yield parameters

Yield is one of the ultimate desired traits in crops, and its quantification can provide an insight on the effectiveness of the treatment at the used conditions, although other parameters may be necessary to explain the reason behind a change in yield.

The yield quantification **(Fig. 3.2)**, done by analysing spike samples taken to the lab showed that the amino acid treatment had a significant effect of yield ($p < 0.001$). Post-hoc analysis showed that only Pro and Asn produced yield that was reduced in a statistically significant way compared to the control ($p < 0.001$ and $p = 0.048$ respectively). Between these two, Pro yield was less than the one of Asn (reduced by 25 % and 11 % compared to the control respectively). Of the treatments that could not be statistically differentiated from the control, PGA, Ala and Asp were the only treatments that had higher seed dry weight per m^2 than the non-treated plants. These produced significantly more yield compared to Arg and Gly.

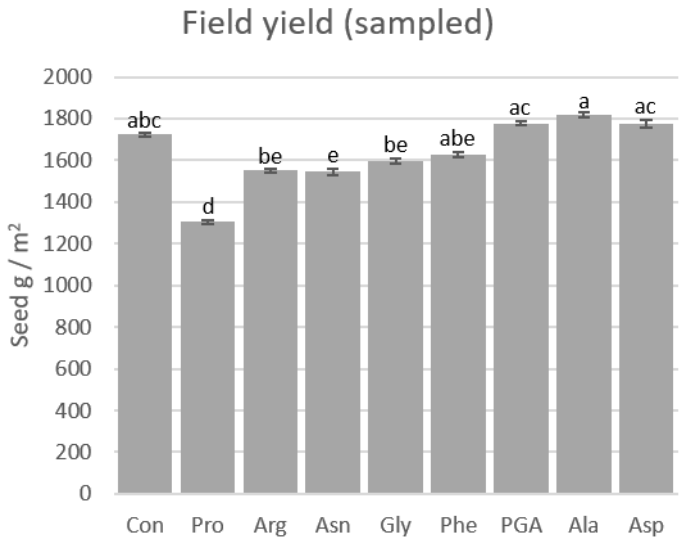


Fig. 3.2. Yield of field-grown wheat, measured as spike samples taken to the lab, separating the chaff from the seeds, calculating seed weight and extrapolating it to the number of spikes per ha. Columns represent means ± standard error (n = 50) for each amino acid treatment. Different letters indicate statistical differences (α = 0.05).

The yield was calculated by sampling spikes from the field and is dependent on the number of spikes in a given area. Yield per area may be influenced either by productivity per spike, the total number of spikes per area, or a combination of the two. When comparing the dry yield produced by each spike among all treatments **(Fig. 3.3A),** there were significant changes between treatments ($p < 0.001$), although the post-hoc test reveals there was a there was no

treatment that was statistically different to the control. However, Asn and Phe-treated plants had lower yield trends compared to all other treatments, albeit having statistical differences with only Arg ($p = 0.031$ and $p = 0.038$ respectively), Ala ($p = 0.014$ and $p = 0.017$ respectively) and Asp ($p = 0.038$ and $p = 0.046$ respectively).

The main factor that appeared to be influencing the yield difference among treatments was spikes per area or spike density (**Fig. 3.3B**), which changes significantly between treatments ($p < 0.001$). This parameter showed a very similar profile across treatments to the yield results shown in **Fig. 3.2**, with Pro standing out by having a significant decrease compared to Con (27 % reduction) and most other amino acid treatments and evidencing that the yield variation between treatments was not due to a difference in yield per spike but a difference in spike density in the field.

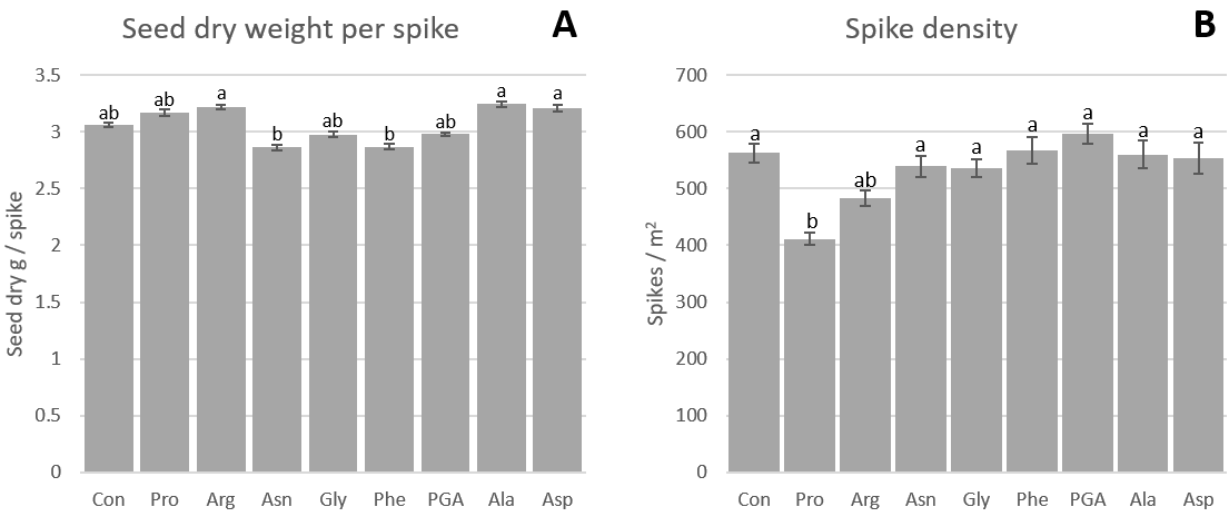


Fig. 3.3. Measurement of parameters that make up yield in field-grown wheat after amino acid application: dry yield per spike (from the harvested grain weight divided the number of spikes the grain came from) **(A)** and spike density in the field (as counted in 1 m^2 areas) **(A)**. Columns represent means ± standard error (n = 50) for each amino acid treatment. Different letters indicate statistical differences ($\alpha = 0.05$).

3.3.2. Seed, spike and plant characteristics

Seed production can be altered by a series of plant characteristics that affects the yield in a given field. Plants will have to use energy to overcome abiotic and biotic stresses in the field with the available resources. These conditions will determine how much energy and biomass the plant can use for developing spikes and seeds that will give a final yield. The characteristics of the spike, such as the length, (**Fig. 3.4A**), the number of spikelets in each spike (**Fig. 3.4B**) and the total fresh weight of spikes (**Fig. 3.4C**) can provide data about how much seed these

spikes might be able to contain and how developed spikes themselves are. At the same time the dry weight of each seed across treatments (**Fig. 3.4D**) can complement the spike and yield information with how much seed is actually being produced in those spikes.

Spike characteristics in this study, measured by cutting off several spikes along each of the treatment areas, showed very similar results for the control and amino acid-treated plants, with minor differences between different treatments. However, the ANOVA identified a significant effect of treatment ($p = 0.002$). Spike length (**Fig. 3.4A**) was similar in most of the treatments, but Pro and Arg had longer spikes than the control ($p = 0.002$ and $p = 0.006$ respectively) and all other amino acid treatments apart from Asn.

The number of spikelets per spike (**Fig. 3.4B**) was also affected by the amino acid treatments, with the ANOVA analysis showing statistical significance ($p < 0.001$). The post-hoc analysis revealed that the control treatment was not statistically different to any other. Pro and Arg were the treatments that showed higher means than the control and all other treatments, albeit only being significantly higher to Gly ($p = 0.011$ for both), Phe ($p = 0.015$ for both) and Asp ($p < 0.001$ and $p = 0.001$ respectively). Asp was also lower than Asn ($p = 0.034$). PGA plants were classified in a different statistical group than Pro and Arg, although the p value was slightly above the established α threshold ($p = 0.052$ for both) due to differences in the statistical power between the ANOVA and the post-hoc analysis.

Total fresh spike weight was relatively consistent among treatments, although the ANOVA showed statistical differences among them ($p < 0.001$). No treatments produced significantly lighter or heavier spikes than the control and PGA treatment produced spikes that were significantly lighter than some other treatments, specifically Arg ($p = 0.013$), Ala ($p = 0.008$) and Asp ($p = 0.020$).

Data for the dry weight of each seed (**Fig. 3.4D**) showed how all amino acid treated plants gave very similar spikes to the non-treated plants, with an inter-group p value of 0.237. Plants for all treatments showed similar dry weight per each seed with very slight and non-significant differences among them.

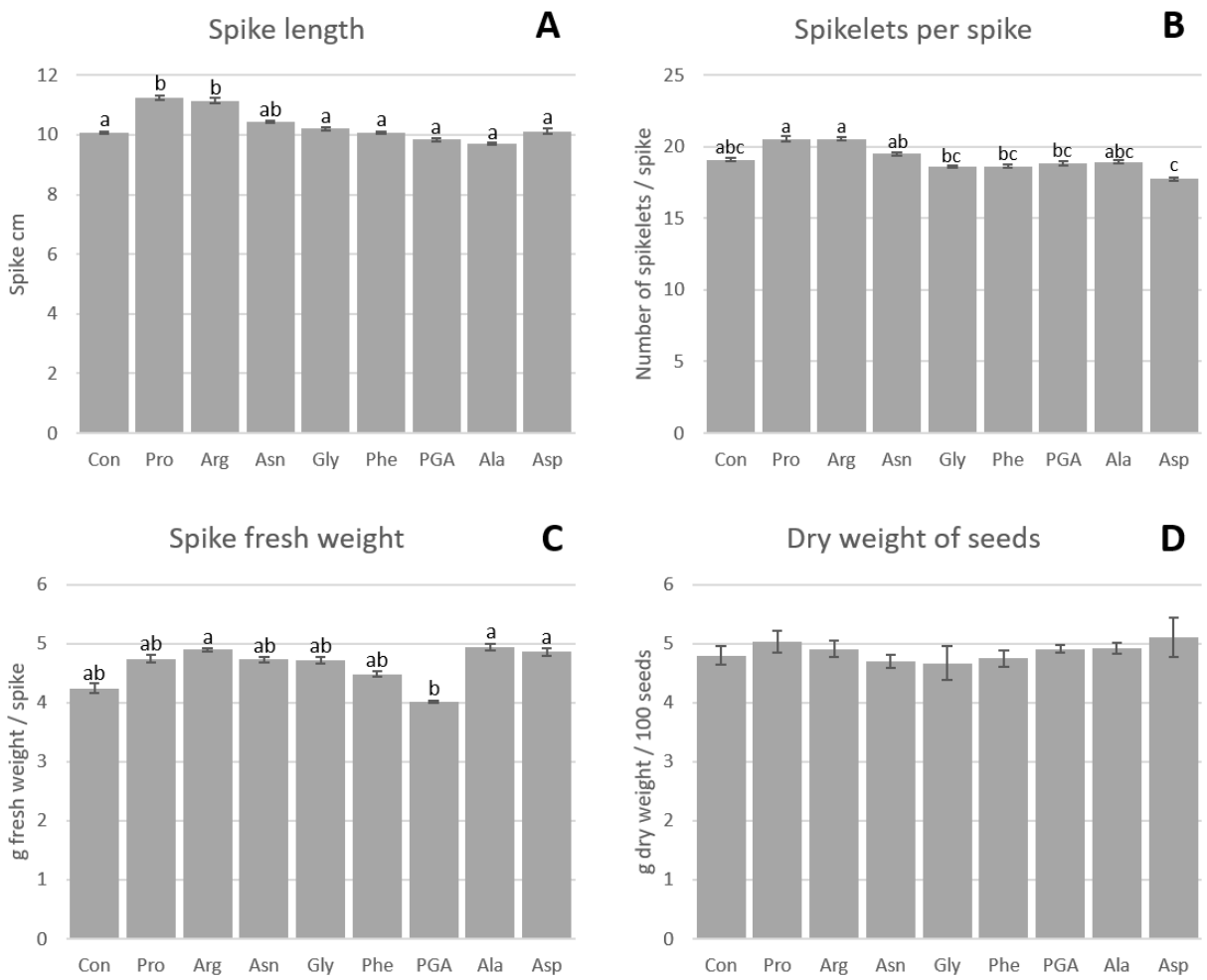


Fig. 3.4. Spike and seed data of field-grown wheat after the application of amino acid treatments: spike length (as measured) **(A)**, spikelets per spike (as counted) **(B)**, spike fresh weight (as weighed) **(C)** and seed dry weight (as weighed after separation from chaff and 48h oven drying) **(D)**. Columns represent means ± standard error (n = 50). Different letters indicate statistical differences for the cases were the ANOVA post-hoc generated different groups (α = 0.05).

Plant height is important in crops as lodging can decrease photosynthetic capacity in leaf tissue and affect yield, historically being a parameter that has been optimised since the green revolution (Evans, 1998). ANOVA analysis showed differences between treatments in plant height (**Fig. 3.5**) ($p < 0.001$). Control plants were the tallest, showing statistical differences in the post-hoc analysis with only PGA ($p < 0.001$) and Pro ($p < 0.001$) treatments, which were shorter.

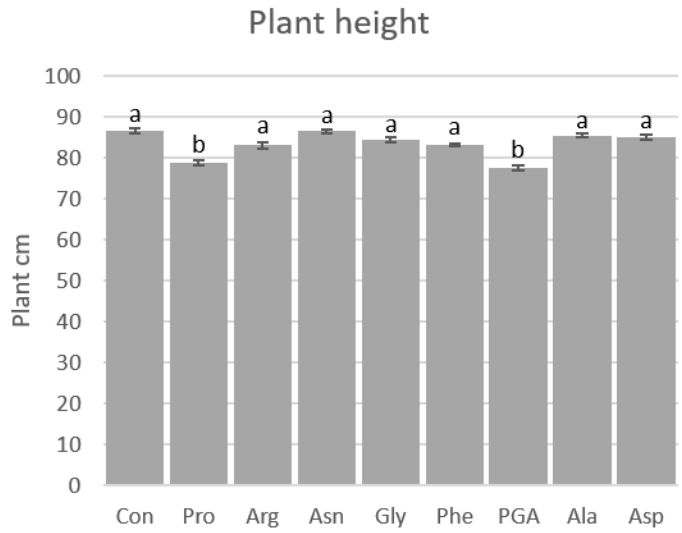


Fig. 3.5. Plant height (length) in field-wheat for each amino acid treatment as measured in the field, using a metre rule without touching the plant. Columns represent means ± standard error (n = 40). Different letters indicate statistical differences (α = 0.05).

3.3.3. Physiological properties that may affect yield

Chlorophyll, the green pigment present in plant photosynthetic tissues, is necessary to perform photosynthesis and convert light energy into chemical energy, ultimately fixating inorganic CO_2 to produce organic carbohydrates that the plant will be able to use for growth. Higher chlorophyll content enables plants to absorb more light and have a higher photosynthetic activity.

SPAD measurement showed a relative chlorophyll quantification by spectrophotometrically analysing leaves. The data gathered with this method (**Fig. 3.6**) showed inter-group differences ($p < 0.001$), with the post-hoc analysis showing that Pro was the only treatment significantly reducing chlorophyll quantity in flag leaves, being lower than Asn ($p < 0.001$) and Gly ($p = 0.023$) at flowering stage. The mean difference between the treatments with highest and lowest SPAD values, which were for Asn and Pro respectively, was of 8%.

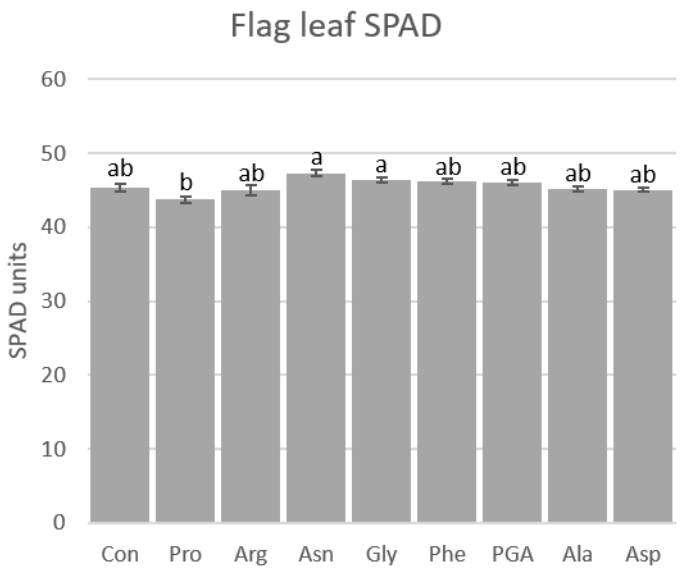


Fig. 3.6. Flag leaf SPAD data at flowering stage (Zadoks 65-69) measured with a SPAD 502 chlorophyll meter for wheat plants grown in the field. Columns represent means ± standard error (n = 50). Different letters indicate statistical differences (α = 0.05).

Chlorophyll quantification performed after extraction was measured at a later stage in plant development, correspondent to flag leaves of plants in grain filling stage. Data showed a large intra-group variability for all treatments, and no statistical differences for either chlorophyll a (Ca) (**Fig. 3.7A**), chlorophyll b (Cb) (**Fig. 3.7B**) or carotenoid (C(x+c)) (**Fig. 3.7C**) content. Chlorophyll a and b were independently measured with different wavelengths according to protocol and it was chosen to display them separately to give information of each of them as well as confirm the very similar patterns they show for each treatment. On the other side, the means were quite different between treatments, showing an evidently higher mean for Pro and PGA-treated plants, which indicates greener plants for these treatments at the measured stage. This may suggest that these two treatments can help plants stay greener for longer and delay leaf senescence. At the development stage in which these samples were taken, all treatments were statistically similar for chlorophyll content for all chlorophyll a, chlorophyll b and carotenoid content.

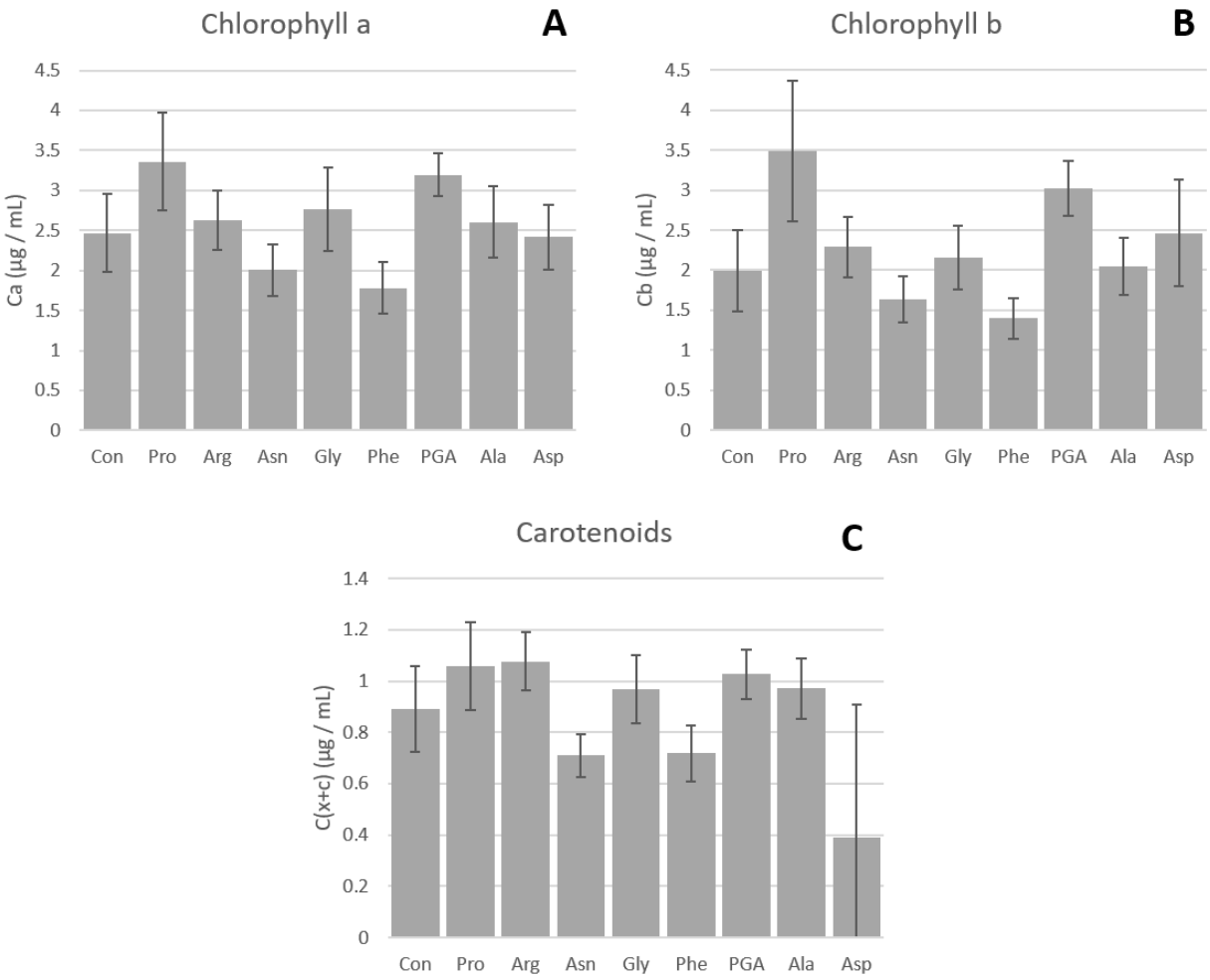


Fig. 3.7. Spectrophotometrically measured chlorophyll content for each of the amino acid treatments at grain filling stage (Zadoks stage 77-83) of plants grown in the field, including chlorophyll a **(A)**, chlorophyll b **(B)** and carotenoids (C(x+c)) **(C)**. Columns represent means ± standard error (n = 10). The ANOVA post-hoc generated no significant groups for any of the graphs shown in the figure (α = 0.05).

The length of leaf tip yellowing, measured by visual observation at flowering stage and at the same time that SPAD measurements were taken showed that flag leaves (**Fig. 3.8A**) had a very similar amount of yellow in them for each of the treatments, with the ANOVA showing no intra-group or inter-group statistical differences (inter-group $p = 0.082$). Similarly, the relative amount of yellow senescence in lower leaves of plants for each treatment (**Fig. 3.8B**) showed statistically similar results for all but two treatments. These were the control and Asp treatments, ($p < 0.001$ for both treatments compared to all others).

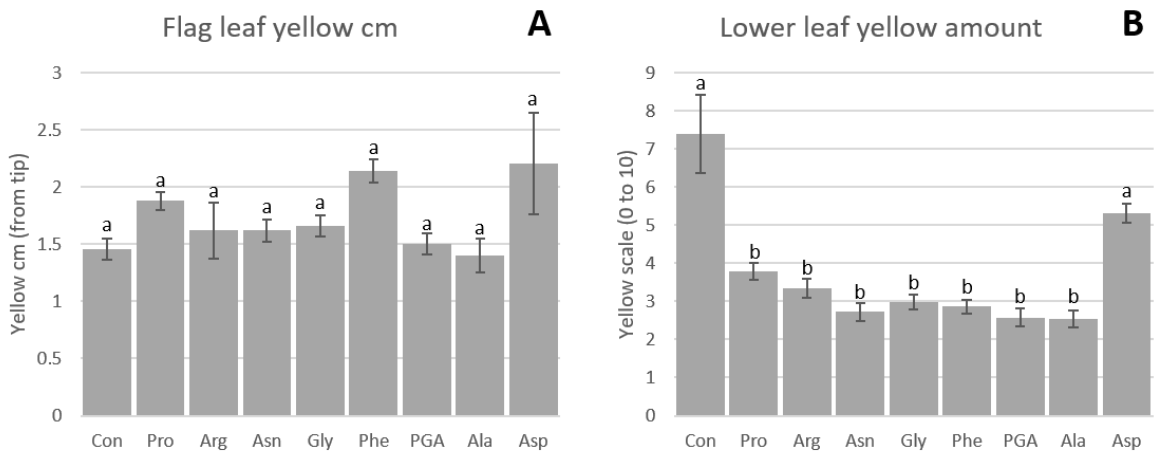


Fig. 3.8. Leaf colour status for health and senescence measurement at flowering stage: lower leaf yellow amount **(A)** and flag leaf yellow cm **(B)**. Columns represent means ± standard error (n = 40). Different letters indicate statistical differences (α = 0.05).

3.3.4. Nitrogen assimilation pathways

N metabolism is key for plant development and can play an important part in determining crop yield. N assimilation and metabolism can affect a wide range of molecules in different metabolic pathways and in different tissues that will ultimately affect plants and their adaptation to environmental conditions. To investigate this, protein content was quantified, and two key N assimilation steps, catalysed by GS and GDH enzyme activities, were measured for understanding how the different amino acid treatments affect the uptake of N and its metabolism, as well as understanding the mechanisms that are leading to these changes.

Protein content in plant tissue is related to N assimilation, as more N in the form of amino acids will enable more protein to be available. Quantification of flag leaf soluble protein at grain filling stage (**Fig. 3.9**) showed that Arg and Asn treated plants had an evidently lower protein content in leaves, with a decrease of 38 and 24% respectively in relation to Con treatment, although the results of the inter-group ANOVA remained non-significant with a p of 0.079.

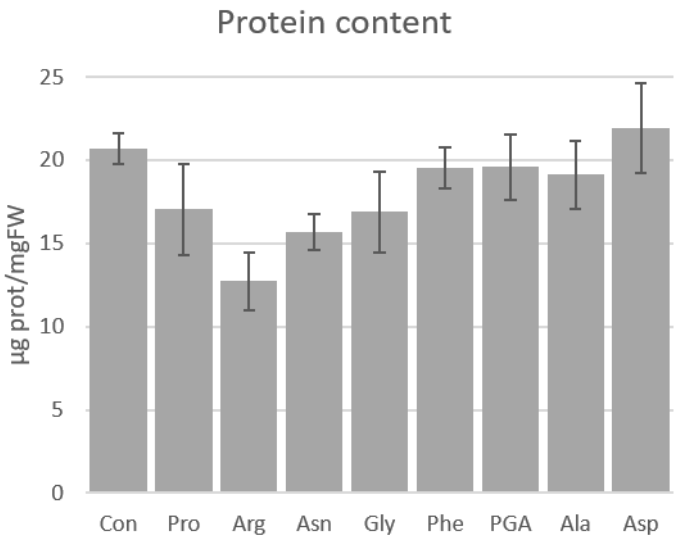


Fig. 3.9. Flag leaf protein quantification. Columns represent means ± standard error (n = 4). The ANOVA post-hoc generated no significant groups (α = 0.05).

N is necessary for wheat growth and will be used for the development of the canopy required for photosynthesis, which will drive yield. The GS-GOGAT cycle is considered to be responsible for a majority of N assimilation (Grzechowiak *et al*., 2020) and its activity in leaves during grain filling has been related to grain protein content (Zhang *et al*., 2017). **Fig. 3.10** shows GS activity at grain filling stage for all treatments. The ANOVA analysis showed differences between treatments for the activity of this enzyme with an inter-group p value of 0.001, with means similar to the control in Pro, Arg, Asn and Gly treatments while Phe, PGA, Ala and Asp treatments showed lower activities.

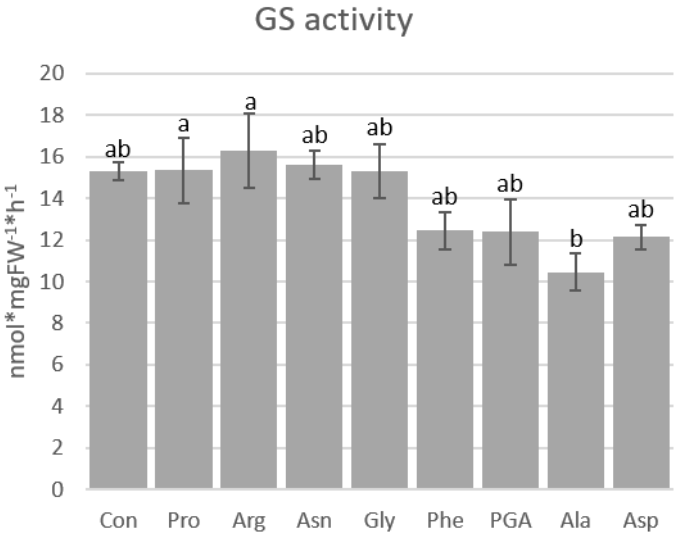


Fig. 3.10. Flag leaf GS enzyme activity. Columns represent means ± standard error (n = 5). Different letters indicate statistical differences (α = 0.05).

GDH is a reversible enzyme that converts 2-OG to Glu in its aminating direction consuming NAD(P)H and introducing a NH_4^+ molecule to the reaction. In its deaminating direction, Glu will be formed from 2-OG while reducing NAD(P) and releasing a NH_4^+ molecule. For this, GDH stands at a junction point between C and N metabolism. Not being considered a major N assimilation contributor, its main *in vivo* role is considered to be deaminating (Fontaine *et al*., 2012). It has been linked to NH_4^+ homeostasis mechanisms during specific conditions for its aminating activity (Skopelitis *et al*., 2006) and C regeneration for the tricarboxylic acid in its deaminating direction when additional C is needed (Fontaine *et al*., 2012).

The activity of GDH in the aminating direction, consuming NADH (NADH-GDH) and NADPH (NADPH-GDH) is shown in **Figs. 3.11A** and **3.11B**, with NADH-GDH being different between treatments (inter-group $p = 0.029$) but showing no difference for NADPH-GDH (inter-group $p = 0.429$). The specific activity of NADPH-GDH was only around 10% of the activity of the NADH-GDH form, indicating that the latter was the main active aminating GDH isoform. NADH-GDH activity was highest for PGA and Asp treatments, with 48% and 55% higher activity than control respectively, although only Asp was statistically different from the control.

Figs. 3.11C and **3.11D** show GDH activity in its deaminating direction, consuming NAD (NAD-GDH) and NADP (NADP-GDH). In this case, both isoforms had comparable activities, and both showed statistically significant differences among treatments ($p = 0.029$ and $p < 0.001$ respectively). In both cases, Asp treated plants were the only ones with a statistically significantly higher value compared to Con plants, with an 81% increase in NAD-GDH and 49% in NADP-GDH. There was also a notable difference in Phe and PGA plants in NADP-GDH, showing an evidently lower activity when compared to all other treatments, although only being statistically lower than Ala and Asp. GDH activities overall suggest a higher total deaminating activity (towards 2-oxoglutarate recovery), as well as higher NAD(H) activities compared to NADP(H).

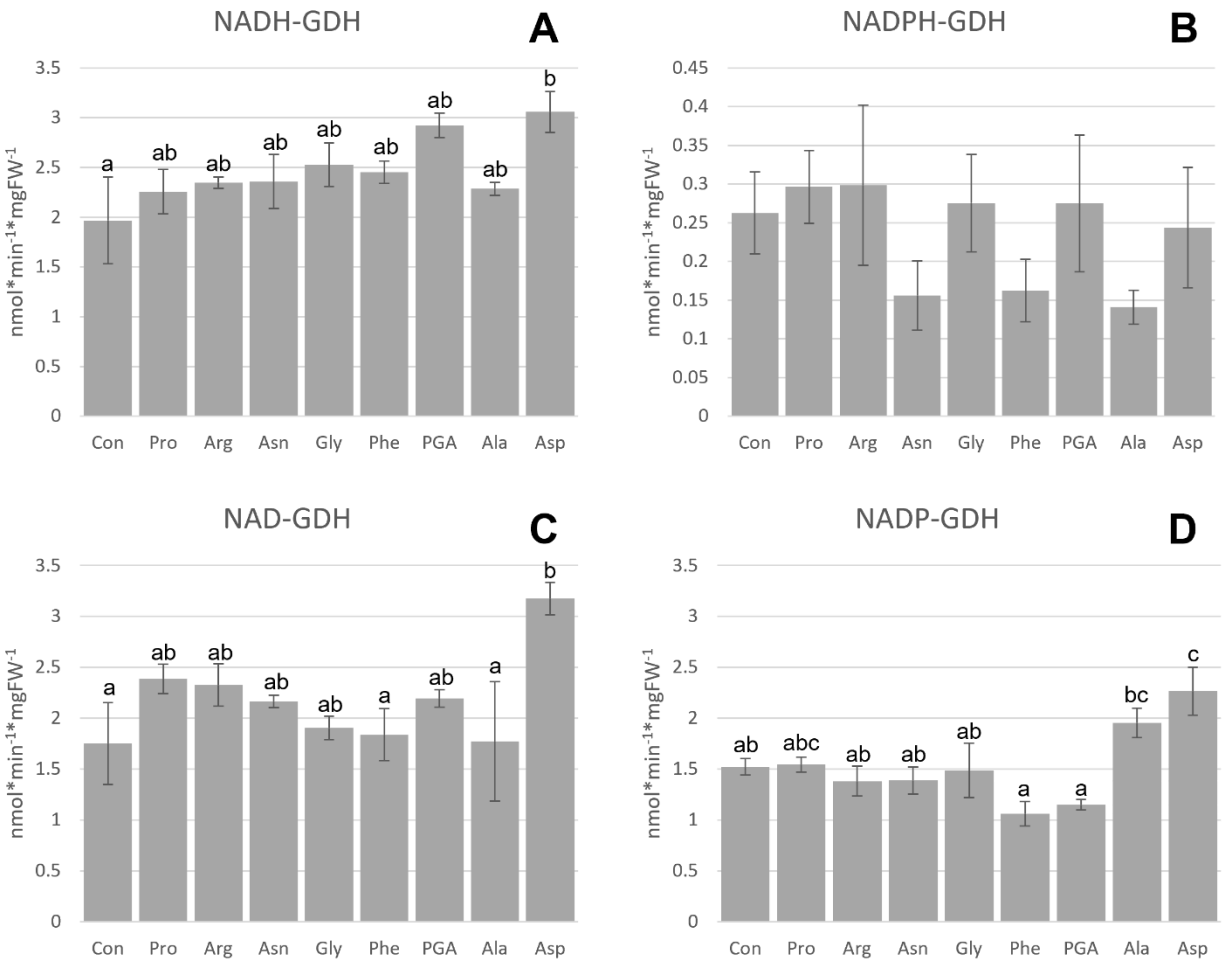


Fig. 3.11. Flag leaf GDH enzyme activities. **(A)** NADH-dependent GDH **(B)** NADPH-dependent GDH **(C)** NAD-dependent GDH **(D)** NADP-dependent GDH. Columns represent means ± standard error (n = 4). Different letters indicate statistical differences (α = 0.05).

3.3.5. Relationships between morphological, physiological, and biochemical parameters

From all parameters measured at different growth stages and shown in **Figs. 3.2-3.11**, it is of interest to see which parameters correlate with each other positively or negatively to pinpoint and understand possible causalities leading to these correlations. For that, **Fig. 3.12** shows the Pearson product-moment correlation coefficients (r) between all parameters measured in this work.

This data evidenced that yield was correlated to several spike measurements, such as having more spikes per area (r = 0.88), shorter spikes (r = -0.88) and less spikelets per spike (r = -0.73). Higher yield was also correlated to metabolic traits at gran filling stage, which included having more protein content (r = 0.61, although not reaching statistical significance with p = 0.08) and lower GS activity (r = -0.83).

As is to be expected, most of the parameters that correlated with yield correlated with each other as well. However, and although these correlations are evident, they sometimes had a p marginally above 0.05, like in the case of protein and GS activity, which shared an r value of -0.65 for which the p value was just over the α threshold at 0.058.

Plant height was not correlated to yield, but it was correlated to both chlorophyll a and b levels during grain filling (r = -0.72 and -0.77 respectively) and these two chlorophylls also correlated with each other. Chlorophyll levels at grain filling interestingly had some moderately negative correlation with SPAD measurements at flowering (r = -0.57 with chlorophyll a and -0.67 with chlorophyll b). Some of the GDH activity values did correlate with each other, in line with the bidirectional nature of this enzyme.

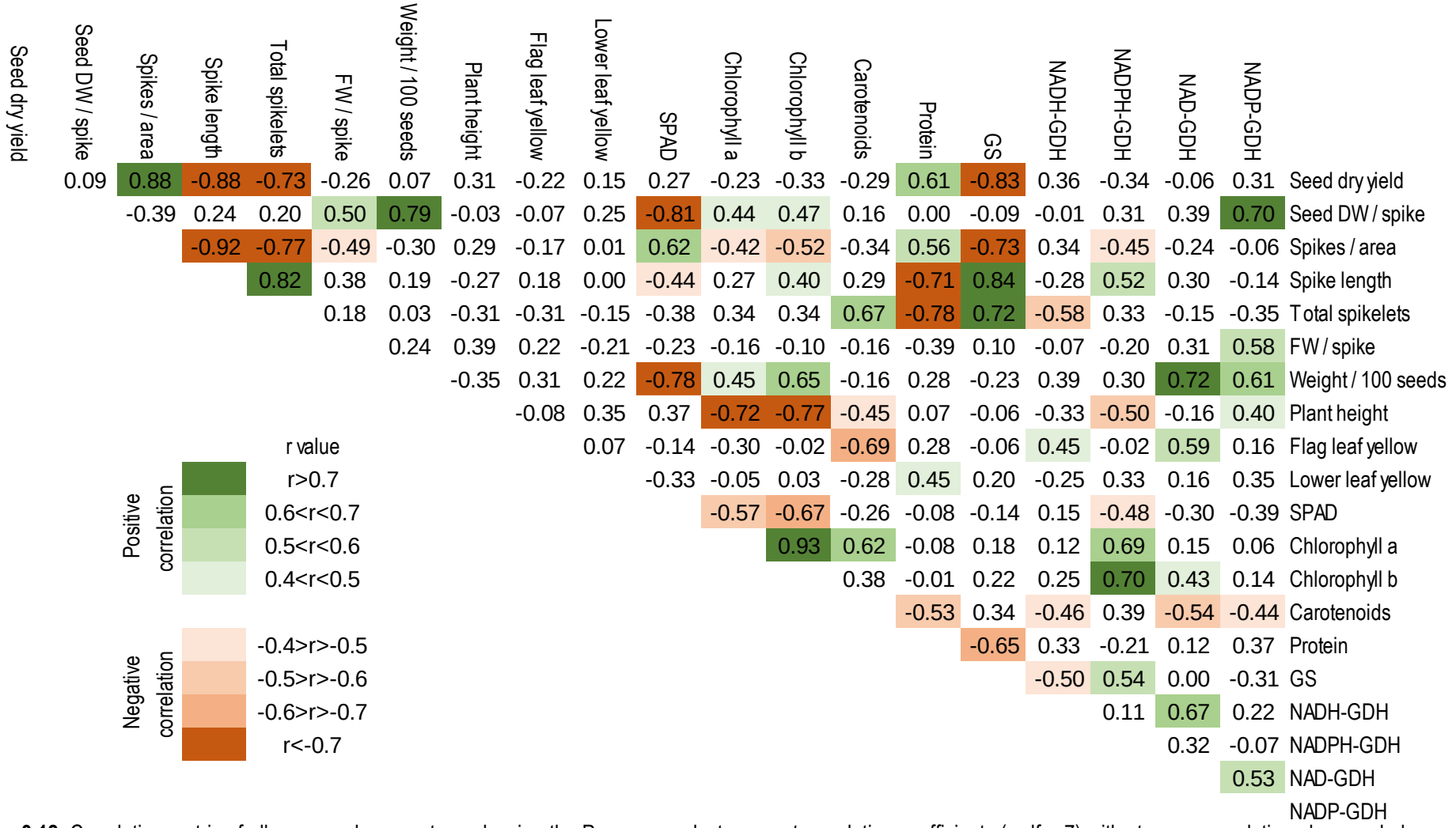


Fig. 3.12. Correlation matrix of all measured parameters, showing the Pearson product-moment correlation coefficients (r, df = 7) with stronger correlation above or below r = ±0.4 indicated by a darker shading of green (positive correlation) and orange (negative correlation). r coefficients of ≥ 0.67 will have p value of < 0.05. Description of parameters as well as the units used for each and the developmental stage in which they were measured are described in previous Figs. for said parameter.

3.4. Discussion

The study of crops in the field can help understand the conditions of these crops when they are grown for commercial purposes. Novel ways of improving crop production need to be feasible under field conditions, and amino acids have shown potential for having biostimulant effects that can benefit crop production (Popko *et al*., 2018). Previous field studies of biostimulant activities have typically focused on adding a mixture of amino acids (Morales-Payan and Stall, 2003; Yakhin *et al*., 2017), with a few recent studies focusing on the addition of individual amino acids at specific concentrations (Garde-Cerdan *et al*., 2014; Wahba *et al*., 2015; Teixeira *et al*., 2018). This study aims to characterise the effect on several growth, yield, physiological and metabolic parameters measured at specific times during the development of wheat after applying individual different amino acid treatment, in order to identify biostimulant effects of amino acids that can improve field crops. Results show different effects for different amino acids, with changes that range from different spike characteristics and yield to altered N metabolism and photosynthetic capacity.

3.4.1. Effect of the spatial distribution in the results

During the field trial it was impossible to measure all desired parameters at all developmental stages of interest, but it was possible to cover a large number of parameters measured at some important time points with field conditions. Not all measured parameters were altered between treatments, but some of them presented important differences. In a field experiment where few parameters are controlled, factors like the environment for crop growth and the soil can have a significant impact on the measurements, either by making differences larger or by homogenising the results. The lack of a controlled environment also leads to a higher variability between biological samples, increasing variance and reducing statistical power.

The experimental design of the field trial together with the results presented in this work make it necessary to address the distribution of treatments in the field and its possible influence in differences shown between treatments. At the time of the trial, it was only possible to apply the treatments in the way shown in **Fig. 3.1A**, with the tractor applying each treatment in a straight line across the entirety of the field. The lack of a randomised spatial distribution of the treatments makes it unavoidable to discuss the possibility of this affecting the results obtained.

The field distribution of treatments (**Fig. 3.1A**) shows that the strip for each column was quite narrow at 25 m wide, while the length of each strip was much greater, at 400 m. The whole field was flat and visually homogeneous. Although plants from the middle of the 25 m strip

were chosen for sampling to avoid the possibility of two different spray treatments having landed on them, there was little space between them and peculiarities of a certain land point can potentially affect results. However, the total number of plants sampled for each measured parameter were taken with ample distance between each other to cover the entirety of the 400 m length, trying to minimise the effect a specific land patch might have on plants.

Some of the treatments showed similar trends for a number of parameters which may be associated to their spatial proximity in the field. **Figs. 3.1-3.12** in the results section show the treatments ordered by prior selection, previous to applying the treatments and with the control first for reference. To allow visualisation of the trends in the data relative to field position, all results have been summarised in a heatmap in **Fig. 3.13**. Looking at this figure, there is not a clear trend across treatments that indicates an obvious effect spatial effect. There are some trends that go together with spatial proximity, but this are generally just pairs of treatments.

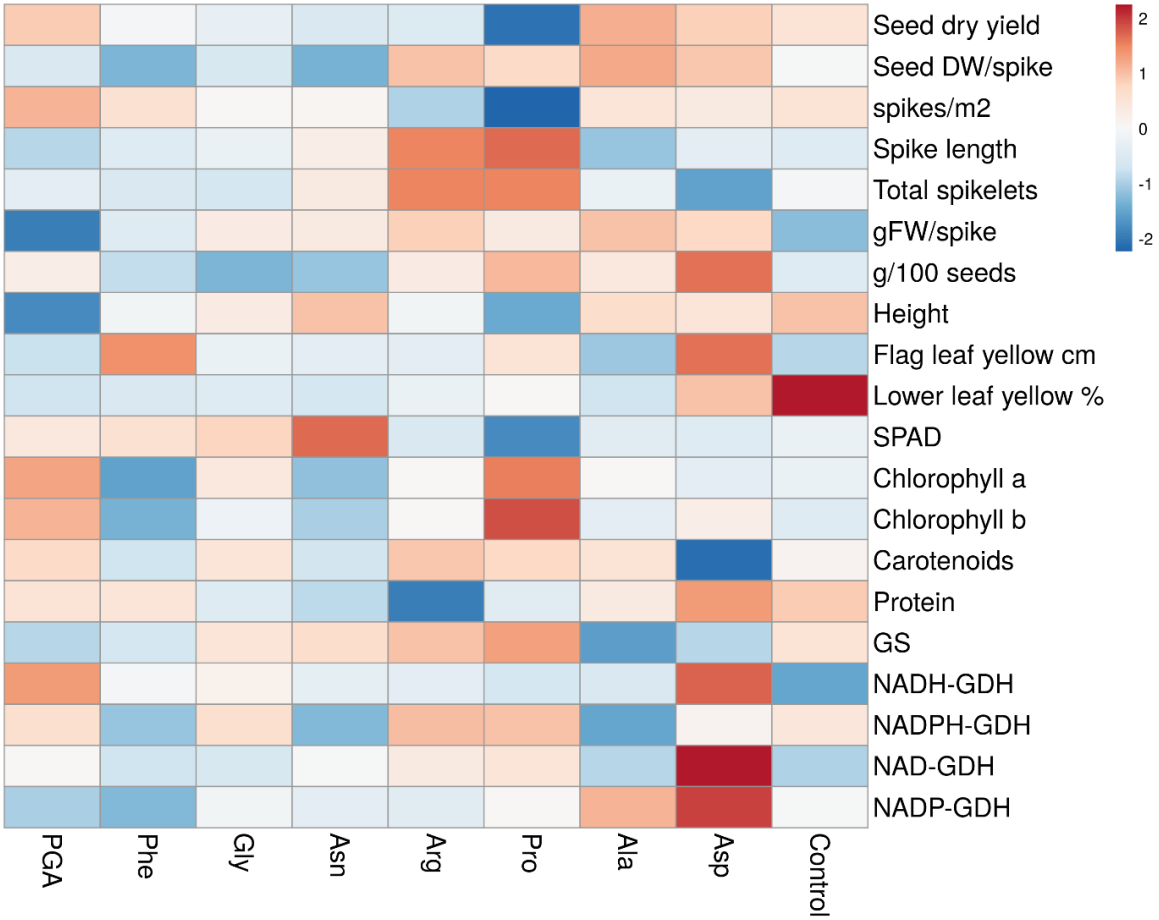


Fig. 3.13. Results of all measured parameters in Figs. 3.1-3.12 in spatial order of the field (South to North), with no clustering for treatments nor parameters (from all parameter data processed in ClustVis as explained in **Section 3.2.6**). The colour scale is assigned from the values 2 (higher values) to -2 (lower values) representing the degree of variation within a particular parameter compared to the overall variation in all parameters individually. The colour of each cell represents the deviation of each parameter for each treatment above or below the mean.

Comparing the treatment distribution along the field (**Fig. 3.1A, 3.13**) with the rest of the results, we detected that in the spike length and spikelets per spike data (**Figs. 3.4A, 3.4B**), the two treatments that stood out with the longest spikes and most spikelets per spike are Arg and Pro, which are next to each other in the field. In a similar way, the four treatments that showed the least seed dry weight per spike (**Fig. 3.3B**) are Asp, Gly, Phe and PGA, which were coincidentally also located next to each other on the field.

Metabolic and biochemical data also showed some trends that may be associated with spatial distribution of the treatments, as Pro, Arg, Asn and Gly treatments did show some very similar values for GS enzyme activity at the time of analysis (**Fig. 3.10**), and their location in the field happened to be adjacent. At the same time, measured protein content (**Fig. 3.9**) showed smaller values for these four treatments, with a dip that was most prominent in Arg treated plants. These four treatments showed similar GS activity to Con plants, which were measured on the other end of the field, but have clearly higher activities than PGA, Ala, Phe and Asp treatments. For protein measurements, the control was similar to the values of PGA, Ala, Phe and Asp plants.

As mentioned, and by the limitations of the experimental design, it is impossible to completely discard the position of treatments as a factor that affected the results to the point of altering some parameters significantly, and if so, if this was due to changes created by treatments or the physico-chemical characteristics of the land. **Chapter 4** focuses on the changes in soil microbial populations after the application of amino acid treatments in field wheat.

3.4.2. Effect of amino acid treatments in plant growth and yield

One of the primary aims in agricultural improvement practices is to improve crop yield while optimising the use of resources. For this, the yield comparison between treatments shown in **Fig. 3.2** is perhaps the one of the most important results from this chapter. Other measured characteristics are often of relative interest and serve for providing an explanation for the changes that lead to different yield outputs. In this study, the yield related parameters showed some interesting results after applying the amino acid treatments. Pro treated plants showed reduced yield per area compared to other treatments, as well as a reduced spike density **(Fig. 3.3A)**. It is inevitable that these two parameters go hand in hand, as yield per area is a function of spike density and spike grain weight. Expectedly, there was a high positive correlation index ($r = 0.88$) (**Fig. 3.12**) between yield and spike density. On the other hand, there was a no correlation between yield and grain weight per spike, showing that yield differences were because of the difference in spike number per area and not because each spike gave different

yield. It is particularly interesting to see Pro treated plants being the ones with lowest yield (**Fig. 3.2**), since Pro is the most studied amino acid that has been shown beneficial to yield in several studies across different species (Garde-Cerdan *et al*., 2014, Wani *et al*., 2016). However, the concentrations in which Pro has been studied for improved stress resilience and yield enhancement are usually much higher than the ones used in this study, using concentrations of 20 mM, 30 mM and above for these purposes and often not specifying the rate of spray application (Ali *et al*., 2007; Wani *et al*., 2016). In this study the concentration of Pro in the mist spray was much lower at 2.17 mM and an entire ha received a relatively low amount of spray at 3 L. It is reasonable to deduce that the effect of Pro will be different at this lower concentration (and probably lower application rate), enabling the observation of the effect it has at a lower concentration, probably as a signalling molecule or some biostimulant function that differs from Pro accumulation.

Grain yield also correlated with a number of other parameters that are not mathematically related to it, as shown in **Fig. 3.12**. The treatments with higher yield showed differential spike characteristics, having a very strong negative correlation with the spike length data shown in **Fig. 3.4A** ($r = -0.88$) and the number of spikelets per spike in **Fig. 3.4B** ($r = -0.73$). Spike weight and the number of spikelets per spike were strongly positively correlated ($r = 0.82$). This is similar to other studies (Genaev *et al*., 2019) and it is predictable that these two variables go hand in hand. At the same time, there were no significant differences between treatments in characteristics such as the weight of the full spikes (**Fig. 3.4C**) and of each grain (**Fig. 3.4D**). The number of spikelets per spike and the yield are determined in two different stages, with the spikelets being determined between the times of floral meristem induction and the terminal spikelet forming (Sreenivasulu and Schnurbusch, 2012) and the yield mostly being determined at later stages (Distfield *et al*., 2014). Since the weight of grains was not correlated to spike length, weight or number of spikelets, it is reasonable to think that the differences in spike characteristics and grain and yield characteristics were not altered together by the treatments as part of a single process but that there are two factors that varied independently after the amino acids are applied.

Height is also associated to growth and yield, with a lower height typically being of interest for its reduced lodging (Evans, 1998) and semi-dwarf varieties having been used for increasing yield (Zanke *et al*., 2014). Height comparison in this trial (**Fig. 3.5**) showed a similar plant height for all treatments with two outliers that show shorter plants in Pro and PGA treated plants. There was no correlation between yield and plant height (**Fig. 3.12**) (r = 0.31) with Pro being the treatment with least yield and PGA being on the other side with one of the highest yields. It is possible that applying PGA helps allocate nutrients to grain at later stages, improving the harvest index. There was however a strong negative correlation between plant height and chlorophyll a (r = -0.72) and b (r = -0.77) during grain filling, suggesting that plants with lower height can maintain a higher photosynthetic activity at this stage. It could be argued that this is related to the plants staying upright during this time, which enhances the leaf photosynthesis capacity as it has been seen in other cereal studies, contributing to achieve greater yields (Shi *et al*., 2020). PGA was one of the treatments that had a biggest yield, but Pro has significantly less (**Fig. 3.2**), while both Pro and PGA were the treatments with highest chlorophyll a and b quantities at grain filling (**Figs. 3.7A, 3.7B**). It does seem that these two treatments had enhanced photosynthetic potential at this stage, and the height of the plant could be related to it.

3.4.3. Photosynthetic pigments after amino acid application

When chlorophyll was measured in this study, it was done at two different stages using two different methods; indirectly using a SPAD meter at the flowering stage and at grain filling stage after extracting flag leaf samples. The first of these measurements (**Fig. 3.6**) showed most treatments having similar levels of chlorophyll, with Pro being the outlier being below other treatments. At this stage the flag leaf and lower leaves were also observed to quantify how yellow they were, but this quantification was very homogeneous in flag leaves (**Fig. 3.8A**) and only Con and Asp stood out in lower leaves (**Fig. 3.8B**), which did not seem to be correlated to chlorophyll or any other parameter measured in this work.

The chlorophyll (**Figs. 3.7A, 3.7B)** and carotenoid analysis performed with older plants (**Fig. 3.7C**), as discussed earlier shows the opposite, with Pro treated plants being an outlier again, but in this case having a higher mean than other treatments for both chlorophyll a and chlorophyll b. It is possible that Pro-treated plants have the capacity to stay greener for longer. Pro plants were the ones that had the least yield (**Fig. 3.2**) which is of interest as other authors have seen a reduction of biomass allocation to the grain related to the stay-green phenotype (Derkx *et al*., 2012).

PGA was the treatment with the second highest chlorophyll a and b (**Figs. 3.7A, 3.7B**), only after Pro. It is well-known that Pro can be helpful to improve photosynthetic rate, particularly under salt and water stresses where this amino acid is accumulated (Hayat *et al*., 2012), by protecting RuBisCO (ribulose-1,5-biphosphate carboxylase-oxygenase) activity and the mitochondrial electron transport chain complex II (El Moukhtari *et al*., 2020). Since these PGA and Pro are the treatments with the highest chlorophyll at grain filling stage, it is possible that the effect of adding PGA is being similar to the effect of Pro addition because of the metabolic conversion of PGA to Glu and then to Pro. The main role of PGA as historically been one of a metabolic intermediary, and this has been discussed both in the previous **Chapter 2** of this work and in similar works that use PGA as a biostimulant (Jiménez-Arias *et al*., 2019). This could explain the similarity in chlorophyll presence at this stage of development compared to the rest of amino acid treatments, as well as the control treatment.

3.4.4. Nitrogen metabolism in amino acid-treated wheat

The way crops uptake N and use it to generate amino acids and proteins can influence plant characteristics, triggering metabolic changes and influencing the flow of nutrients between organs at certain growth stages. Although most studies have been performed with mixtures of amino acids that do not facilitate identifying the effect of individual molecules (Colla *et al*., 2015), individual amino acids can beneficially alter and improve aspects of N metabolism both in controlled environment conditions and in the field (Teixeira *et al*., 2018). In this study, flag leaves at grain filling stage showed differential N metabolism characteristics. The changes that metabolism and biochemistry undergo and their impact on other parameters like yield can help explain changes between treatments.

The amino acids in this study were spray-applied to wheat together with a NPK solution, where the N addition of each extra amino acids was much lower than the amount in the NPK fertiliser itself. This ensured that the effects of adding the amino acid treatments were not simply due to the increase of N or amino acid concentration in the plant and there was a biostimulant effect created by this addition.

Proteins are essential to perform a multitude of functions in plants. Being formed by chained amino acids, changes in protein amount can be related to the assimilative potential of N metabolism and the amount of free amino acids in tissues. Protein content in flag leaves at grain filling stage showed a positive correlation to yield (**Fig. 3.12**), although not statistically significant ($r = 0.61$, $p = 0.08$). Compared to the control treatment, all amino acid treatments

showed similar or lower protein content **(Fig. 3.9)**. There were no statistical differences between treatments in the one-way ANOVA, although Arg and Asn treated plants showed an evident decrease in protein content. These Arg and Asn treatments seemed to also have less chlorophyll content than treatments like Pro, PGA and Asp (**Figs. 3.7A, 3.7B**) at the same developmental stage, hinting at senescence being more advanced in these two treatments as the photosynthetic tissues are degraded and proteins are remobilised.

GS-GOGAT is considered to be the pathway by which 95% of NH_4^+ is assimilated (Lea and Miflin, 2011) and GS is responsible for the whole plant N management (Kichey *et al*., 2006). During leaf senescence a part of the proteins will be degraded to be transported to grain in the form of amino acids (Gregerson *et al*., 2008). GS plays a key role in N remobilisation to grain (Habash *et al*., 2007) and its activity is a marker of N remobilisation to the grain (Kichey *et al*., 2007).

In this study Con, Pro, Arg, Asn and Gly treated plants showed higher GS activity levels during grain filling when compared with Phe, PGA, Ala and Asp treatments (**Fig. 3.10**). From the treatments with more GS activity, Arg and Asn have already been discussed for having less protein content (**Fig. 3.9**) and less chlorophyll content (**Fig. 3.7A, 3.7B**), which together with higher GS activity may indicate that these treatments are able to remobilise metabolites to grain as leaves progress into senescence. Proteolytic activities are associated with senescence in leaves, and a part of the proteins degraded into NH_4^+ is reassimilated to be exported from the senescing leaves in the form of amino acids (Gregerson *et al*., 2008). It has been suggested that during early grain filling, GS activity (in both cytosolic GS1 and plastidial GS2 isoforms) is increased (Ma *et al*., 2019), while at mid grain filling the expression will decrease, specially the GS2 isoform (Zhang *et al*., 2017) as the photosynthetic tissues are degraded. After this point GS1 will be responsible for N remobilisation to grain in the later stages of grain filling (Distfield *et al*., 2014). Pro treatment showed less protein content that most other treatments and one of the highest GS activities at grain filling, but the chlorophyll content was also the highest so it looks like there was still a high photosynthetic potential at this stage.

Overall, there was also a considerably strong negative correlation between protein content and GS activity (**Fig. 3.12**) ($r = -0.65$, $p = 0.058$), hinting at a relation between these two parameters, probably tied to N remobilisation and senescence timing. GS enzyme activity also presented a significant negative correlation with yield ($r = -0.83$), further indicating that the timing of leaf

senescence and remobilisation of metabolites to grain was different between treatments and affects the final yield.

GLRs are thought to be potential amino acid sensors in plants (Price *et al*., 2012), and antisense plants for AtGLR1.1 show a number of changes in transcript abundance of N metabolism enzymes, with GS1 among them (Kang and Turano, 2003). It is possible that some of the treatments here led to signalling effects due to the increased amount of certain amino acids and that was altering GS activity levels and affecting development and yield. We know that plant senescence is heavily influenced by plant hormones and environmental factors, and that a reduction in N uptake leads to N remobilisation to seeds and senescence during maturity or stress conditions (Hajibarat and Saidi, 2022). As results from this work point at a change in N remobilisation timing, it may be interesting to further study the effects of amino acids as signalling molecules that alter N metabolism and ultimately lead to changes in the plant senescence process.

Other studies have shown that applying amino acids (free or in protein hydrolisates) can upregulate genes related to the metabolism of NO_3^-, NH_4^+, PO_4^{3-}, Mg and Fe (Santi *et al*., 2017), with Teixeira *et al*. (2018) suggesting an increase in N assimilation by increasing nitrate reductase activity. In this work none of the amino acid treatments had significantly higher GS activities than the control at grain filling stage, and with GS-GOGAT being the main NH_4^+ assimilation pathway in plants it can be assumed that NH_4^+ assimilation was not increased in this study. NO_3^- reduction to NH_4^+ via nitrate reductase, the step of N assimilation increased in the work of Teixeira *et al*. (2018), was not measured here, but since NH_4^+ assimilation via GS was not increased it is reasonable to think that NO_3^- was not being reduced at a higher rate either, as that would increase the concentration of NH_4^+ to stressful or toxic concentrations.

GDH enzyme is part of the N assimilation cycle that performs the bidirectional (de)amination of NH_4^+ using NAD(P)(H). The activity for this enzyme was assayed *in vitro* in both directions and using both NAD(H) and NADP(H) as substrates for each direction **Figs. 3.11A, 3.11B** in the aminating direction and **3.11C, 3.11D** in the deaminating direction). Results from these assays showed overall GDH activity was higher for the aminating direction of the enzyme, mainly because NADPH activity was an order of magnitude lower than the other three measured activities. The other deaminating direction, NADP-GDH, however, showed lower but comparable activities to the aminating ones. For both directions on the NAD(H)-GDH, Asp treated plants were the only ones that showed significantly higher activities compared to the

control and the rest of amino acid treatments. It is important to bear in mind that these assays were performed *in vitro*, where the activities tend to be historically higher for the deaminating direction (Miflin and Habash, 2002) even though the primary role of this enzyme under normal conditions *in vivo* is considered to be deaminating (Fontaine *et al*, 2012), functioning as a 2-OG recovery and remobilisation mechanism. The assays performed in this work are *in vitro* and show the activity of the enzyme in both directions, which is very similar for both in NAD(H) enzyme. The evident increase in Asp treatment in NADH, NAD and NADP-GDH might suggest different conditions in the plants. Stress conditions have been shown to affect GDH activities, with increased activities under different stresses (Skopelitis *et al*., 2006; Zhou *et al*., 2015). It is a possibility that Asp treated plants were showing increased GDH activities because they were under a form of stress. The experiments presented in the following **Chapter 4** also showed that Asp plants were also considerably different regarding their microbial populations, remarking the differences of this amino acid treatment. Overall, it is reasonable to speculate there was some stress factor impacting the homeostasis of the Asp treated plants leading to increased GDH activity. However, the yield for Asp treated plants was not affected by any of these changes as it was one of the treatments with the highest grain production (**Fig. 3.2**).

3.4.5. Future work

This chapter presents a good first step for describing the effects of multiple amino acids when adding them to a crop of commercial interest, identifying the changes in growth, yield and metabolism, and comparing the effects between different amino acids. However, the results of this chapter represent only a fraction of all the information that we can gather to expand our understanding of amino acids as biostimulant in crops. Many of the limitations in this study have already been mentioned in previous sections of the discussion, such as the lack of randomisation of the treatments and the developmental stages in which sampling was carried out.

Looking into future field trials that can expand into the baseline established by this work and taking into account the results in this chapter and current knowledge of amino acid effects, there are some points to be taken into account the next steps into this topic. Evidently, future trials would benefit from a randomised block design in which each treatment could be sprayed in several different blocks surrounded by different random treatments to eliminate the possibility of a position effect due to any characteristic of the plants or specific soil patches. Aside from that, the effect of amino acids in this work has been evaluated at specific developmental stages. Increasing the points in which different measurements are taken, both before amino acid

application and after application, short and long term (for example, sampling 1h, 8h, 24h and 48h after application, as well as regular monitoring longer term), can help determine the timing of changes and if these last in time. This would be particularly beneficial in N metabolism parameters, which can have a short-lived effect on the plant and would help elucidate the optimal frequency of amino acid addition. In this way, it would also be possible to see at which stages does metabolism change, being able to more accurately link the treatments with particular changes in the plant regarding development and yield.

Based on the results from this chapter, analysing additional parameters would be of interest: quantification of individual amino acids, for example via high-performance liquid chromatography (HPLC), would be useful to determine if there is an increase after treatment application and if this stays longer term, and which amino acids change over in each treatment, giving us information about metabolic conversion of them.

Measuring stress markers would also be useful for determining the homeostasis of the plant after treatment application and if there is a specific elicitation of amino acid metabolism. For this, Pro is a molecule typically used as a stress marker as its in-tissue concentration increases under a variety of stress conditions. This would be an appropriate marker to use as it is an amino acid, and it is to be expected that amino acid application will influence amino acids metabolism specifically. When considering Pro as a candidate stress marker, it must be taken into account that Pro is a treatment itself in this experiment and will likely be in any future amino acid study using a wide range of treatments. However, the low concentration in which amino acids are applied would mean that any considerable Pro concentration spikes would be not because of assimilation of the amino acid in the treatment but because of a biostimulant effect. Other candidates for stress markers would be antioxidants such as phenolic compounds or flavonoids, that could also indicate a biostimulant action after treatment application if their levels are altered.

3.5. Conclusions

The present field trial addresses the effect of individual amino acids in field wheat and describes a range of changes that can be described as biostimulant effects. Results showed that applying amino acid treatments to field-grown wheat leads to differences in the crop with effects that go beyond the addition of these molecules to the metabolic pool of the plant. The application of different amino acids led to different effects, with the spike characteristics of the crop being altered and the yield being different. The study of biochemistry and N metabolism also showed that some of the treatments can alter the presence of photosynthetic pigments and N assimilation metabolism at flowering and grain filling stages, suggesting an impact on the timing for senescence and nutrient remobilisation from source leaves to sink grain.

The findings from this work do not completely identify the effect each individual amino acid has in wheat. The lack of a randomised layout in the field regarding treatments evidences the need to support these results with further studies that addresses the possible effect of this. A further trial which controls metabolic parameters at a larger number of developmental stages is also necessary to fully characterise the changes in N metabolism after adding individual amino acids, as well as expanding the scope to other metabolic routes and stress markers. Despite this, this work exemplifies the potential of adding amino acids to field crops as biostimulants to improve agricultural practices.

Chapter 4. Amino acid application to field grown wheat has differential impacts on the rhizosphere microbiome

4.1. Introduction

The microbial community present in the soil affects plants by means of the biological and chemical relation between these two players of the same ecosystem. As part of the plant-microbe relationship, the microorganisms present in the soil can significantly impact plant growth and health, in the same way that plants affect the microorganisms in the soil forming a tightly interdependent and specific community (Nihorimbere *et al*., 2011; Tkacz *et al*., 2020). The rhizosphere, being the soil which is in direct contact with plant roots, has a greater impact on the plant than the rest of bulk soil and presents a different microbial composition (Schreiter *et al*., 2014).

Microorganism associations with plant life have widely been studied for several microbes of interest such as leaf pathogens, symbiotic rhizobia and mycorrhizal fungi. However, although essential for crop development and productivity, current knowledge of most microorganisms in the rhizosphere is scarce or even non-existent (Mendes *et al*., 2013). The challenge of accurately describing the microbiome of a specific environment is due to the high number of species present in the soil, the low abundance of some groups and the difficulty of identifying, characterising and establishing cultures for some species (Spain *et al*., 2009). The complexity of the microbiome and difficulty of its analysis makes it currently difficult to link soil microbial processes to specific taxa.

Despite the difficulties described above, advances in metagenomic methodology have helped improve the understanding of the processes occurring in the plant microbiome (Fierer, 2017), enabling the bypassing of isolation methods to identify unculturable microorganisms (Suyal *et al*., 2019) and providing a very high-throughput, identifying the vast majority of soil microbes (Kaushik *et al*., 2020). Despite its limitations, such as identifying relic DNA from dead or dormant cells (Carini *et al*., 2016), metagenomic methods remain a remarkable tool for identifying the characteristics of the soil microbiome in relation to agricultural practices.

Soil composition and physicochemical characteristics also shape the microbial community in the underground ecosystem and will dictate the root-microbe relationships (Schreiter *et al*., 2014; Xiong *et al*., 2021). Agricultural inputs can directly and indirectly affect soil microbial populations, as they can modify the availability of key components in the soil, such as N and P, and alter soil characteristics such as the pH (Zhang *et al*., 2013). This change in soil characteristics can favour some species over others and change the profile of the microbial population around plant roots (Treonis *et al*., 2010; Geisseler *et al*., 2014).

Amino acid addition in agricultural input often presents limited effect on crops even at high amounts, as the rhizosphere microflora can outcompete the plants with a rapid uptake and mineralisation of free amino acids by soil microbial species, limiting the quantity available for plants (Owen and Jones, 2001). However, plants can uptake enough from liquid fertilisers to induce changes that affect plant growth and can change microbial populations (Wang *et al*., 2019).

When amino acids are applied as biostimulants, they are not used with the purpose of changing the overall available amount of N in the plant and soil and are expected to alter other parameters that may affect plant development. Few studies have been made with individual amino acids acting as biostimulants, and even fewer focused on how this may affect plant microbial populations. Teixeira *et al*. (2018) recently showed how the addition of diluted amino acids improve N metabolism variables in soybean.

The addition of amino acids in fertilisers can impact the rhizosphere microbiome altering the balance between species. This can be by direct addition of amino acids to the soil, changing its chemical composition and affecting the balance of the microbial populations, but also by indirect changes via the alteration of the plant biology. Plants may respond to amino acid application by undergoing different physiological processes, including altered responses to biotic and abiotic stress as seen in the previous **Chapters 2** and **3**, as well as in several amino acid effect studies (Pratelli and Pilot 2014, Seifikalhor *et al*., 2019, El Moukthari *et al*., 2020). It is also possible that changes in biochemistry triggered by foliar amino acid application may alter the secretion of specific substances (amino acids or others) to the rhizosphere that in turn have a direct influence on the soil microbiome.

Studying what alterations amino acids provoke in the rhizosphere bacterial population is key to understanding how the addition of these molecules can ultimately affect crops through changes in rhizosphere microflora. The aims of this chapter are to determine if distinct amino acid treatments affect rhizosphere soil microbial populations, and if so, to what extent and how can those changes affect the plant. For this, rhizosphere samples of field-grown wheat with different amino acid application were taken and DNA was extracted for metagenomic sequencing and subsequent analysis. The presented analysis focuses on establishing differences in bacterial communities of plants where different amino acid treatments were applied. Bacterial community composition between treatments is compared using mathematical approaches that take into account differences in taxa presence and richness, as well as phylogenetic closeness between them, to present results in the form of alpha and beta diversity parameters that give information about specific changes in populations between samples Singular taxa appearing more prominently in specific treatments are also identified, and possible effects of these bacteria in the plant are discussed.

4.2. Materials and methods

4.2.1. Field trial characteristics

The field trial used for this study was the same used in **Chapter 3**, thus sharing the same plant material, experimental design and growth conditions described in **Section 3.2.1**, including the application of amino acids at Zadoks growth stage 32.

4.2.2. Rhizosphere soil sampling and DNA isolation

For isolation of rhizosphere soil, plants were pulled out of the ground and shaken at almost full maturity (10 days before harvest). Roots with adjacent rhizosphere soil (1-2 mm from the roots) were cut off the plant, taking three samples per treatment from the middle of the 25 m wide treatment and with approximately 100 m distance in the strip between samples. These samples were immediately put into 50 mL tubes on ice.

Once in the laboratory, 30 mL sterile phosphate buffered saline (PBS) at a pH of 6.5 and a concentration of 0.1 M NaCl with 0.2% (v/v) surfactant (Tween 20, added after sterilisation) was added to the tubes. These were vortexed for 2 minutes and filtered into another 50 mL tube through a 100 µm nylon mesh. The resulting liquid was centrifuged for 5 minutes at 3,000 *g* and the supernatant was discarded. The pellet was resuspended in 1.5 mL PBS (without surfactant) and stored in 1.5 mL Eppendorf tubes at -20ºC.

DNA extraction of rhizosphere soil was carried out using the Qiagen pro DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany), following manufacturer instructions using approximately 100 mg tissue per sample and accounting for the deviation by adjusting the volume of the extraction buffer.

4.2.3. Sequencing and data analysis

Frozen DNA samples with ≥ 10 ng/mL concentration, ≥ 20 µL volume and A260/280 = 1.8 - 2 were sent to Novogene UK (Cambridge, UK) to perform a bacterial amplicon-based metagenomics sequencing of the 16S ribosomal region (Illumina PE250, Q30 ≥ 75%) as well as taxonomic annotation and alpha and beta diversity data analysis. There, DNA was diluted to 1 ng/µL using sterile water for amplicon generation. Amplicons were generated using V4-V5 regions of the 16S rRNA gene. Each amplicon had a fragment length of 393 bp and the primers used were the following:

Primer 515 **F** (5'- GTGCCAGCMGCCGCGGTAA -3')

Primer 907 **R** (5'- CCGTCAATTCCTTTGAGTTT -3')

PCR reactions were carried out using Phusion High-Fidelity PCR Master Mix (New England Biolabs UK, Hitchin, UK). PCR products were mixed with the same volume of loading buffer containing SYB green and a 2% agarose gel electrophoresis was used for detection and samples with a bright main strip between 400 and 4500 bp were chosen for further experiments. PCR products were mixed at equal density ratios and the mixed products were purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The libraries generated with NEBNext Ultra DNA Library Prep Kit for Illumina and quantified via Qubit and qPCR would later be analysed by Illumina platform.

For operational taxonomic unit (OTU) cluster and taxonomic annotation analysis, sequences were analysed using UPARSE software (Edgar *et al*. 2013) using all the effective tags. Sequences with ≥ 97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database (Wang *et al*., 2007) for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus and species) (Threshold: 0.8~1) (Quast *et al*., 2013). To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE software v3.8.31 (Edgar, 2004) was used to compare multiple sequences rapidly. OTU abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalised data.

Alpha diversity was analysed by studying the complexity in biodiversity of samples using observed species, Shannon index, Simpson index and Goods coverage, using the Tukey post-hoc test to statistically different groups in the first 3. Those indexes were calculated with QIIME v1.7.0 and displayed with R software.

Beta diversity analysis was used to evaluate differences of samples in species complexity. Analysis of molecular variance (AMOVA) statistical analysis (Excoffier *et al*., 1992) was used to determine if two treatments had different community compositions ($p < 0.05$). Beta diversity on both Weighted and Unweighted UniFrac distance metrics were calculated by QIIME version 1.7.0. Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package for graphics in R software. Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualise from complex,

multidimensional data. A distance matrix of Weighted or Unweighted UniFrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by WGCNA package, stats package and ggplot2 package in R. Unweighted Pair-group Method with Arithmetic Means (UPGMA) clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software (version 1.7.0). Linear discriminant analysis (LDA) effect size (LEfSe) analysis was conducted by LEfSe software. AMOVA was calculated by Mothur using the amova function.

Univariate analysis of the microbial population with each of the wheat physiological and metabolic parameters measured in **Chapter 3** was performed with the web software Calypso version 8.72 (Zakrzewski *et al*., 2017), based on R. This generated a high-throughput of correlations of differentially abundant bacteria with each parameter, statistically comparing taxa abundances across sample groups. Spearman's correlation index (r_s) was used in this analysis, basing the coefficient on ranked values for each variable. Chloroplast 16S sequence data and rare taxa with a relative abundance of < 0.01% were removed and the top 3000 taxa were included in the analysis, filtered by mean. Data was normalised via total sum normalisation (read counts divided by number of read in each sample) with square root transformation (Hellinger normalisation).

4.3. Results

When analysing microbial population data collected by sequencing ribosomal 16S ribosomal DNA, there are many different approaches available to determine similarities and differences between samples at population level. Choosing which information to compare and the appropriate analytical approach to use is a key step when processing the multidimensional information inferred from sequencing. Although there is not a consistent and standardised way of selecting which parameters to compare (Kim *et al*., 2017), the presented data aims to provide an accurate representation of the differences between treatment microbiomes and how they may be affecting plants that are in contact with them. For this chapter, the analysis presented represents a selection of all undertaken data analyses, chosen to best reflect the key conclusions from the work and taking into account common trends from similar studies (Souza *et al*., 2013; Xiong *et al*., 2017; Wang *et al*., 2018B; Siegel-Hertz *et al*., 2018, Wang *et al*., 2019; Yin *et al*., 2020; Hassen *et al*., 2020; Bickford *et al*., 2020).

4.3.1. Sequencing results and quality control

The sequencing data for each of the treatments is the result of a high number of sequence reads in each DNA sample. In this study, three DNA samples were used for each treatment, and a total of around 400,000 sequence reads were obtained for each treatment, except for Asp which had considerably less reads at 124,132 (**Table 4.1**). This result addresses the accuracy with which the results represent the actual population in each treatment of the field trial, showing very consistent results for most of the treatments, with a high number of reads. In the case of Asp treatment, the lower number of reads was inconsistent with the rest of the treatments, limiting the data we can infer from these sequence reads and the comparison with other treatments.

Table 4.1. 16S ribosomal DNA sequencing data: number of reads per treatment, as a sum of all three samples per treatment.

Treatment	Sequence reads
Con	395,801
Pro	399,075
Arg	412,406
Asn	391,162
Gly	406,057
Phe	400,292
PGA	407,068
Ala	421,480
Asp	124,132

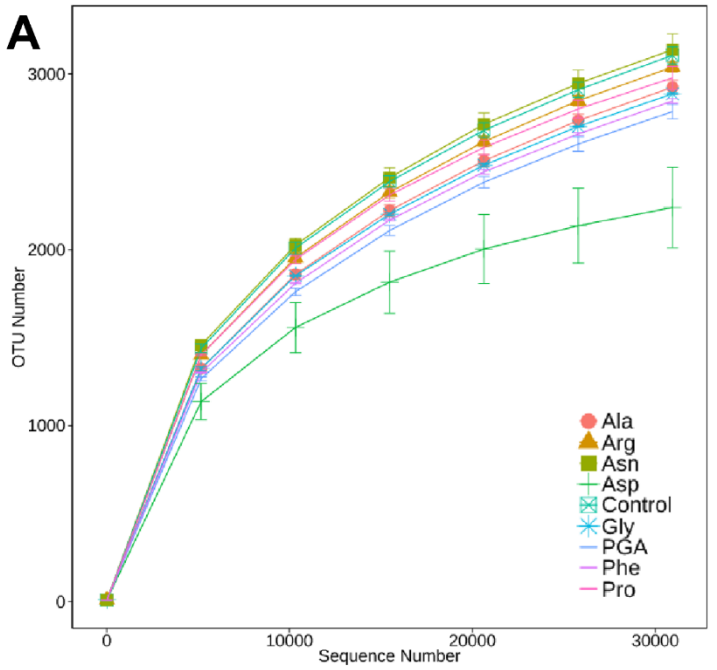
Biodiversity curves (**Fig. 4.1**) are used for indicating the number of analysed OTUs or species in metagenomic samples. This data can give an idea of the potential maximum number of species in an ecosystem and reveal information about how close the sum of analysed sequences is from the real number of species in an environment. Theoretically, analysing an infinite number of samples will give us the sequences of the totality of species in an ecosystem. In practice, the analysis is limited by the sequencing depth and coverage (related to cost) and the aim is to obtain the most complete snapshot of the microbial data as possible (Zaheer *et al*., 2018).

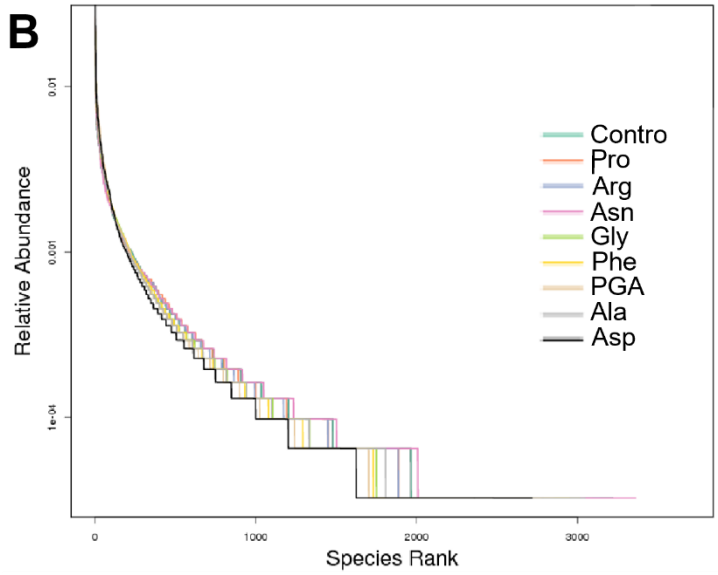
The graphical representation for the rarefaction curve **(Fig. 4.1A)** showed the cumulative number of identified OTU up to 30.000 reads. These sequence reads were taken randomly from the total number of sequences read per treatment to make up a standardised curve of the identified OTU per treatment, comparing the OTU identification process per read. Most treatments showed similar patterns and a similar number of total OTUs, with Asn treatment being the one that had the highest number of them identified with 3,000. Asp treatment was an outlier in this graph, showing a considerably lower number of identified OTUs in the environment of the plants treated with this amino acid with little over 2,000 of them identified. Overall, the rarefaction curves presented in **Fig. 4.1A** show to be approaching a plateau, a sign that a large fraction of the present OTUs were identified, despite not being fully complete and plateaued because of limitations of the method.

Rank abundance plots show a depiction of both species richness and evenness. With the rank abundance in the X axis (with the most abundant species given a rank of one) and the relative abundance in the Y axis, the richness is represented by the number of ranked species and the evenness is depicted in the slopes of the line that can fit the graph. For the present data in **Fig. 4.1B** the relative abundance of the most present species was very similar in all treatments with the biggest differences only being on the lowest rank (lowest abundance) species.

Fig. 4.1C cumulatively indicated the number of bacterial species identified as each of the samples is analysed. As the rarefaction curve in **Fig. 4.1A** was limited to a fraction of the total sequence reads shown in **Table 4.1**, this added accumulation plot serves to further confirm the thoroughness in the species representation of this dataset when depicting the environment. **Fig. 4.1C** expectedly showed a high increase after the first few samples are analysed and evident signs of plateauing after the last samples, an indication of a high level of completeness in the number of species discovered in the analysis.

Good's coverage was also calculated, an alpha diversity index used for quantifying measurement depth. This was > 0.96 for all samples, indicating that less than 4% of the reads from each sample were from OTUs that appear only once in that sample. Good's coverage indicated a high measurement depth for all analysed samples.





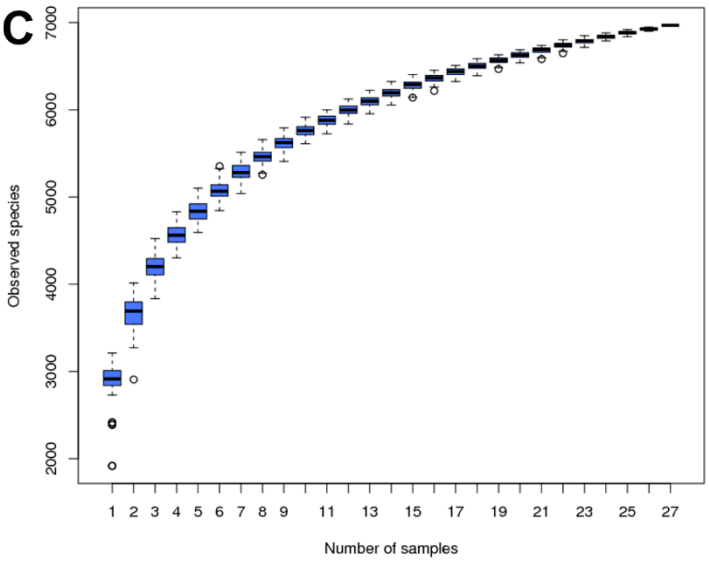


Fig. 4.1. Biodiversity curves for bacterial communities analysed in field-grown wheat under different amino acid treatments. **(A)** Rarefaction curve of the total identified OTUs per treatment over 30,000 sequence reads (n = 3) **(B)** Rank abundance curve: each ranked OTU (from the most abundant, ranked 1) in the X axis has its corresponding abundance in the Y axis **(C)** Total accumulation of observed bacterial species per number of samples analysed (n = 27).

When performing 16S DNA sequencing with metagenomic samples, not all OTUs are defined to species level, this being a limitation when identifying the effect of specific species on the soil and the plant. For analysing this, determination of the taxonomical category to which each OTU was defined is shown in **Fig. 4.2**. From the identified OTUs (that were identified at least for kingdom level) 99.6% of bacteria were identified at a phylum level, 99.0% to class level, 90.7% to order level, 85.0% to family level, 57.9% to genus level and 7.8% to species level.

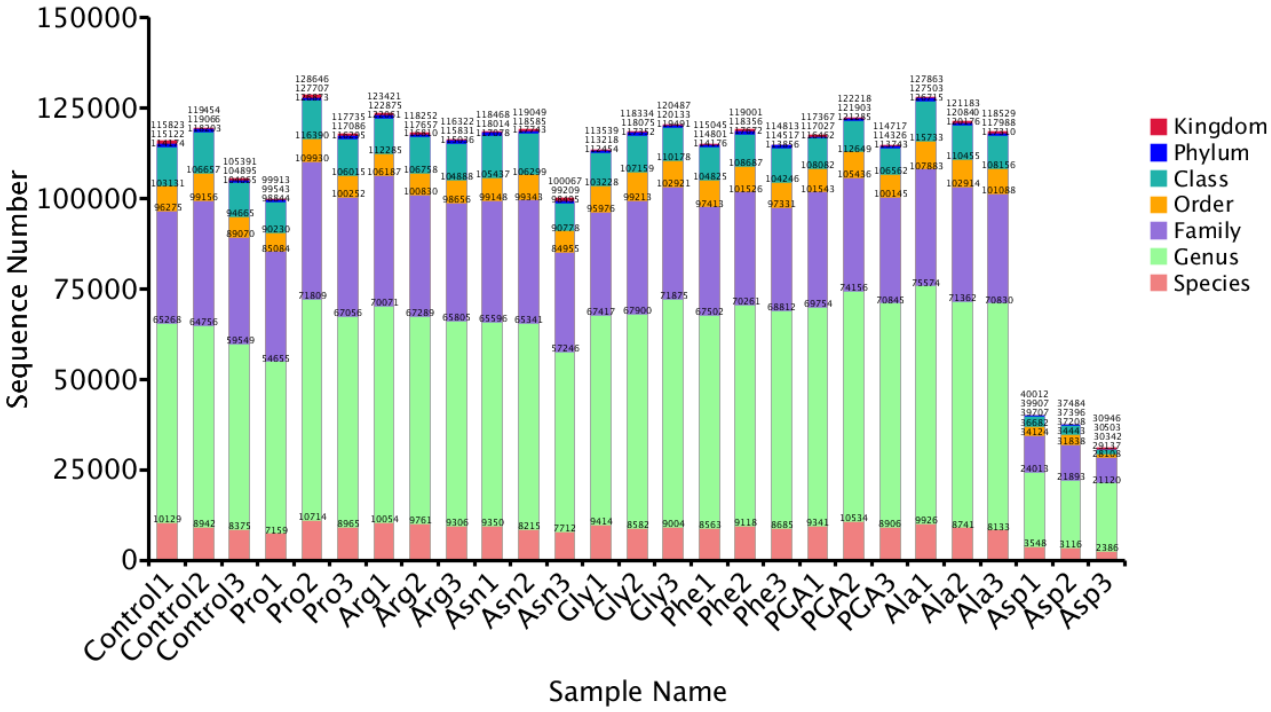


Fig. 4.2. Classification level of each 16S ribosomal DNA sequencing read for each of the samples.

4.3.2. Microbial community composition analysis

Microbial composition differences are often not only in the difference on the number of species detected or on the presence or absence of particular taxa, but in the abundance of these. By identifying the most prominent organisms in the environment and how these change between treatments it is possible to infer the microorganisms that are positively or negatively affected by the differences among them.

The top ten bacterial phyla for each treatment are shown in **Fig. 4.3A**, with the phylum level being chosen for being able to represent all groups in a single legible cumulative graph. This figure evidenced that Proteobacteria is the most represented phylum in all treatments, followed by Bacteroidetes. Together, these two phyla summed up more than 50% of the bacteria in each treatment. Gly, Phe, PGA, Ala and Asp treatments had a lower representation of Cyanobacteria than the rest. PGA and Asp had more Bacteroidetes than the rest. Asp also had more Firmicutes but less presence of Actinobacteria and Proteobacteria.

Fig. 4.3B shows the relative representation of bacteria at a lower classification level by showing predominant genera instead of phyla. It is presented as a heatmap to easily compare a higher number of genera across each treatment and their over- or under-representation. It is clear how Asp deviated a lot from other treatments with a very high number of both over- and under-represented genus. Once again, Gly, Phe, PGA, Asp and Ala shared a high number of genera underrepresented over the others, such as *Reyranella*, *Phaselicystis*, *Gemmata*, *Pirellula*, *Singulisphaera* and *Isosphaera*. Ala clearly had overrepresentation of *Pseudarthrobacter* and Phe had overrepresentation of *Galbitalea* and *Streptomyces*. PGA had quite a few overrepresented genera most evident in this treatment, such as *Bradyrhizobium*, *Rhizobacter*, *Acidibacter*, *Devosia* and *Shinella*. The control treatment, interestingly, was most underrepresented for a number of genus (and many of which PGA had overrepresented): *Rhizobacter*, *Opitutus*, *Acidibacter*, *Devosia* and *Shinella*.

These differences in phylum and genus diversity are very interesting when trying to pinpoint specific microorganisms that may thrive more or less under different amino acid application of wheat. Identifying these and trying to identify the relation (or lack thereof) of a number of these with plants may prove useful for understanding the differences in the plants under these conditions.

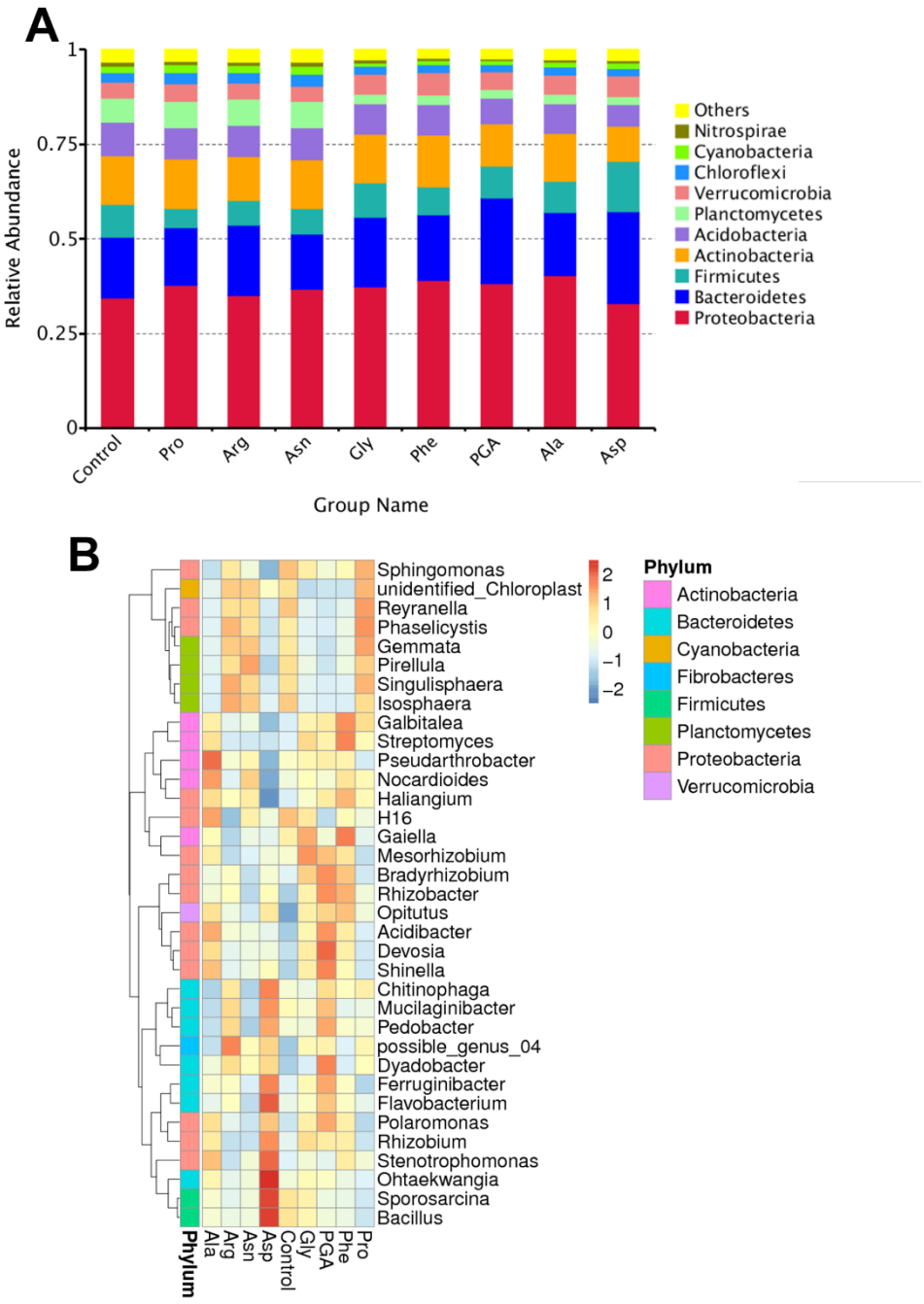


Fig. 4.3. Bacterial community composition analysis of field grown wheat under different amino acid treatments **(A)** Relative abundance of top 10 phylum per treatment **(B)** Heatmap of main bacterial genus and their relative representation across different treatments. The colour scale shows z-scores representing the degree of variation within that particular taxon compared to the overall variation of all the taxa. The colour of each cell represents the deviation of each taxon (Y axis) in each treatment (X scale) above or below the mean. Phyla for each genus are presented on the left for reference.

4.3.3. Alpha diversity analysis

Alpha diversity defines the microbial diversity within a specific ecosystem according to the samples taken from it. This measurement can summarise the structure of an ecological community according to the richness (number of species or OTUs), its evenness (the distribution of the present species or OTUs according to their abundance), or both.

In this work, alpha diversity metrics were calculated to address how the different amino acid treatments comparatively impact the number of bacterial species present in the rhizosphere soil and their abundance.

Species richness for each treatment is shown in **Fig. 4.4**. **Fig. 4.4A** shows a flower diagram with common (core) and exclusive species for each treatment. All treatments had 2,085 common species and a number of exclusive species that varies between 88 (for Asp) and 364 (for Control). Species that appeared in more than one treatment but were not part of the common core of species are omitted in this diagram, and would add up until reaching the total number of species in each treatment shown in **Fig. 4.4B**, in which each treatment showed a different number of bacterial species in the rhizosphere. Asn treatment showed the highest number of detected species, a very similar number to the control treatment, with these two being the only ones that were statistically different to PGA, a treatment with considerably less species identified. Pro and Arg treatments had a similar number of observed species to Asn and Con but were not statistically segregated from PGA. Gly, Phe and Ala on the other side had noticeably less species than Asn, Con, Pro and Arg and were very similar between each other and with PGA. However, the difference in species richness of the discussed eight treatments was small and, in most cases, statistically not significant (with the aforementioned exception of Con and Asn to PGA) comparing it with Asp, which had a lower richness than any other treatment, with a difference of over 300 species with the next treatment (PGA) and was statistically different to all other treatments.

The Shannon alpha diversity index **(Fig. 4.4C)** showed very similar results and statistic groupings to the total observed species. The Simpson index **(Fig. 4.4D)** also showed similar but more homogenised results due to its higher weighing on evenness, with all treatments being statistically equal expect for Asp. .

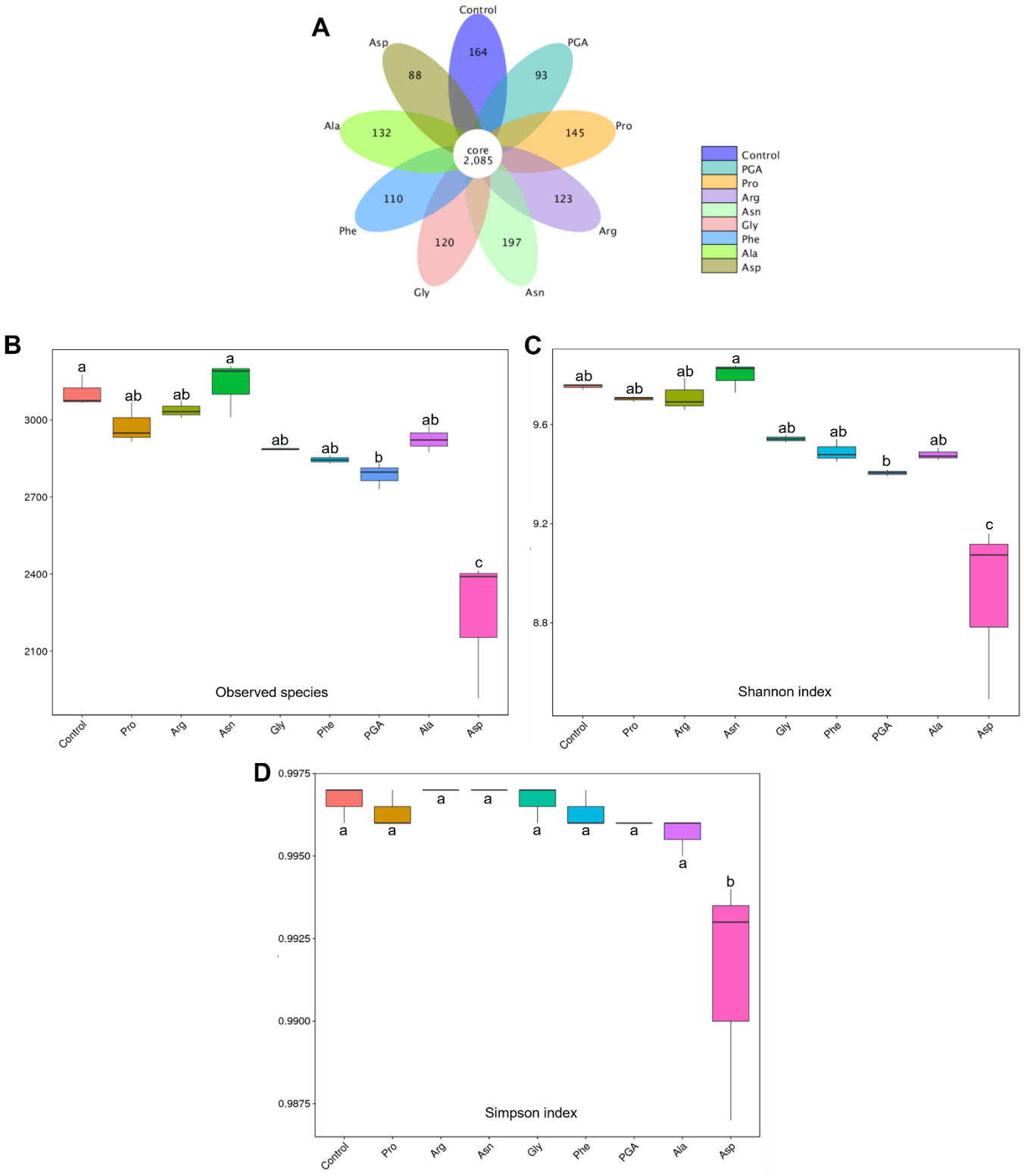


Fig. 4.4. Alpha diversity measurements **(A)** Flower diagram quantification of the number of OTUs exclusive to one of the treatments or common "core" OTUs among treatments and **(B)** Boxplot of total observed species per treatment. **(C)** Shannon index for alpha diversity **(D)** Simpson index for alpha diversity. Different letters indicate statistical differences by Tukey post-hoc test (α = 0.05).

4.3.4. Beta diversity analysis

Beta diversity analysis refers to the degree to which samples are different from one another, the variation of microbial communities between samples defining the extent of change in the community composition (Whittaker *et al*., 1960).

AMOVA (Excoffier *et al*, 1992) was used for determining possible statistical differences between treatments. This is a non-parametric method to determine whether the difference of microbial community structure between groups is significant. The AMOVA analysis gave an inter group p of < 0.001, suggesting significant effects of amino acid treatment.

The multivariant nature of similarities and dissimilarities among samples can make it difficult to identify which samples are closer to each other and which share the largest differences. For simplifying and visualising a range of samples and their differences, there are a number of mathematical approaches available to perform dimensional reductions that allow the separation of samples by using groups of variables that best explain the differences between them. For this purpose, two-dimensional plots can be used in beta diversity analysis for comparing multiple samples. The process of converting a large range of variables into two dimensions will inevitably reduce the factors taken into account and merge others. For this reason, each two-dimensional analysis will be different and limited according to the process of reducing the available data into distances in a plot. Each of the three different two-dimensional analysis performed in this study (**Fig. 4.6**) have been plotted according to different criteria.

Part of the following results are based on UniFrac distances. The UniFrac distance metric is commonly used in microbial ecology to compare different biological communities according to their evolutionary similarity. All taxa for samples are placed in a phylogenetic tree and common branches are branded as shared or unshared depending on if they are leading to taxa present in both samples or not. Evolutionary trees are compared between two samples, and when comparing over two environments, UniFrac distances are calculated using data clustering. Phylogenetic tree differences are calculated as follows:

$$\left(\frac{\text{sum of unshared branch lengths}}{\text{sum of all tree branch lengths}}\right) = \text{fraction of total unshared branch lengths}$$

Equation 4.1. Calculation of phylogenetic tree dissimilarities between two metagenomic samples.

Unweighted UniFrac will only take into account phylogenetic tree distances between treatments, thus only giving information of taxa presence. Weighted UniFrac, however, accounts for both evolutionary tree similarities (OTU presence) and relative abundance of each taxa in the environment. For metagenomic studies of microbial populations, Weighted UniFrac

measurement can help tell apart distinct environments even if microorganism species are largely similar in terms of presence/absence.

PCoA takes elements and structures from a multi-dimensional data set and reduces them to principal coordinates in which each sample is located. In PCoA, Weighted UniFrac and Unweighted UniFrac can be used to assist the analysis, giving PCoA an advantage over other two-dimensional analysis, as ecological distances between samples are part of PCoA analysis. PCoA of Weighted UniFrac distance (**Fig. 4.5A)** showed two very clear clusters of samples, where the ones from Con, Pro, Arg and Asn were much more similar to each other than to the rest of treatments. The same happens with the other four treatments, with samples from Gly, Phe, PGA, Ala and Asp being similar to each other but substantially more different from the previous four. There was a single sample from Asp that greatly deviates from all others in the PCoA based on Weighted UniFrac distance. Notably, although the abscissa axis accounted for a higher variance, mainly due to that Asp sample, and the ordinate axis was the one that, although accounting for less variance, was more effectively separating the samples by treatment.

PCoA based on Unweighted UniFrac distance (**Fig. 4.5B**) showed the same two treatment clusters, but in this case all three Asp treatments were outside them, with one of them being much further than the other two. This is unsurprising, as Asp was a consistent outlier and this treatment had less sequence reads (**Table 4.1**), less identified species (**Figs. 4.1A, 4.2**) and some of the most obvious differences on the bacterial community composition heatmap (**Fig. 4.3B**). **Fig. 4.5B** showed the opposite case to its previous figure, and the Y axis was the one that was not being effective at separating samples because of a rogue Asp sample. Looking at both Weighted and Unweighted UniFrac distance based PCoAs it is evident that the first represented the data much more accurately with 51 and 19% of the variance being represented in each axis (**Fig. 4.5A**), while in the Unweighted one 10 and 7% of the variance was taken into account with over 80% of the total variance not being represented in the plot (**Fig. 4.5B**).

PCA was also performed as an alternative method of data reduction (**Fig. 4.5C**). The samples were clustered in a very similar way to PCoA (**Figs. 4.5A, 4.5B**), with Con, Pro, Arg and Asn having less differences among them and Gly, Phe, PGA, Ala and Asp being part of the other cluster. Due to using different methods for reducing variables into two-dimensional data, distances between samples were not the same as in PCoA, with three outliers, one from each of Asp, PGA and Ala treatments. The rest of the samples were all quite packed in the Y axis and distributed along an X axis that accounted for a relatively low 9.94% of variance.

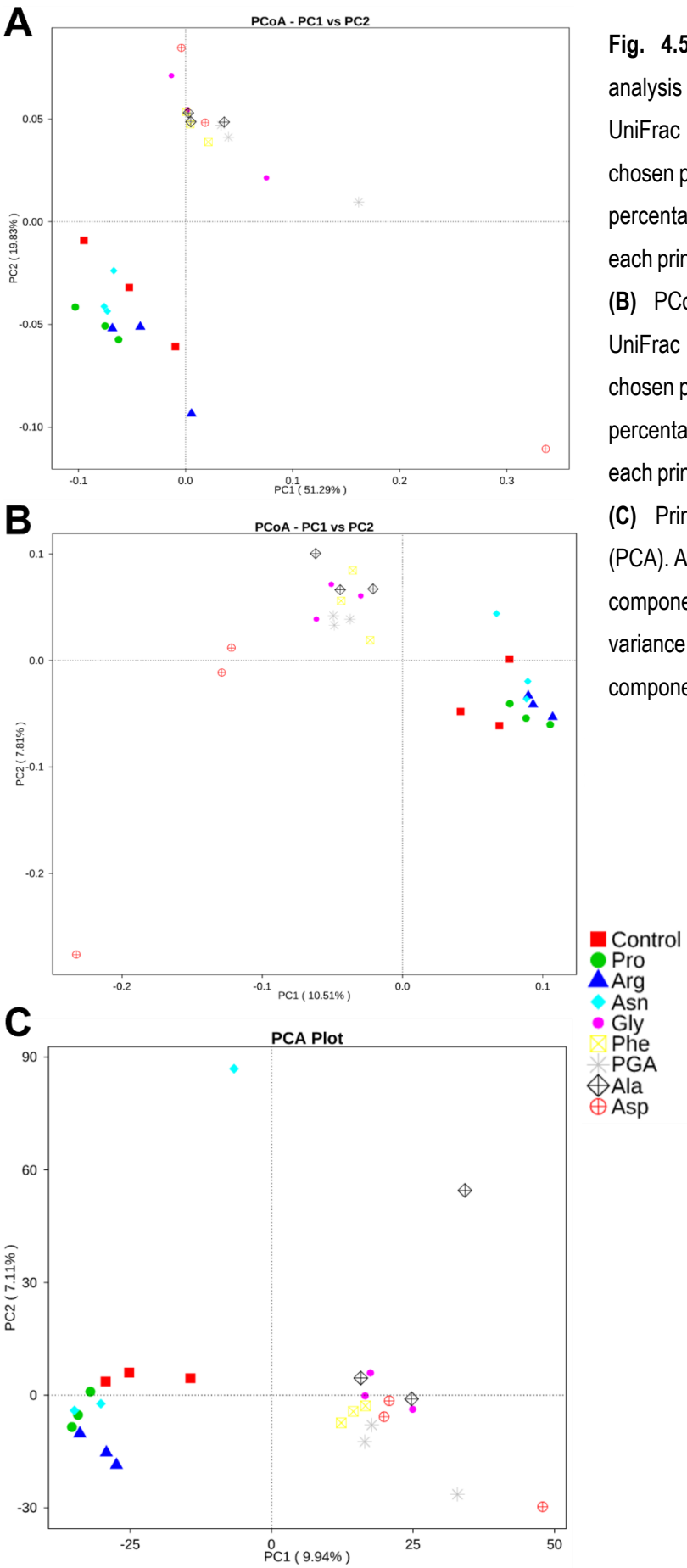


Fig. 4.5. (A) Principal coordinate analysis (PCoA) based on Weighted UniFrac distance. Axes indicate chosen principal coordinates, with the percentage of variance explained by each principal component in brackets. **(B)** PCoA based on Unweighted UniFrac distance. Axes indicate chosen principal coordinates, with the percentage of variance explained by each principal component in brackets. **(C)** Principal component analysis (PCA). Axes indicate chosen principal components, with the percentage of variance explained by each principal component in brackets.

An alternative way to investigate differences and similarities between treatment groups is performing a hierarchical cluster analysis, classifying the treatments according to pre-set differences in order to build a hierarchical tree that shows which treatments are closer to each other. This enables using large datasets such as these to build relatively simple hierarchies that help discern relationships between treatments. To this end, an UPGMA analysis was performed, taking into account both Unweighted (**Fig. 4.6A**) and Weighted (**Fig. 4.6B**) UniFrac distances. For each of these analyses, samples with the closest distance are clustered together forming a new node, with the branching point halfway between the two samples. Then the average distance between the new "clustered sample" (including both samples clustered on the first step) and the rest of samples is compared and the two closest ones will be clustered and will create a new 'clustered sample'. By repeating these steps, we can finally build a complete hierarchical tree with all samples.

The Unweighted UniFrac based UPGMA cluster tree (**Fig. 4.6A**) only gives us a classification based on phylogenetic distances (presence only) while the Weighted UniFrac tree also gives information about the abundance of taxa (**Fig. 4.6B**). Because of this, the hierarchy was slightly different between individual samples and the classification varied slightly when abundance of species was taken into account. However, both trees clearly separate two main treatment clusters: one formed by Control, Pro, Asn and Arg and another one with PGA, Phe, Ala, Gly. In the case of Asp, the Weighted UPGMA showed Asp inside the second described cluster, while in the Unweighted UPGMA it was branched off before the two main clusters, indicating that it was more different to these two clusters than they are to one another for this analysis.

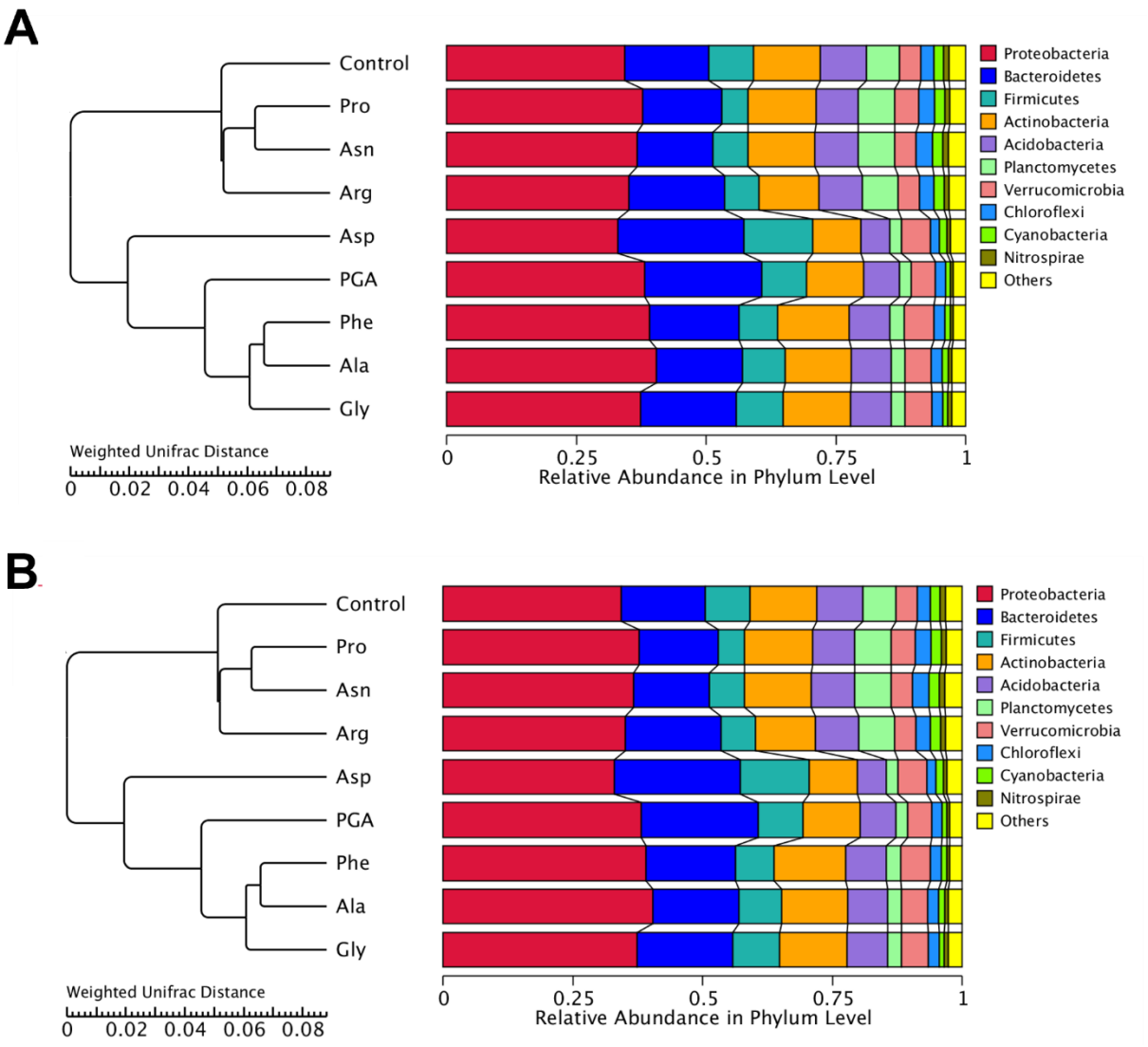


Fig. 4.6. Unweighted pair-group method with arithmetic mean (UPGMA) based on Unweighted UniFrac distances **(A)** and Weighted UniFrac distances **(B),** with the Top 10 most abundant phyla of each analysis and their relative abundances are graphed on the right of each tree for reference, from the data used for each hierarchy tree.

4.3.5. Biomarker taxa and differences in their representation

Because there is evidence for treatment effects of amino acid application on rhizosphere communities, we wanted to identify taxonomic groups that contribute to these differences. LEfSe is a tool developed to determine and find biomarkers between a certain number of groups using relative abundances (Segata *et al*., 2011). These biomarkers can be metagenomic characteristics such as species, OTUs, genes or functions that are most likely to explain the differences between classes. Identification of the biomarkers is done by emphasising statistical significance, biological consistency and effect correlation.

The biomarkers found in the LEfSe study were represented as an overall histogram, a cladogram and additional histograms for identified biomarkers. The main histogram **(Fig. 4.7A)** showed biomarkers with significant differences among treatments, ranked by effect size for each treatment. The cladogram **(Fig. 4.7B)** mapped the biomarkers to a taxonomic tree for analysing a possible phylogenetic hierarchical structure of the biomarkers. Finally, individual histograms were generated for each taxa identified as a biomarker **(Fig. 4.8)** to compare the relative abundance among treatments for each of the biomarkers.

Seven of the groups had taxa identified as biomarkers (**Fig. 4.7A**), whereas two (Phe and Arg) had none. The control treatment had more Acidobacteria than other treatments, with most of it being from the subgroup 6. PGA treatment had overrepresentation of the family Comamonadaceae, which led to over-representation of its order (Burkholderiales) and class (Betaproteobacteria). Pro treatment had Myxococcales (and its class Deltaproteobacteria) as well as Planctomycetes. Gly was overrepresented for Rhizobiales and its class Alphaproteobacteria. Ala was overrepresented for Proteobaceteria as a whole, and specifically for the class Gammaproteobacteria and its order Xanthomonadales. Asn treatment was overrepresented for Planctomycetes (fully composed by the Planctomycetales in the Planctomycetaceae family.

As expected, most of the taxa identified in the LEfSe analysis as biomarkers were represented at multiple classification levels, as shown in the cladogram representation of the biomarkers in **Fig. 4.7B**. So, for example, the genus *Bacillus* was identified as a biomarker and so was its family (Bacillaceae), order (Baccillales), class (Bacilli) and phylum (Firmicutes), all biomarkers of the Asp treatment. The same was true for Acidobacteria and its subgroup 6 for the Con treatment. The Planctomycetes phylum, although identified as a Pro treatment biomarker, had its family Planctomycetaceae and its order and class identified as biomarker of Asn treatment. Finally, the broad phylum Proteobacteria was identified as an Ala biomarker,

but the different classes within it were biomarkers of different treatments; Alphaproteobacteria (a biomarker of Gly), Betaproteobacteria (PGA) and Deltaproteobacteria (Pro). Lower subdivisions of these three Proteobacteria classes were also identified as biomarkers, always of the same treatment as their class.

Gammaproteobacteria and its order Xanthomonadales were a biomarker of Ala. However, within this order the family Xanthomonadaceae and its genus *Stenotrophomonas* were classified as a biomarker of Pro.

In **Fig. 4.8** only histograms for the lowest hierarchical levels identified as biomarkers are shown if all their higher levels are biomarkers of their same treatment, as they all shared very similar profiles across levels. If the higher-level branches into biomarkers of different treatments, then the histogram for the lowest level of each branch is shown as well. All these individual biomarker histograms showed trends seen in previous figures: either a clear difference between the two main clusters identified in the UPGMA hierarchical trees (Myxococcales, Planctomycetes, Comamonadaceae, Rhizobiales, Acidobacteria, Planctomycetaeae) or Asp as a very evident outlier (*Bacillus*, *Stenotrophomonas*, Xanthomonadales).

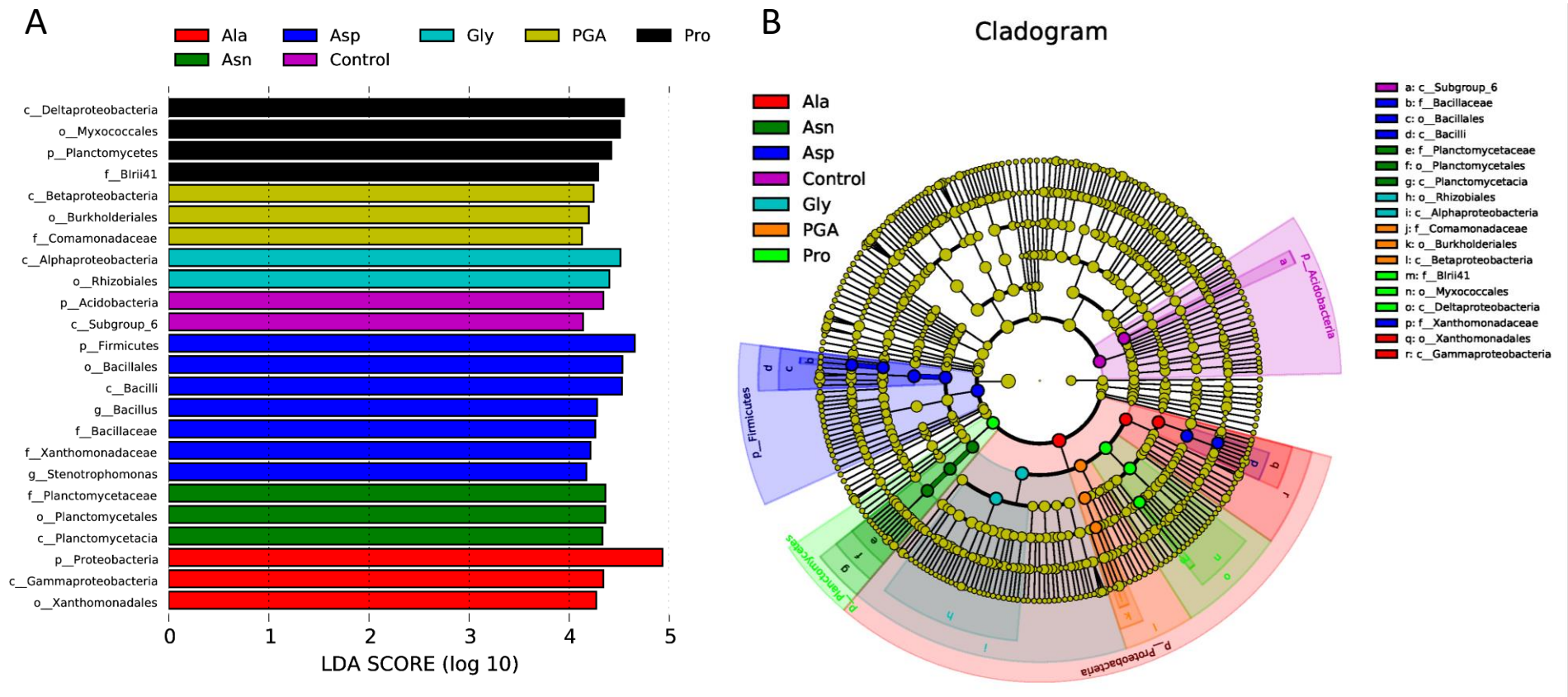


Fig. 4.7. LEfSe (linear discriminant analysis (LDA) effect size) analysis of characteristic taxa for each of the amino acid treatments in which biomarkers were found. **(A)** Histogram of taxa with an LDA score higher than the set threshold of 4 for statistically significant abundance differences about groups. **(B)** Cladogram showing taxonomic ranks from phylum (inner side) to genus (outer side). The diameter of each circle proportionally represents the relative abundance of each taxon. Coloured backgrounds with the written phylum or attached letters signify taxonomic ranks related to biomarkers, with the ranks below phylum having the same background colour as the taxa legend on the right side of the cladogram with its corresponding letters. Coloured nodes represent taxa with increased representation in the treatment of the colour in the legend on the left side of the cladogram.

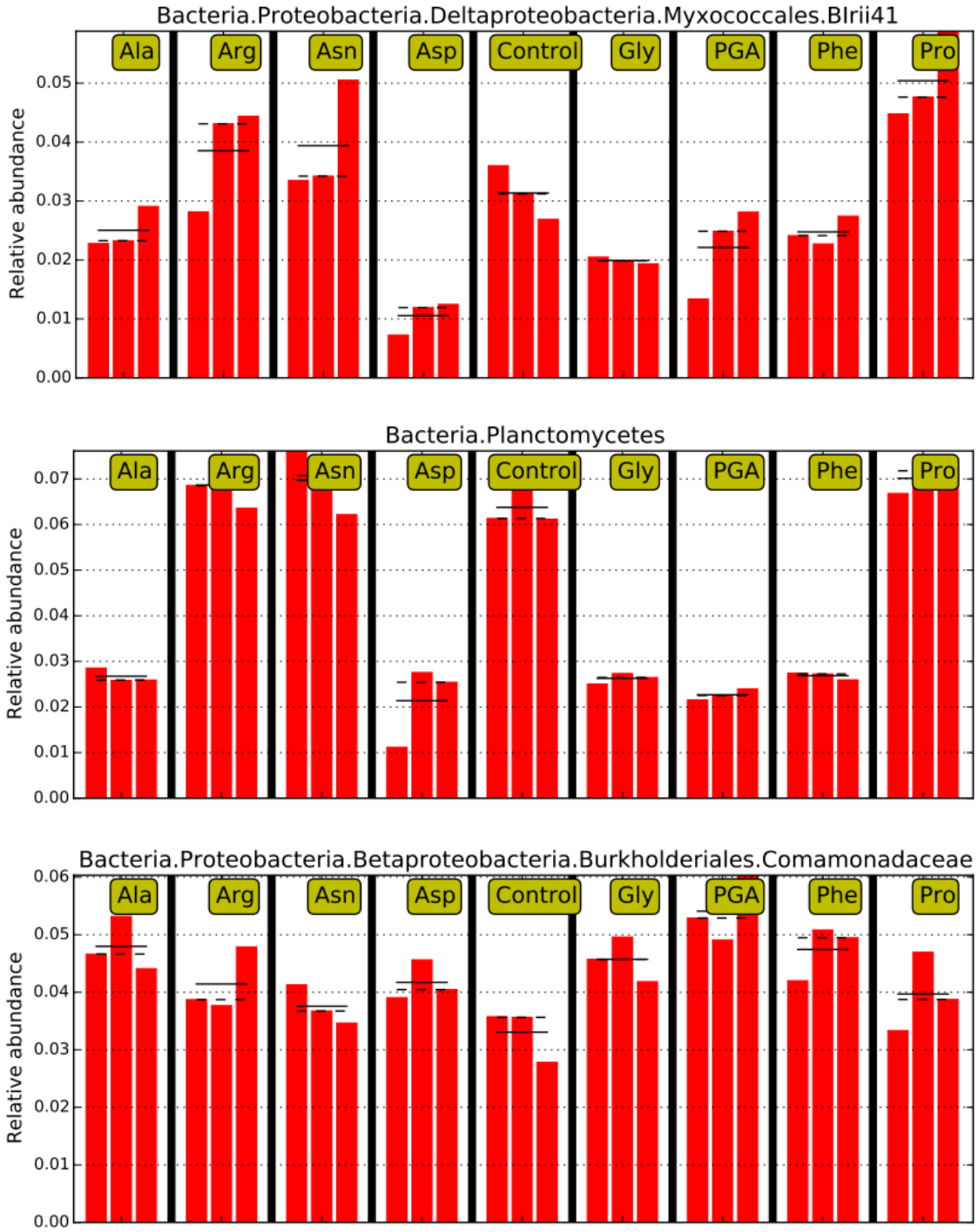


Fig. 4.8. Individual relative abundance histograms for biomarkers identified in the LEfSe analysis, only showing the lowest classification level for each biomarker present at various levels. All three samples per treatments are shown, with solid lines representing the mean and dashed lines representing the median for each treatment.

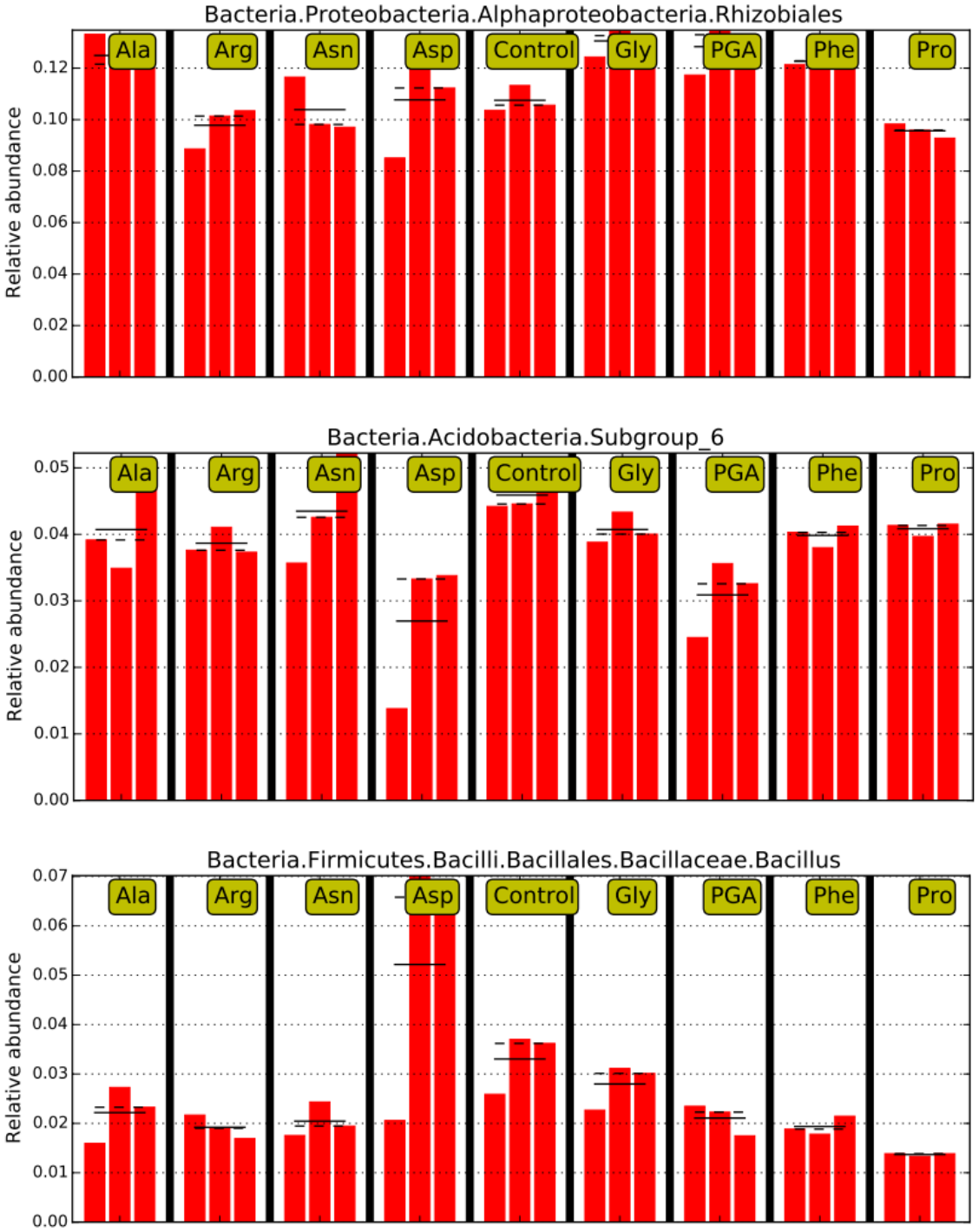


Fig. 4.8 (Continuation)

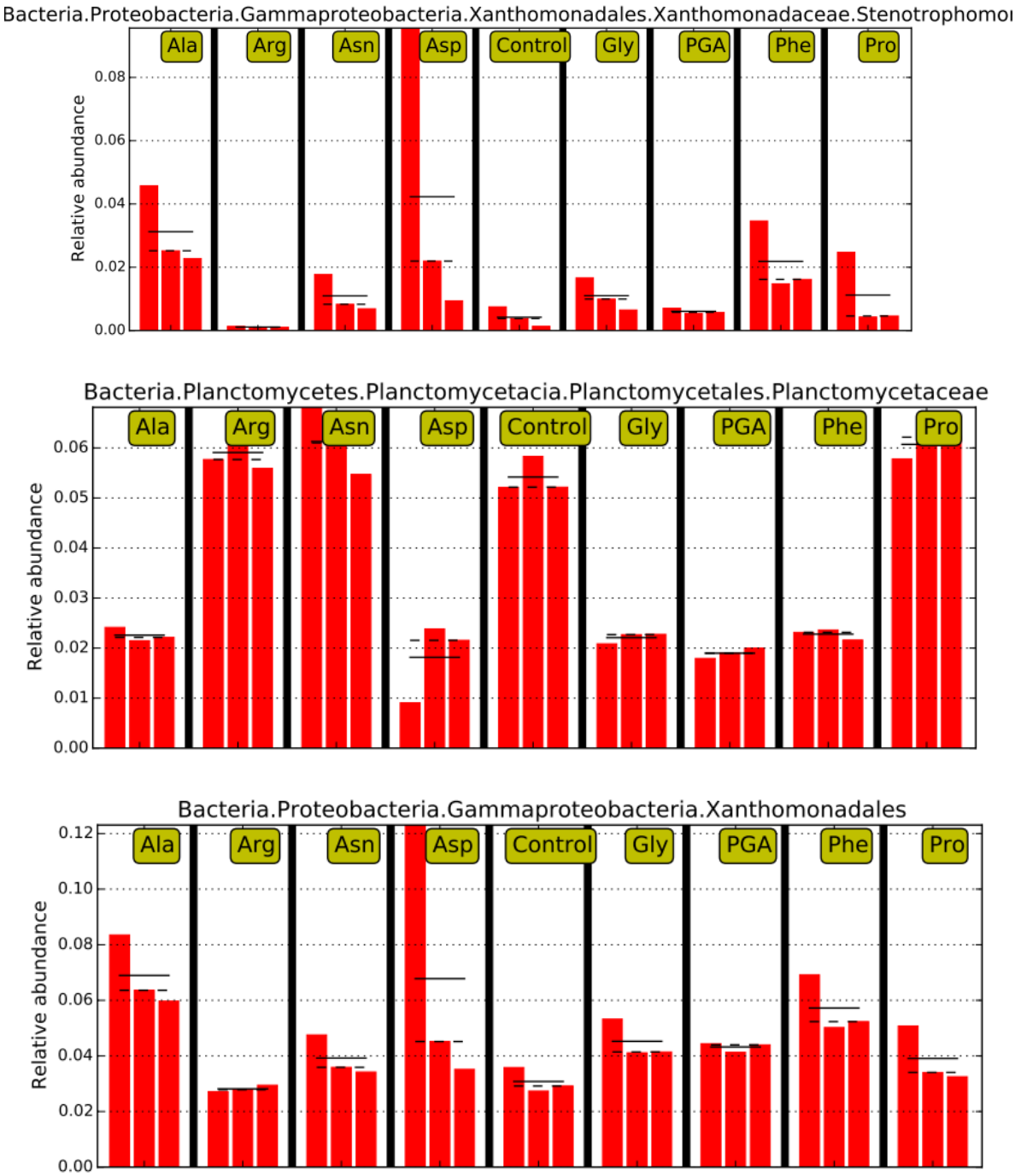


Fig. 4.8 (Continuation)

4.3.6. Correlation between microflora and plant traits

As well as a direct comparison of microbial communities between treatment sites, it is of interest to investigate possible links between the microbiome and plant traits that vary in response to amino acid treatment. Therefore, correlations between different growth and metabolic parameters measured in **Chapter 3** and the abundance of specific taxa were tested. This enabled identification of potential taxa that may directly or indirectly be affecting the crop, helping the understanding of the ways in which microbial community is related to crop performance.

Table 4.2 shows the genera with the strongest correlations (positive or negative) between their abundance and each of the aforementioned parameters. The two genera that showed correlation to most parameters are *Gemmata* and *Roseiflexus*, with five parameters being related to each. Interestingly, both of these were strongly correlated with spike length, spikelets and GS activity (positively), as well as to yield (negatively). *Gemmata* was also negatively correlated with NADH-GDH activity, while *Roseiflexus* was negatively correlated with the number of spikes per area.

A further six genera (*Aetherobacter*, *Phaselicystis*, *Micromonospora*, *Zavarzinella*, *Reyranella* and *Sorangium*) were significantly correlated with four parameters each. These showed very similar characteristics, with all being negatively correlated to yield and positively correlated to the number of spikelets and GS activity, with the first three of them also being positively correlated to spike length and the last three being negatively correlated to NADH-GDH activity. Most genera that correlated with multiple parameters showed mostly similar trends, with some exceptions, such as *Polaromonas* and *Caulobacter*, which were both negatively correlated to the number of spikelets and GS activity, with *Polaromonas* being positively correlated to yield and *Caulobacter* being positively correlated to NADH-GDH activity.

Interestingly, some parameters were strongly correlated to a high number of genera, while others had a much lower number that passed the statistical threshold. g FW/100 seeds and SPAD measurement, for example, only showed *Tumebacillus* as a significantly correlated genus, while the aforementioned association of *Roseiflexus* with spikes per area was the only one that made the cut for that parameter.

Table 4.2. Univariate analysis of bacteria genus correlated with each growth, yield and metabolic parameter measured in the study shown in **Chapter 3**. The shown r_s represents Spearman's rank correlation coefficient. The genus present for each parameter are the ones with a Bonferroni adjusted p of < 0.05. The parameters seed DW/spike, g FW/spike, lower leaf yellow, chlorophyll a, chlorophyll b, carotenoids, protein and NADPH-GDH are not shown here as they did not show any taxa with p < 0.05.

Yield		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Gemmata	-0.8	0.00019
Aetherobacter	-0.77	0.00073
Phaselicystis	-0.77	0.00083
Unclassified.MBA2108	-0.75	0.002
Micromonospora	-0.72	0.0062
Polaromonas	0.72	0.008
unidentified_Planctomycetaceae	-0.71	0.0083
Roseiflexus	-0.71	0.01
Zavarzinella	-0.7	0.014
Reyranella	-0.7	0.016
Sorangium	-0.69	0.02
Unclassified.Mitochondria	-0.68	0.028
Gemmatimonas	-0.67	0.034

Spikes/m^2		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Roseiflexus	-0.69	0.018

Spike length		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Gemmata	0.69	0.019
Roseiflexus	0.69	0.02
Phaselicystis	0.68	0.026
Aetherobacter	0.68	0.026
unidentified_Planctomycetaceae	0.68	0.032
Micromonospora	0.67	0.033
Unclassified.MBA2108	0.67	0.037

Total spikelets		
Taxa (genus)	r_s	p (Bonferroni adjusted)
Sorangium	0.93	1.50E-09
Unclassified.Sandaracinaceae	0.92	4.20E-09
unidentified_ML635J21	0.9	4.30E-08
Unclassified.BIrii41	0.9	5.20E-08
Zavarzinella	0.89	2.00E-07
Unclassified.mle127	0.89	2.50E-07
Glycomyces	0.85	6.00E-06
Caulobacter	-0.83	2.10E-05
Phaselicystis	0.83	2.90E-05
Unclassified.FW13	0.81	7.80E-05
Singulisphaera	0.81	8.30E-05
Unclassified.Planctomycetaceae	0.81	0.00012
Unclassified.SBR2076	0.8	0.00013
Gemmata	0.8	0.00013
Unclassified.Tepidisphaeraceae	0.8	0.00015
Planctomyces	0.8	0.00017
RB41	0.79	0.00025
Bdellovibrio	0.79	0.00033
Geobacter	0.79	0.00035
Actinoplanes	0.78	0.00047
Unclassified.Anaerolineaceae	0.78	5.00E-04
Unclassified.Pla1_lineage	0.78	5.00E-04
Pirellula	0.78	0.00055
Unclassified.Xanthomonadales	-0.78	0.00057
Turicibacter	-0.77	7.00E-04
Unclassified.Subgroup_2	0.77	0.00076
Unclassified.Mitochondria	0.77	0.00086
Unclassified.Gemmatimonadaceae	0.76	0.00092
Unclassified.Polyangiaceae	0.76	0.00093
unidentified_Planctomycetaceae	0.76	0.00097
Unclassified.SJA15	0.75	0.0019
Frondihabitans	0.75	0.0022
Gemmatimonas	0.74	0.0033
Polaromonas	-0.73	0.0038
Reyranella	0.73	0.0039
Unclassified.OM190	0.73	0.0045
Unclassified.Holosporaceae	0.73	0.0045

G55	0.72	0.0052
Unclassified.Cytophagaceae	-0.72	0.0058
Clostridium_sensu_stricto_1	-0.72	0.006
Polyangium	0.71	0.0081
Unclassified.Latescibacteria	0.7	0.013
Romboutsia	-0.7	0.013
Unclassified.env.OPS_17	0.7	0.013
Rhizobium	-0.69	0.016
Candidatus_Alysiosphaera	0.69	0.02
Clostridium_sensu_stricto_13	-0.68	0.021
Isosphaera	0.68	0.022
Luedemannella	0.68	0.024
X1124	0.67	0.029
Terrisporobacter	-0.67	0.029
Roseiflexus	0.67	0.03
Unclassified.MBA2108	0.67	0.033
Micromonospora	0.67	0.033
Ruminiclostridium_1	-0.67	0.037
Aetherobacter	0.66	0.043

g FW/100 seeds		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Tumebacillus	-0.77	0.00079

Plant height		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Unclassified.Subgroup_22	0.74	0.0035

Flag leaf yellow cm		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Unclassified.Alphaproteobacteria	-0.68	0.034

SPAD		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Tumebacillus	0.75	0.0019

GS		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Phaselicystis	0.87	1.10E-06
unidentified_Planctomycetaceae	0.84	1.10E-05
Gemmata	0.84	1.10E-05
Aetherobacter	0.83	2.40E-05
Sorangium	0.83	2.40E-05
Unclassified.mle127	0.83	2.60E-05
Zavarzinella	0.82	4.30E-05
Polaromonas	-0.81	7.30E-05
Unclassified.MBA2108	0.81	7.40E-05
Micromonospora	0.81	7.40E-05
Reyranella	0.81	7.90E-05
Gemmatimonas	0.81	9.00E-05
Singulisphaera	0.81	9.10E-05
Unclassified.Mitochondria	0.79	0.00022
Bdellovibrio	0.79	0.00029
Unclassified.Gemmatimonadaceae	0.79	3.00E-04
Glycomyces	0.78	0.00036
Unclassified.Sandaracinaceae	0.78	0.00039
unidentified_ML635J21	0.78	0.00048
Unclassified.Tepidisphaeraceae	0.78	0.00052
Unclassified.SJA15	0.78	0.00055
Isosphaera	0.77	0.00077
Pirellula	0.77	0.00088
Roseiflexus	0.76	0.0012
Unclassified.Pla1_lineage	0.76	0.0012
Unclassified.BIrii41	0.76	0.0012
Unclassified.FW13	0.76	0.0014
Luedemannella	0.75	0.0017
Candidatus_Alysiosphaera	0.75	0.0017
Unclassified.Latescibacteria	0.75	0.0017

RB41	0.75	0.0018
Frondihabitans	0.73	0.0035
Unclassified.Subgroup_2	0.73	0.0036
Unclassified.Xanthomonadales	-0.73	0.0039
Unclassified	-0.72	0.0051
Planctomyces	0.72	0.0052
Caulobacter	-0.72	0.0068
Shinella	-0.71	0.0077
Unclassified.03196A21	0.71	0.0077
Rhizobium	-0.71	0.0079
Unclassified.SBR2076	0.71	0.0081
G55	0.71	0.0085
Turicibacter	-0.71	0.0088
Ruminiclostridium_1	-0.71	0.01
Unclassified.env.OPS_17	0.71	0.01
Clostridium_sensu_stricto_13	-0.7	0.013
Actinoplanes	0.7	0.014
Unclassified.Planctomycetaceae	0.69	0.016
Unclassified.Subgroup_6	0.69	0.017
unidentified_Acidobacteriaceae_Subgroup_1	0.69	0.018
Unclassified.OM190	0.69	0.018
Paraclostridium	-0.68	0.025
Unclassified.Saccharibacteria	-0.68	0.027
Ohtaekwangia	-0.68	0.028
Clostridium_sensu_stricto_1	-0.68	0.028
Porphyrobacter	0.67	0.028
Unclassified.Fimbriimonadaceae	0.67	0.03
Methylotenera	-0.67	0.03
Pseudoxanthomonas	-0.67	0.031
Nordella	-0.66	0.039
Unclassified.Cytophagaceae	-0.66	0.041
Unclassified.PHOSHE51	-0.66	0.044

NADH-GDH		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Candidatus_Alysiosphaera	-0.76	0.0015
RB41	-0.76	0.0015
Unclassified.Subgroup_6	-0.75	0.0023
Unclassified.Planctomycetaceae	-0.74	0.0026

Unclassified.OM190	-0.73	0.0038
Glycomyces	-0.73	0.0042
Zavarzinella	-0.72	0.0082
Reyranella	-0.71	0.0093
Sorangium	-0.71	0.011
Unclassified.Subgroup_11	-0.71	0.011
Unclassified.BIrii41	-0.71	0.012
Unclassified.Gemmatimonadaceae	-0.7	0.012
Actinoplanes	-0.7	0.012
Frondihabitans	-0.7	0.013
Unclassified.Sandaracinaceae	-0.7	0.013
Unclassified.Latescibacteria	-0.7	0.013
Polyangium	-0.7	0.015
Unclassified.Polyangiaceae	-0.69	0.017
Unclassified.Cytophagaceae	0.69	0.021
X1124	-0.68	0.024
Unclassified.SJA15	-0.68	0.027
Pirellula	-0.67	0.038
Unclassified.Tepidisphaeraceae	-0.67	0.039
Unclassified.Pla1_lineage	-0.67	0.039
Unclassified.03196A21	-0.67	0.04
Caulobacter	0.67	0.04
Gemmata	-0.66	0.042
Unclassified.Holosporaceae	-0.66	0.042
Bdellovibrio	-0.66	0.043
Planctomyces	-0.66	0.047
Luedemannella	-0.66	0.047

NAD-GDH		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Unclassified.Nitrosomonadaceae	-0.73	0.0039

NADP-GDH		
Taxa (genus)	**r_s**	**p (Bonferroni adjusted)**
Rhodomicrobium	-0.74	0.0026

4.4. Discussion

The relationship between plants and rhizosphere microbiota can impact crop performance, altering growth and yield parameters. Changes in the plant-soil ecosystem such as the addition of specific molecules like amino acids will affect both plants and soil directly and indirectly, dynamically changing the relationship and altering the whole ecosystem. Since amino acids in field crops do impact the dynamic holobiome, studying how the microbial communities are impacted by this addition can help understanding the overall effect of amino acids in the crop. By identifying diversity changes, trends and specific taxa characteristic of one or more of the treatments we can improve our understanding of how adding amino acids will ultimately impact crop performance through the microbiome.

When taking a rhizosphere metagenomic sample, just a snapshot of the actual rhizosphere is taken, and data from the entire environment will have to be inferred from the sample. Current metagenomic analyses allow for an accurate representation of present species, although the dynamic nature of the rhizosphere means the presence and distribution of population will only be representative of the time (and plant developmental stage) in which the sample was taken. In the case of this analysis, the metagenomic samples were taken in a single point in time, at full maturity of the plants. This enables the analysis of the long-term effects of amino acid addition but also presents some limitations since it does not make it possible to compare the soil microbiome at different points in time, from before amino acid addition to short term after application, to different developmental stages after. Time, monetary and field availability issues in this field trial meant that it was not possible to sample and analyse samples at different stages, but this is something to take into account for future field trials and will lead to more informative results where the comparison between stages will give extra information of when and how amino acid addition are affecting the soil microbiome, as well as if this effects last in time.

The selected data treatment will also shape and limit the information inferred from the results. Sequence reads can identify 16S ribosomal DNA information pertaining to specific taxa in a certain environment, but the large number of taxa present and the differences between samples often being relatively small makes it tricky to establish comparisons. To date, analysing microbial diversity and changes from environmental samples remain fairly descriptive (Siegel-Hertz *et al.*, 2018) and it is difficult to pinpoint specific causes and consequences of changes in the microbiome, which would be the next step in this kind of studies but presents notorious difficulties due to the large amount of data in metagenomic samples and the relatively scarce knowledge we have of the organisms in the microbiome and their interactions between them

and with plants. Nevertheless, present descriptive analyses, alongside correlational approaches, can be useful to generate hypotheses that can be used to design experimental approaches, determining the functional relevance of particular taxonomic groups.

With the present field wheat data and after amino acid application, it is expected that samples of different treatments will present differences in terms of rhizosphere microbiota, this being part of the reason behind the plant physiological changes analysed in **Chapter 3**. These changes may be related to the ability of specific microorganisms to influence factors such as plant N metabolism and stress response mechanisms.

The analysis chosen for this study was a bacterial microbiome analysis, which can identify most bacterial species present by sequencing of the 16S rRNA gene. However, this is not the only variable present in the soil. Other microorganisms, such as fungi or archaea are also present in the soil and can present alterations from their environment and after the addition of compounds such as amino acids. These microorganisms can affect plants substantially: fungi can form symbiotic relationships with plants providing N and P for them (Chen *et al*., 2021) and archaea can activate plant immunity after colonising roots (Jung *et al*., 2020). Although all microorganisms could not be analysed here due to monetary limitations, it is important to have in mind that there are other parameters at play in the rhizosphere, either in the form of other microorganisms as mentioned, or physico-chemical characteristics of the soil that can also be affected after some compounds are applied via agricultural methods and change over time.

4.4.1. Sequence data represents good coverage of the rhizosphere bacterial microbiome

When analysing metagenomic data, evaluating the samples to establish the accuracy of them is an important step to determine the confidence with which conclusions can be inferred from them (Kim *et al*., 2017). Validating the data quality ensures that the analysed sample data is complete and accurately represents the environment. Results from **Fig. 4.1** are a sign that most species in the environment were detected, with plateauing graphs both for the number of OTUs per treatment (**Fig. 4.1A**) and the number of species detected between all analysed samples (**Fig. 4.1C**). It is also worth mentioning that **Fig. 4.1A** contains 30,000 reads per treatment to normalise the results across treatments and the total reads per treatment are between 145,132 and 421,480 **(Table 4.1)**. It is therefore reasonable to assume that the curve would be completely plateaued if the entirety of the reads were considered. Furthermore, **Fig. 4.1B** evidenced how the predominant species shared a very similar abundance profile across all treatments. This homogeneity continued for a relatively large number of species, although after

200 of them there is a more evident differentiation. This result was to be expected, as all treatments shared adjacent soil, the same plant species and the same climatic conditions and thus it was not expected that the amino acids would completely overhaul the microbial distribution. Other studies with similar measurements like Wang *et al*. (2018B) show higher differences in all the mentioned parameters used in this work for data validation, which is also to be expected as it analyses the rhizosphere microbiome of different plant species from the same genus. Here the conditions between treatments are more similar, explaining the higher homogeneity of the data, especially in predominant species. The larger variation in presence/absence of the least abundant species was most likely due to these species appearing only in one or some of the treatments. Taken together, the information from **Fig. 4.1** makes it reasonable to assume that the analysed samples capture a very complete picture, accurately representing the microbiota present in each environment.

From all analysed treatments, there was one that stands out in almost all analysed parameters: Asp samples were the only ones that show a high deviation from the rest in terms of sequencing reads (**Table 4.1, Fig. 4.2**), all three samples for this treatment had a lower score on alpha diversity indexes (**Figs. 4.4C, 4,4D**) and less species were determined for it (**Fig. 4.4B**). Nevertheless, the rarefaction curve (**Fig. 4.1A**) indicated that a high proportion of species present had been captured by the sequencing data. This treatment also showed the strongest over- and underrepresentation of specific genera, contrasting with all the rest (**Fig. 4.3B**), although the main phylum relative abundance was only slightly altered (**Fig. 4.3A**), with an increase in Bacteroidetes and Firmicutes and a decrease in Proteobacteria, Actinobacteria and Acidobacteria. Beta diversity bidimensional plots (**Figs. 4.5A, 4.5B, 4.5C**) showed that two Asp samples were much more similar to several treatments (Gly, Phe, PGA and Ala) while there was a single one that deviates greatly from all other samples. Since all three samples were affected in the deviation of this treatment in respect to the others, it is not likely that this difference was due to a technical error. The samples were taken with a long distance between them (100 m, four times the distance between a sample of a treatment and the same sample of the treatment next to it), so it is unlikely that this was due to a specific patch in the field presenting natural differences in soil. It is more likely that Asp treatment led to changes that are not recollected in this bacterial analysis, such as more domination of other microorganisms like fungi, or changes such as plant stress specific to this treatment.

4.4.2. Rhizosphere microbial diversity differs after amino acid treatment

The data suggests quite similar microbial presence in most of the treatments, although some notable differences appeared, differentiating some treatments from others. Alpha diversity indexes aim to determine the diversity of an environment based on their species richness and evenness, here measured by the two widely used indexes to measure this parameter (Kim *et al*., 2017): Shannon and Simpson indexes. The Shannon index, more focused on the number of present species (species richness) (Spellerberg and Fedor, 2003), showed all treatments except for Asp had very similar diversities (**Fig. 4.4C**), and the distribution was very similar to the observed species in **Fig. 4.4B** with the only statistical difference (apart from Asp) being between Asn and PGA. Whilst non-significant, Con, Pro and Arg tended to be closer to Asn while the rest of the treatments were more similar to PGA. The Simpson index (**Fig. 4.4D**), which focuses more on species evenness (Simpson *et al*., 1949), showed all treatments except for Asp had similar values, underlining the homogeneity between treatments when considering the relative abundance of species, also shown in the similar distribution of the main present phyla shown in **Fig. 4.3A.**

The overall alpha diversity indexes from these treatments in terms of species richness and evenness was much more similar than the one observed in other works (Wang *et al*., 2018B, Praeg *et al*., 2019), which was to be expected in samples of treatments located in the same field. Furthermore, any noticeable difference in the alpha diversity between treatments (except Asp) resided in the presence of different species across treatments and not in the evenness of them. There were, however, specific genera with altered representation in some of the treatments (**Fig. 4.3B**), with a notable underrepresentation of certain Planctomycetes and Proteobacteria genera in Ala, Asp, Gly, Phe and PGA treatments, although, as mentioned, the effect in the main phyla composition was minor (**Fig. 4.3A**). The number of species that were only identified in one of the treatments (**Fig. 4.4A**) did not alter the main composition of treatments either, most likely related to their very minor presence or to the fact that most identified OTUs were not identified to species level (**Fig. 4.2**).

All beta diversity plots taken together underline that there is no easy identifiable main aspect in which most treatments can be segregated with; even after identifying principal coordinates and principal components those accounted for a quite low total variance, with less than 11% for each axis in Unweighted PCoA (**Fig. 4.5B**) and PCA (**4.5C**). Even in the PCoA based on Weighted UniFrac distances (**Fig. 4.5A**), which accounted for much more variance in their axis with 51.29 and 19.83% respectively, most of the segregation power was lost to a single Asp

sample that differs a lot from all others (including other Asp samples). Similar studies where UniFrac distances were used as a measure for beta diversity tend to show more difference between samples for Weighted and Unweighted UniFrac distances (Wang *et al*., 2018B). However, these more evident differences in beta diversity from other studies are once again due to the usual comparison in rhizosphere metagenomic analyses being between environments from different locations or conditions *e.g.,* elevation (Fontana *et al*., 2020), or between different species (Wang *et al*., 2018B). In this study we compared soil from the same field in adjacent locations which are expected to be all naturally the same and the only difference is the application of an amino acid at a single point during plant development, so it is reasonable to think there was a strong common baseline of microorganisms and the changes created by the amino acids were more limited. This is evident when comparing the range of differences in beta diversity between treatments to the range of the other similar studies cited above.

4.4.3. The soil rhizosphere from the analysed amino acid treatments forms two treatment clusters

As mentioned, it was expected that for plants in the same field with the only difference of amino acid treatments rhizosphere diversity would be similar. The observed differences were small but significant, with the AMOVA analysis confirming differences between the rhizosphere of each treatment. Interestingly, many of the results tended to form two main groups that present very similar results for many of the analysed variables. This was already hinted when looking at the alpha diversity and particularly the Shannon index (**Fig. 4.4C**), which presented similar values for all treatments except Asp but also tended to form a group of treatments with higher and very similar Shannon index values for Con, Pro, Arg and Asn treatments. Gly, Phe, PGA and Ala had slightly lesser values from the first group and were also very homogeneous within the group. These two groups were not statistically segregated, as only Asn and PGA showed significant differences, but there was a trend that matched the beta diversity analysis plots.

Two-dimensional plots presented to determine how similar or different samples were from one another made these groupings much more obvious: PCoA based on Unweighted UniFrac distances (**Fig. 4.5B**) presented two tight clusters with the same treatments each: Con, Pro, Arg and Asn in one and Gly, Phe, PGA and Ala in the other, in this case adding Asp as well, with two of its three samples being very much integrated in this cluster and the other one being very deviated from both. PCoA based on Weighted UniFrac distances (**Fig. 4.5A**) showed a very similar pattern with the addition of some deviation for one sample each for Arg, Gly and PGA, although they still were closest to their cluster. A similar thing happened with the PCA plot on

Fig. 4.5C, with only one sample of Asn and PGA being relatively deviated in this case. Taken together, the alpha and beta diversity comparisons do suggest that there were two clusters of related microbiomes. This was further supported by the treatment hierarchy established in the UPGMA analysis, based both in Unweighted (**Fig. 4.6A**) and Weighted (**Fig. 4.6B**) UniFrac distances, which established the same two clusters (except for Asp in Fig. 4.6B, which did not take into account species relative abundance). For the remainder of the discussion, we will refer to these groupings as Cluster 1 (Con, Pro, Arg and Asn treatments) and Cluster 2 (Gly, Phe, PGA, Ala and Asp treatments).

Having analysed the difference in soil microbial diversity between treatments, it is important to understand the possible reasons behind those differences and how the application of amino acids may come into play. For this all the possible factors in the plant rhizosphere of each sample must be considered to establish hypotheses of what is happening in the plant microbiome that may impact the development of the crop.

Similar to the study in **Chapter 3**, the location and distribution of each treatment in the field is again a relevant point of discussion, as the lack of a randomised distribution for the treatments made it a possibility for some of them to have similar characteristics due to their physical proximity, potentially showing a position effect between treatments close to each other in the field. When sampling each of the three biological replicates per treatment, these were taken from the first quarter, middle and last quarter of the longitudinal 400 m per treatment, with around 100 m between each sample, trying to get a representative sample of the rhizosphere across the entire length of each treatment strip. Due to the inability to randomise them, it is possible that the distribution of the treatments in the field influenced the microbial populations present in each.

When comparing the cluster distribution described in the last **Section 4.4.3** against the distribution of treatments in the field (**Fig. 3.1A**) we see some of the treatments clustered together while being next to each other in the field (starting from the left side of the figure, South-East to North-West: first PGA-Phe-Gly from Cluster 2, then Asn-Arg-Pro from Cluster 1). However, the treatments after that were from Cluster 2 again (Ala-Asp) and then the last treatment was the control which was again from Cluster 1. In short, cluster distribution along the field was 2-2-2-1-1-1—2-2-1, with treatments of Cluster 2 being separated by as much as four strips, totalling 400 m, with treatments of Cluster 1 in between. Similarly, Asn, Arg and Pro were also quite far away from Con but in the same Cluster. The fact that treatments so far apart in the field showed such similar beta diversities and so close in reduced-dimensionality

plots (**Fig. 4.5**) does suggest that the field location was not a determinant factor in the differences presented in this work and changes in amino acid metabolism did generate an effect in the plants after being treated.

The goal of this chapter was to observe and analyse microbiome differences after amino acid application, and they did seem to create some significant differences between treatments. A reasonable hypothesis for the differences between treatments in this work is that the metabolic pathway locations of different amino acids (**Fig. 1.4**) play a role in the way the treatments are affecting the soil microbiome. Notably, although in different locations in the field, all treatments of amino acids that are metabolically derived from glycolysis pathway (Phe, Gly and Ala) were part of the same Cluster 2. On the other hand, amino acids metabolically linked to TCA cycle had their treatments divided between the two clusters; Asn is derived from oxalacetate and was in Cluster 1 while Asp, derived from the same molecule, was in Cluster 2. In the same way, PGA is linked to 2-OG and was in Cluster 2 while Pro is also derived from the same molecule and was in Cluster 1.

The distribution of treatments in the field and the metabolic pathway location of each of the amino did not seem to be linked; plants treated with amino acids derived from glycolysis pathway and TCA cycle were not always next to each other; there were instances where more metabolically linked amino acids next to each other belong to different clusters, as well as treatments from the same cluster and next to each other being more distant in their metabolic location.

4.4.4. Biological relevance of microbiome changes after amino acid treatments

Soil microbiome is very dynamic and sensitive to changes. When any of the other characteristics in the rhizosphere changes, the microbial community can be altered and a new balance will be established. Some microorganisms will be overrepresented respective to the previous condition while others will have less presence. The reason for these changes can range from changes in plant exudates to variation in soil physical characteristics. In this study, bacterial taxa representation changed among treatments (**Fig. 4.3A**), somewhat varying the phylum profile too (**Fig. 4.3B**) and some overrepresented taxa were identified as biomarkers of a certain treatment (**Figs. 4.7, 4.8**). Taxa representation was also linked to the changes in growth and yield parameters shown in the previous chapter (**Table 4.2**).

Despite metagenomics enabling the identification of most present microorganisms in an environment, there is not always a readily available characterisation of all microbial taxa of

interest in regard to plant life and the rhizosphere. This makes connecting biomarkers or any taxa with concrete effects in the ecosystem difficult. However, even if there is sometimes little data about the impact of specific microorganisms in the rhizosphere, some other studies have used rhizosphere comparisons between different environments, observing if the presence and representation of some genera changes between those environments and using this data to partially infer the relevance of certain taxa.

Revealingly, biomarkers identified by the LEfSe analysis, although being identified as a biomarker of a single treatment (**Fig. 4.7A**), showed abundance profiles with very similar segregation to Clusters 1 and 2, as seen in **Fig. 4.8**. This way, Myxococcales, Acidobacteria (and specially its subgroup 6) and Planctomycetes (and specifically the family Planctomycetaceae) had higher abundances in Cluster 1 treatments. Myxococcales, a biomarker for Pro treatment, can utilize amino acid containing growth substrates such as peptones, using it to assimilate inorganic N from the soil (Dawid, 2000). Acidobacteria are ubiquitious in terrestrial environments, including different soils (Kalam *et al*., 2020), and are able to alter the activity levels of N metabolism enzymes (Eichorst *et al*., 2018), as well as biosynthesis of secondary metabolites including non-ribosomal peptides (Crits-Christoph *et al*., 2018). Planctomycetes also play a role in global N cycles, with its capacity to perform anammox reactions, appearing in relatively high abundances in soil (Buckley *et al*., 2006). It is interesting that Cluster 1 treatments, of which these taxa were biomarkers and had distinctively higher amounts, were also the ones with highest GS enzyme activity in the results from the previous chapter (**Fig. 3.10**). It can be hypothesised that plants from treatments in Cluster 1 were having increased N availability in the soil due to having higher amounts of a number of microorganisms that are directly related to the N cycle, affecting N assimilation by plants at grain filling stage. Cluster 2 also had taxa associated to N cycle that could positively impact plant growth (Rhizobiales), although its difference of abundance between treatments of different clusters was much smaller. The control treatment was in this Cluster 1, meaning this would be the baseline without amino acid application and the treatments in Cluster 2 would be the ones that potentially affect N assimilation, potentially because of the altered microbiome. However, as mentioned in **Chapter 3**, the changes in N assimilation did not lead to higher yields as some of those treatments were the ones with less yield. Furthermore, multitude of genera phylogenetically related to the biomarkers showed a similar effect on wheat developmental parameters (**Table 4.2**), with *Gemmata*, *Zavarzinella*, *Planctomyces*, *Pirellula*, *Singulisphaera* and *Isosphaera* (all Planctomycetes), as well as *Sorangium* (Myxococcota) presenting a positive correlation with

the number of spikelets and GS activity as well as a negative correlation with yield and NADH-GDH. This suggests that several related bacteria genera changed their representation in the same way when in the soil of the same treatment.

Cluster 2 also had biomarkers of which all treatments in the cluster showed increased presence over Cluster 1 treatments. However, as mentioned above, for biomarkers of Cluster 2 treatments the abundance difference was less that for biomarkers of Cluster 1 treatments. Xanthomonadales has little amount of literature related to its presence in soil and its relation with plant physiology, mainly appearing in other whole metagenomic studies (Lasa *et al*., 2019) and having shown to be more abundant in disease-suppressive soil than in disease-conducive soil (Mendes *et al*., 2011). Rhizobiales can provide nutrient, phytohormones and metabolite precursors to plants (Erlacher *et al*., 2015), with some genera also playing a role in N fixing both in leguminous and non-leguminous plants (Fischer *et al*., 2012). Comamonadaceae includes plant pathogen genus such as *Acidovorax* (Burdman and Walcott, 2012). The genus *Polaromonas*, from the family Comamonadaceae, was identified in **Table 4.2** as being the complete opposite to most other genus that correlate with growth parameters: it was positively correlated to yield and negatively to the number of spikelets and GS activity. This makes this bacteria genus interesting for the possible causes of its correlation with yield, while also emphasizes that higher GS assimilation at grain filling stage is not necessarily correlated to higher yields, potentially being a symptom of the need of the plant of assimilating N at later stages, making up for not having done so in earlier growth stages.

Some biomarkers, as is the case of *Bacillus* and *Stenotrophomonas*, appeared disproportionately overrepresented solely in Asp treatment. These taxa are both considered plant growth promoting rhizosbacteria (PGPR), with several species of the genus *Bacillus* playing a role in N fixation, phosphorus nutrition and phytohormone production, as well as protecting plants from abiotic stressors and against pathogens (Saxena *et al*., 2020) and producing secondary metabolites (Kim *et al*., 2011; Palaniyandi *et al*., 2013). *Stenotrophomonas*, also considered a PGPR, is ubiquitous in plant and soil environment and is related to biological plant disease resistance, as well as protection from nematodes (Hayward *et al*., 2010) also having shown to have a positive impact for plants in N starvation stress (Alexander *et al*., 2019). Interestingly, in the case of *Stenotrophomonas* it was a single sample that was peaking much higher than all others (Asp1), while in the case of *Bacillus* it was the other two (Asp2 and Asp3) so it was not an issue of a single sample or specifically the sample that had very different microbial composition than the rest in **Figs. 4.5A, 4.5B** and **4.5C**. The results from Asp treatment are

certainly unexpected and likely associated with a parameter that was not analysed in this dataset. The difference in bacterial diversity and composition as well as the lower number of sequenced reads hints at difficulties in the sequencing or analysing process that might have altered the results for this treatment.

It is possible that amino acid addition can impact plant signalling processes. These signalling processes and their relation to amino acids have been studied in a number of plant related studies, showing that these molecules not only act as protein building blocks and N storage, but that they can also act as signalling molecules, often in small concentrations, to trigger and help a variety of processes from metabolic processes to biotic stress responses (Forde and Lea, 2007).

Ca^{2+} is considered to be a signalling molecule in plants (Choi *et al*., 2016) and has been related to various biotic stress resistance such as fungal pathogens (Kang *et al*., 2006) and insects (Browse, 2009). Ca^{2+} influx activated by bacterial PAMPs has been proven as part of the immune response (Ma *et al*., 2012). Ca^{2+} conduction has also been linked to amino acid-gated ion channels, related to Glu receptors (Vincill *et al*, 2012). Plant GLRs can use not only Glu but a wide range of amino acids as ligands (Qi *et al*., 2006; Tapken *et al*., 2013). In the model plants *Arabidopsis*, *Arabidopsis thaliana* GLR (*AtGLR*) mutant knockouts have shown to be hypersensitive to *Pseudomonas syringae*, suggesting a relationship between these receptors and bacterial pathogen resistance (Li *et al*., 2013). Ca^{2+} influx as part of the GLR response gated by as many as eight amino acids has also been seen, as described by Vincill *et al*. (2012). The same authors suggested that local amino acid patterns created by plant exuded amino acids and by microbial metabolism could create specific amino acid patterns in the rhizosphere that could be transduced by Ca^{2+} permeable GLRs, influencing root physiology and development.

It is unknown if this happens exactly as described by Vincill *et al*. (2012), but there is evidence of exuded amino acid reaching the rhizosphere quickly being mineralised by microbial metabolism (Owen and Jones, 2001), arguably influencing the bacterial populations and the available amino acid and N available in the soil for plants to take up, influencing their growth and development.

The work presented here evidences changes in the rhizosphere bacterial populations after the application of different amino acids in the soil. These changes also vary between amino acids and some amino acids reacted similarly in the groups described as Clusters 1 and 2. It is possible that the amino acid signalling via GLR and maybe mediated by Ca^{2+} influx is affected by the

rhizosphere environment derived from the amino acid addition and the plant exudates themselves as a consequence of this addition. It is worth mentioning that the Ca^{2+} current created by each amino acid in Vincill *et al*. (2012) is different, and the trends of each amino acid does not seem to correlate to Clusters 1 and 2 in this work.

Determination of biological activities using 16S rRNA genes from metagenomic samples is challenging, as it is a very high-throughput analysis of a snapshot that does not provide information on individual specific groups of microorganisms but compares abundances of them. Culturing soil samples in selective growth media is a method to identify microbial species that perform certain biological activities and can therefore survive and reproduce in a specific environment. This, although low throughput, enables a more direct link to a specific activity being performed in the soil. Jiménez-Arias *et al*. (2019) provide a good example of this, counting colony forming units (CFUs) in different selective media, and determining an increase in phosphate-solubilising microorganisms after PGA treatment in lettuce.

For this work, a similar complementary approach would be a logical next step after the information gathered in the metagenomic sequencing analysis. Particularly, it would be interesting to establish selective growth media linked to the changes seen in this chapter as well as **Chapter 3** in terms of N metabolism, as well as other alterations between treatments identified in the metagenomic analysis, such as Acidobacteria growth. Using selective media and quantifying the reproduction of different bacteria in them would allow for the confirmation of biological activity, which is something that is difficult to measure in metagenomic analyses.

4.5. Conclusions

This chapter assesses the impact of amino acid application in the rhizosphere bacterial community of field wheat, aiming to identify differences between different amino acid treatments. Experimental results evidenced changes between microbiomes of different treatments. Species richness and evenness was altered between treatments, with differences mainly laying on the presence of less abundant microorganisms. Overall composition of the microbiomes also differed between treatments and two clear clusters were formed, each with treatments that showed very similar microbial composition within the cluster but with much bigger differences between the clusters. Characteristic taxa for a specific treatment or cluster were also identified, and the correlations between taxa and plant growth and yield parameters seen in the previous chapter were analysed.

Due to the highly complex plant-soil interactions it is not possible to conclude direct cause-effect mechanisms between the application of amino acids and specific changes of rhizosphere microbiome. However, by observing the differences between different treatments we propose different hypothesis about the significance of a number of taxa in plant physiology linked to amino acid application. Although there are still considerable knowledge gaps in plant-soil-microbe interaction and how different microorganisms can directly or indirectly affect plant life, this study enables to link changes in said interactions with the effects of amino acid addition.

Chapter 5. General discussion

5.1. Key findings

This thesis aims to expand the available information concerning the effects of amino acids in plants, helping the identification of amino acids with potential agricultural interest. To this end, differences in plant growth, yield, N metabolism and rhizosphere microbiome were analysed. *Arabidopsis thaliana*, as a model plant, and wheat, as a crop of agricultural interest, were used in controlled and field condition experiments, both in the presence and absence of stress. Results show that individual amino acid effects, positive or negative, depend on environmental conditions and stress. Some of the findings here progress the knowledge of amino acids as plants biostimulants. The key findings of this thesis are as follows:

Regarding the use of PGA as a biostimulant:

- External PGA addition to *Arabidopsis* seedling causes severe stress after a certain threshold, with 10 mM preventing germination altogether.
- Low concentration external PGA addition affects plant growth and yield, particularly under stress and in field conditions. In wheat, effect is non-significant in glasshouse conditions, while significant in the field. In *Arabidopsis*, effects become much more evident under salt stress conditions.
- The effect of PGA on *Arabidopsis* roots is distinct from Glu and Pro, and although PGA conversion to Glu may occur, this does not prevent PGA toxicity. When PGA conversion to Glu is blocked and plants are under salt stress, external PGA causes exaggerated growth reduction, while external Glu or Pro demonstrate stress alleviation capabilities.

Regarding the screening for identifying amino acids of interest for their biostimulant action:

- In *Arabidopsis*, low concentrations of Pro, Glu and Gly show a positive impact in root growth under salt stress, with PGA and Arg having negative impacts with and without salt. Ala negatively impacts root growth only under salt stress.
- Under optimal greenhouse growth conditions, amino acid spray treatments do not affect wheat growth or yield.
- In field-grown wheat, amino acid treatments lead to growth and performance changes, positive or negative depending on the applied amino acid, which correlate with changes in protein content and N metabolism at grain filling stage.

Regarding the effect of amino acid treatments in field crop soil rhizosphere microbiome:

- Rhizosphere bacterial diversity is affected after amino acid application, with some changes in taxa relative abundance but more notably in taxa presence.
- Amino acids Pro, Arg and Asn have bacterial profiles similar to each other, while Gly, Phe, PGA, Ala and Asp are different from the first group but similar among each other.

5.2. Potential of pyroglutamate as a plant biostimulant amino acid

Unlike many other amino acids, PGA effects in plants have not been thoroughly studied and there are many responses to PGA that need to be assessed yet. Its application in plants has been related to stress resistance and secondary metabolism stimulation in recent years (Bilska *et al*., 2018, Mejri *et al*., 2019), and conversion of PGA to Glu and then to Pro has been suggested as a mechanism to combat stress (Jimenez-Arias *et al*., 2019). However, the effect of PGA as an amino acid that has biological effects on plants on its own and regardless of its metabolism has not been confirmed. Characterising the effect of PGA in plants and discerning if the effect is of PGA itself or an effect of its metabolism is one of the main aims of this thesis. This was explored using experiments with PGA and its metabolically related amino acids Glu and Pro, as well as using *oxp1* mutant plants to cut PGA conversion to Glu.

Results in *Arabidopsis* (**Figs. 2.6 to 2.9)** evidence differences between the effects of external application of PGA compared to Glu and Pro, two products of PGA metabolism. WT plants with added PGA at the tested low concentrations decreased root growth similarly in non-stressed and salt-stressed plants. This contrasts with Glu and Pro application, which do not decrease growth in non-stressed plants and slightly increase growth in salt-stressed plants with Glu and Pro comparing it with the control treatment. *oxp1* mutants that cannot metabolise PGA showed a similar decline when applying PGA, and an even sharper one when salt-stressed, while Glu and Pro led to better growth in both cases. These results evidence that, even though PGA can be metabolised to Glu and Pro in nature, there is a biological effect of PGA in plants beyond being a metabolic intermediary, even when the conversion cannot occur. The effect of PGA is even accentuated under certain conditions when it cannot be metabolised, further demonstrating the inherent effect of this amino acid. On a wider scope, the effect of adding other amino acids such as Gly, Ala and Arg to *oxp1* mutants in **Chapter 2** is also altered by the lack of PGA conversion to Glu, suggesting a wider role of PGA that affects more than its most directly linked metabolites. Additionally, when trying to understand the mechanisms behind the effect of PGA in plants, it is worth noting that, although PGA metabolism within the GSH cycle is seemingly simple and the only known degradation pathway is via 5-OPase to obtain Glu, all

steps of the cycle described in **Fig. 1.5** are not fully confirmed in plants and there may be an alternative pathway that is being affected after disrupting 5-OPase or adding external PGA.

Assessing what kind of changes PGA provokes in plants has been a central point along this thesis. The concentrations used in *Arabidopsis* root growth assays in **Chapter 2** as well as in wheat in greenhouse assays and in the field in **Chapter 3** represent a negligible fraction of the total N and amino acid pool available to the plants. Thus, rather than PGA exclusively having an effect by its assimilation or conversion to other molecules, we hypothesise that there is a hormone or signalling effect of this amino acid. Probable candidates for this are GLRs, which as mentioned in previous chapters are known to have ligand promiscuity in plants, being able to bind to a large number of amino acids (Forde and Roberts, 2014). Although not carried out in this thesis, specific tests to determine a potential role of PGA as GLR ligand and its involvement in Ca^{2+} signalling will be necessary to confirm the role of this amino acid in known signalling processes and how it can differ from other amino acids. Observing potential root inhibition with PGA addition in *Arabidopsis* GLR mutants could also be useful to study this association.

When trying to discern the effects of PGA in plants its concentration, target species and growth conditions must be considered. In soils, amino acid concentrations are generally 0.1 – 10 µM (Jones *et al*., 2005), meaning that the negative effects observed in **Chapter 2** after applying PGA in *Arabidopsis* are unlikely to occur in field crops when amino acids are applied in a very diluted fashion in spray form. Glu has proven to be useful for improving plant stress response (Chang *et al*., 2010) but has also been reported to reduce root growth in *Arabidopsis* seedlings (Walch-Liu *et al*., 2006; Forde, 2014). In a similar way, it is expected that PGA will have different effects under different circumstances. The experiments carried out in this thesis show that effects of PGA are consistently more evident under some form of stress: in wheat growth assays under limited watering (**Fig. 2.2**) and in *Arabidopsis* in the presence of salt (**Figs. 2.7, 2.9**). In non-stressed plants this was less evident for both species (**Figs. 2.1, 2.2, 2.6, 2.8**). Remarkably, water-limited two-month-old plants (**Fig. 2.1**) did not show significant differences after PGA application, while four-month-old ones did (**Fig. 2.2**), suggesting a long-term effect that is more noticeable later in development rather that right after application. In the field, and although no artificial and specific stress was applied, there were differences between treatments and PGA was one of the highest performing treatments regarding yield related parameters (**Figs. 3.2, 3.3, 3.4**). We hypothesize that the conditions of the field, as opposed to controlled environments, are more challenging and not all the potential of the crop is achieved in all plants,

making it more evident when a treatment makes a difference under the inherent stress on the field. Jiménez-Arias *et al*. (2019) showed the efficacy of externally applied PGA in field trials (via soil drenching with 0.5 kg/ha irrigation in lettuce), and a very recent study from the same group (Jiménez-Arias *et al*., 2022) demonstrated that using PGA in a cereal such as corn can increase yield, although not in a cost-effective way and other alternatives may be more advantageous for commercial use.

5.3. Screening of amino acids as biostimulant candidates

The effects of amino acid accumulation and external application have been widely reported in studies that assess the effects of one or more of them in *Arabidopsis* (Forde, 2014) and in different crops (Teixeira *et al*., 2018; Wang *et al*., 2019). This thesis screens the effect in wheat comparing the controlled environment and field conditions through foliar spray, as well as in *Arabidopsis* comparing non-stressed and salt-stressed seedlings in solid media plates. Aside from the specific effects of PGA discussed in **Section 5.2**, the variety of screened amino acids have interesting effects under specific conditions.

The results for amino acid screening in *Arabidopsis*, greenhouse-grown wheat and field-grown wheat all serve the purpose of analysing a range of amino acid under the same experimental conditions, contrasting with the majority of amino acid effect studies that focus on one or very few of them making the comparison difficult (Wahba *et al*., 2015; Teixeira *et al*., 2017; Jimenez-Arias *et al*., 2019). WT non-stressed *Arabidopsis* seedlings showed a very slight growth increase with some amino acids (**Fig. 2.6**), such as 500 µM Gly and 200 and 300 µM Ala, and the negative effects of PGA and Arg on growth were evident. Salt stress however led to more consistent differences in growth with respect to the control in Pro, Glu, Gly and low concentration Ala, but also more aggressive growth reductions in Arg and high concentrations of PGA and Ala (**Fig. 2.7**). Although these concentrations are much higher than amino acid concentrations generally present in nature (Jones *et al*., 2005), they do hint at positive effects of Pro, Glu and Gly for growth. Gly at higher concentrations (Han *et al*., 2018) and Glu from 50 µM (Walch-Liu and Forde, 2007) have been reported to inhibit root growth, opposite to our results and underlining the gaps in the understanding of amino acid related mechanisms. The mentioned studies propose phytohormone and signalling mechanisms to explain the complex behaviour after the application of these amino acids. This is a reasonable and possible hypothesis, but there is a need of further research to confirm it and characterise specific action mechanisms.

Pro is the amino acid that has been most studied for its effects in plants, generally being considered as positive for stress responses (Ali *et al*., 2007) and crop productivity (Špoljarević *et al*., 2011). Since results from WT *Arabidopsis* seedlings in **Chapter 2** show that low concentrations of Pro up to 500 µM led to little to no differences in root growth in conditions of no stress (**Fig. 2.6**) and the root growth was higher than without Pro in salt stress conditions (**Fig. 2.7**), it seems that at the very low concentrations studied here, root growth changes due to Pro are not due to simple overaccumulation from external Pro but because of a stress resistance trigger at low concentration that may be associated to signalling.

The field study in **Chapter 3** also shows Pro as the worst performing crop, contrasting with previous studies which show Pro improving plant performance even in non-stressed plants (Wani *et al*., 2016). Here PGA, Ala and Asp were the treatments that produced higher yield than the control, and even then, differences were statistically non-significant (**Fig. 3.2**). The results from grain filling protein and N assimilation enzymes suggest differences in the timing of senescence and nutrient transport to grain for different treatments (**Figs. 3.9, 3.10, 3.11**), which correlates with yield (**Fig. 3.12**).

The lack of effect in greenhouse-grown wheat for all amino acid treatments in **Chapter 2** contrast with some interesting differences in field-grown wheat in **Chapter 3,** reinforcing the hypothesis of the role of amino acids in stress response. In the greenhouse, conditions are close to ideal and the absence of abiotic and biotic stressors will not trigger the amino acid-related stress defence mechanisms that can help improve yield and that have been demonstrated (Batista-Silva *et al*., 2019). It is also possible that when plants are in the best possible conditions in the greenhouse, they are already achieving optimal performance, and the changes after amino acid application in low concentrations do not have the power to induce improvements.

When comparing key results from wheat experiments in this thesis, it is necessary to mention that different wheat varieties were used for each chapter, with spring wheat being used in **Chapter 2** and winter wheat in **Chapter 3**. Spring wheat was used in the glasshouse experiments so that it could be grown to flowering without vernalisation. Furthermore, winter wheat was the only one available to use in the field at the time the field trial took place, and it does bring some limitations in terms of comparing the results from both chapters, as the timing of sowing and harvesting of spring and winter wheat differ. However, both spring and winter wheat are known to share similar physiology and their growth and yield are subject to the same

prediction models (Cann *et al*., 2019). Except for direct comparison of quantitative results, it is reasonable to compare results from both varieties.

5.4. Effects of amino acid treatments in rhizosphere microbiome in field-grown wheat

The plant-soil-microbe ecosystem is complex and tightly interconnected, and changes to any of these parameters will affect the balance of the whole system. Spray amino acid addition is expected to affect plants directly through foliar uptake and indirectly through the soil, where microbiota will also be affected. Some proposed roles of amino acids in the rhizosphere and in the context of plant-soil interactions have been previously described (Moe *et al*., 2013), and the use of metagenomics has been used to identify differences between treatments, fields or environments in recent years (Siegel-Hertz *et al*., 2018; Praeg *et al*., 2019; Hassen *et al*., 2020; Tuo *et al*., 2020). With the aim of determining if amino acid treatments affect plants via potential changes in the soil microbiome, **Chapter 4** focused on addressing differences in rhizobacterial populations after the spray application of said treatments.

The results of the metagenomic analysis overall strongly suggest that, although differences do not majorly overhaul the microbiome, there is some impact of amino acid treatments in balance of bacterial populations. Although not being able to discern major changes that are directly responsible for changes in crop performance, the diversity indicators used suggest some differences between some of the treatments in terms of diversity and some taxa representation. Said differences are quite specific, but the groups generated in the beta diversity analysis show how some of the amino acid treatments led to similar effects, with Con, Pro, Arg and Asn on one cluster and Gly, Phe, PGA, Ala and Asp in the other. These clusters may be related to amino acid metabolism, as amino acids derived from the glycolysis pathway are all from the same cluster. However, this does not completely align with all treatments, as amino acids derived from TCA cycle appear in both clusters, with PGA and Pro for example being in different clusters. Further analysis of amino acid effects in crop field soil are required to confirm the changes seen here and link them to specific causes.

The complexity of plant-soil-microbe ecosystem adds additional difficulty to obtain consistent results that clearly explain the effects of amino acid addition. In **Chapter 4**, the position of each treatment is addressed and there was no clear gradient of results aligned with the positions, but the pre-treatment balance might be very different in different geographical locations and fields. If similar experiments were to be performed in the future, the chemical and biological

characteristics of the field previous to treatment would most likely be considerably different, which could significantly affect how the microbiome will change and affect plants after applying treatments; the action mechanism of biostimulants is not as straightforward as traditional chemical fertilisers and will depend on the ecosystem, complicating the characterisation of their effect. It is also worth studying if changes in rhizosphere microbiome are due to the spray treatments reaching the soil, or if it is the plants that undergo physiological changes when treated and this alters their root exudates, altering the microbiome.

Furthermore, **Chapter 4** focuses on the rhizosphere microbiome, but impact of microorganisms otherwise related to the plant after treatment application is also to be considered and could be an interesting direction to enhance the understanding of plant-microbe relationship. The impact of amino acid including fertilisers in leaf microbiota has been previously analysed, although using amino acid mixtures (Wang *et al*., 2019). Similar studies with individual amino acids could help elucidate if leaf microbiota changes after amino acids are applied and if this is a significant factor for crop performance.

5.5. General conclusions and final words

The data produced during this thesis helps understand biostimulant functions of amino acids in the context of plant physiology and crop science.

Knowledge on PGA is expanded by studying the relevance of its metabolism via 5-OPase in its effects, showing changes on a wider amino acid metabolism level. The importance of stress to trigger PGA effects is addressed, as well as toxicity symptoms by accumulation of this molecule.

In terms of the use of amino acids as biostimulants in crops, the data presented using a wide range of amino acids evidences changes in performance, as well as in some other parameters that might be affecting yield such as protein and N metabolism. The rhizosphere microbiome also changes after amino acid application in the field, suggesting an effect in this part of the holobiome as part of their biostimulant effects.

Significant progress has been made in the last years regarding biostimulants and specifically amino acids used as such. The findings in this thesis contribute to this progress, while at the same time underlining that there are gaps in the basic science of biostimulants that need to be understood to use them more effectively in agriculture.

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