Improving Crop Growth by Enhancing 5-Aminolevulinic Acid Production by Plant-Growth-Promoting Bacteria

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TABLE OF CONTENTS

TABLE OF CONTENTS		i
ABSTRACT		iv
1	**GENERAL INTRODUCTION AND LITERATURE REVIEW**	1
1.1	Introduction	1
1.2	The biosynthesis of ALA	9
1.3	Microbial production of ALA	10
1.4	Commercial product of ALA	12
1.5	The application of ALA	13
1.6	Effect of ALA under stress conditions	15
1.7	Research rationale	18
1.8	Research aims, objectives and hypotheses	19
2	**BACTERIA: IDENTIFICATION AND QUANTIFICATION OF ALA PRODUCTION**	21
2.1	Introduction	21
2.2	Materials and Methods	23
2.2.1	Bacterial identification	23
2.2.2	Establishing bacterial growth curve and ALA concentrations	23
2.2.3	Determining the effect on bacterial ALA production of adding a precursor and an inhibitor	25
2.2.4	Statistical analysis	26
2.3	Results	26
2.3.1	Bacterium identification	26
2.3.2	Bacterial growth curve and ALA concentrations	26
2.3.3	The effect on bacterial ALA production of adding a precursor and an inhibitor to the incubation medium	28
2.4	Discussion	30
2.4.1	Bacteria identification	30
2.4.2	Bacterial growth curve and ALA concentrations	31
2.4.3	The effect on bacterial ALA production of adding a precursor and an inhibitor to the incubation medium	33
2.5	Conclusion	34
3	**EFFECT OF *BACILLUS PARAMYCOIDES* INOCULATION ON GROWTH OF CRESS (*LEPIDIUM SATIVUM*) AND KALE (*BRASSICA OLERACEA* VAR. *ACEPHALA*)**	35
3.1	Introduction	35
3.2	Materials and Methods	38
3.2.1	Experiment 1: Cress germination and growth	38
3.2.1.1	Preparation of the bacterium	39
3.2.1.2	Seed preparation	39
3.2.1.3	Experiment 1.1: Application of various dilutions of bacterial Culture medium on cress germination and growth	39

3.2.1.4	Experiment 1.2: Effect of culturing the bacterium in different concentrations of LB broth and consequences for cress growth	40
3.2.1.5	Cress harvesting and sample processing	40
3.2.2	Experiment 2: Effect of bacterial metabolites and ALA on kale growth	41
3.2.2.1	Experimental approach	41
3.2.2.2	Kale seed preparation	42
3.2.2.3	Experimental set-up	43
3.2.3	Statistical analyses	44
3.3	Results	45
3.3.1	The vegetative characteristics of cress following addition of various dilutions of bacterial supernatant	45
3.3.2	The plant properties of cress following the application of bacterial supernatant cultivated in various LB concentrations	46
3.3.3	The vegetative characteristics of kale following addition of Various dilutions of bacteria supernatant	49
3.3.4	The vegetative characteristics of kale following addition of ALA	50
3.4	Discussion	52
3.4.1	The effect of various dilutions of bacteria supernatant on vegetative characteristics of cress	52
3.4.2	The effects of various dilutions of bacteria supernatant on vegetative characteristics of kale	55
3.4.3	The effects of ALA as a seed dressing on the vegetative characteristics of kale	58
3.5	Conclusion	58
4	**THE RESPONSE OF CRESS (*LEPIDIUM SATIVUM*), KALE (*BRASSICA OLERACEA* VAR. *ACEPHALA*) AND RADISH (*RAPHANUS SATIVUS*) TO DIFFERENT APPLICATION METHODS AND CONCENTRATIONS OF ALA**	59
4.1	Introduction	59
4.2	Materials and Methods	61
4.2.1	Experimental approach	61
4.2.2	Seed preparation for germination and growth in Petri dishes	62
4.2.3	The effects of ALA on the growth of kale, radish and cress	63
4.2.3.1	Seed and foliar ALA treatments	63
4.2.3.2	Experimental set-up	63
4.2.4	Statistical analyses	64
4.3	Results	65
4.3.1	The growth response of cress and radish to a range of ALA concentrations; Petri dish experiments	65
4.3.2	Growth parameters of kale, radish and cress following the Addition of ALA solutions; soil grown	69

4.3.2.1	SPAD readings for kale, radish and cress	69
4.3.2.2	Vegetative growth of kale, radish and cress	72
4.4	Discussion	76
4.4.1	The growth response of cress and radish to a range of ALA Concentrations; Petri dishes	76
4.4.2	Effects of ALA solutions on the growth parameters of kale, radish and cress	79
4.4.2.1	Effects of ALA solutions on SPAD values	79
4.4.2.2	Effects of ALA solutions on growth of kale, radish and cress	81
4.5	Conclusion	82
5	**EFFECTS OF ALA ON KALE (*BRASSICA OLERACEA* VAR. *ACEPHALA*) FOLIAR YIELD, NUTRIENT CONTENT AND BIOCHEMICAL DIFFERENCES MEASURED BY FTIR**	84
5.1	Introduction	84
5.2	Materials and Methods	85
5.2.1	Experimental set-up	85
5.2.2	Plant harvesting and analysis	86
5.2.2.1	Acid digestion	87
5.2.2.2	Multi-element analyses by ICP-MS	87
5.2.2.3	Extractable carbon and nitrogen	89
5.2.3	Statistical analyses	89
5.3	Results	90
5.3.1	Effects of ALA treatment on SPAD values	90
5.3.2	Effects of ALA treatment on biomass	91
5.3.3	Effect of ALA treatment on the FTIR spectra of kale leaves	92
5.3.4	Effects of ALA treatment on the macro- and micro-nutrients in kale leaves	96
5.4	Discussion	101
5.4.1	Effects of ALA treatment on SPAD values and growth of kale	101
5.4.2	Effects of ALA treatment on the foliar FTIR profiles	102
5.4.3	Effect of ALA treatment on macro- and micro-nutrient concentrations in kale leaves	105
5.5	Conclusion	108
6	**GENERAL DISCUSSION**	109
6.1	ALA production by *Bacillus paramycoides* as a potential plant growth promotor	111
6.2	The effect of ALA on the vegetative growth of kale, radish and cress	114
6.3	The effects of ALA on foliar elemental concentrations	116
6.4	General conclusions and recommendations	118
7	**REFERENCES**	119

ABSTRACT

There is an increasing need to produce crops sustainably and one way of doing that is to use plant growth promoters and/or natural products which will encourage crop growth whilst maintaining soil health. A nonprotein amino acid called 5-aminolevulinic acid (ALA) has been shown to enhance plant growth when used at low concentrations, although it causes oxidative stress at high doses. ALA is used in medicine and in food production and whilst ALA can be synthesised chemically, the expense of doing so is driving research into developing microbial production systems primarily utilising photosynthetic bacteria.

Prior to the start of this project, an unidentified bacterium which demonstrated plant (rice) growth promoting ability that was thought to be due to natural production of ALA, was isolated from a peat soil. The aims of the current study were to test the potential of this bacterial isolate to promote plant growth and to evaluate the consequences of applying ALA to three contrasting brassica species.

The bacterium was identified as *Bacillus paramycoides* following 16S sequencing and a series of bacterial growth experiments were conducted to measure ALA production as a function of cell number (expressed as optical density at 600 nm). There was a close relationship between ALA concentration in the growth medium and OD_{600}. ALA production by *Bacillus paramycoides* was optimised in a further set of experiments during which glutamate and levulinic acid (a reaction precursor and an inhibitor of ALA dehydratase respectively) were utilised. The maximum ALA concentration measured was around 200 µM which is in line with published concentrations.

Following confirmation that the bacterium produced ALA, the bacterial culture medium containing metabolites was used to moisten filter papers on which cress (*Lepidium sativum*) seeds were sown. Presence of metabolites only increased germination and seedling biomass relative to metabolite-free medium when highly diluted. No effect of the bacterial metabolites on growth of kale (*Brassica oleracea* var. *acephala*) in peat-based soil was observed, although kale responded positively to a low concentration (0.0012 mM) of ALA applied as a seed dressing and negatively to a higher concentration (0.12 mM). Radish was less responsive to ALA than either cress or kale.

Overall, the plant response to ALA amendment was species and dose-dependent, with low ALA concentrations generally initiating a positive response, whilst high concentrations were often deleterious. FTIR spectra for plants subjected to a seed soaking ALA treatment before sowing, or to a foliar spray at the 3-4 true leaf stage, presented differently from one another. Spectra for seed-treated plants appeared to be indicative of a stronger biochemical response to higher concentrations of ALA than plants given the foliar spray instead. Seedling/plant sensitivity and stage of growth should therefore be considered when treating plants with ALA.

The findings here corroborate the general view that ALA benefits plants at a low concentration, but, is phytotoxic at high doses. However, the data also suggest that the ALA response is species specific and varies with application method as well as being dose dependent. The spectra highlight that ALA may cause damage to plants which is not overtly evident because it may not translate into altered biomass.

1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

For several decades, the world has faced the environmental effects of agricultural chemical inputs. According to FAO figures, world fertilizer consumption (WFC) of three main plant macro-nutrients, nitrogen (N), phosphate (P) and potassium (K) is estimated to grow annually on average by 2.4 per cent and is expected to reach 201.66 million tonnes by the end of 2020 (FAO, 2017). In 2002, the total fertilizer consumption of America, Asia and Europe was 95% of the WFC (Guler, 2006). Based on data from 2012, the majority of chemical fertilizer consumers are the Netherlands (665.5 kg ha^{-1}), Egypt (624.8 kg ha^{-1}), Japan (373.2 kg ha^{-1}), China (301.5 kg ha^{-1}), Britain (287.5 kg ha^{-1}), Germany (205.4 kg ha^{-1}), France (180.1 kg ha^{-1}), USA (160.8 kg ha^{-1}), Italy (126.4 kg ha^{-1}), India (121.4 kg ha^{-1}), Greece (115.4 kg ha^{-1}), Indonesia (106.9 kg ha^{-1}), (Figure 1.1; Savci, 2012).

The introduction of chemical fertilizers has contributed to increased crop production to feed the growing population; however, excessive use results in pollution of the natural environment, and affects soil fertility, soil microbial communities, soil organic matter and water resources, which impacts long-term sustainability (Zhang *et al.*, 2018). Leaching of nutrients into water leads to eutrophication, resulting in algal growth, oxygen depletion and fish mortality. Human health is also impacted as a consequence of consuming polluted water (Killebrew and Wolff, 2010).

Since overuse of chemical fertilizers is a potential threat to the environment, animals and to human health, there is an urgent need to substitute it with eco-friendly alternatives. The challenge is to find ways to sustainably increase

agricultural production to keep pace with the growing world population while maintaining a healthy environment (Zhang *et al.*, 2012). Sustainable agriculture using green technology offers the solution by alleviating chemical based agricultural products whilst concurrently attaining agricultural needs (Dwivedi *et al.*, 2017).

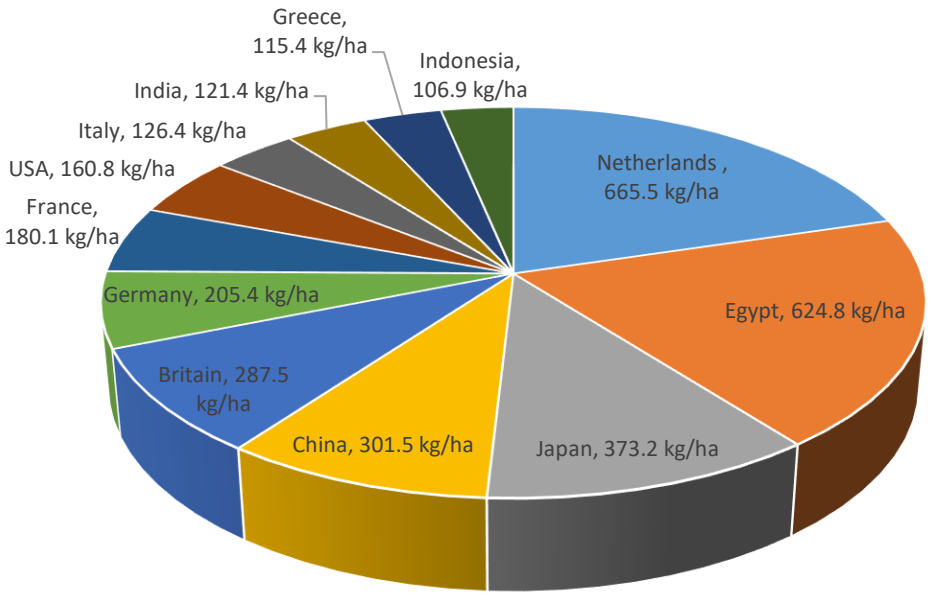

Figure 1.1. The major 12 countries utilizing chemical fertilizer in 2012 (Savci, 2012).

Biotechnological approaches have been selected as green technology to solve the problem by using microorganisms to improve agricultural productivity and reduce the global dependency on agricultural chemicals (Ahemad and Kibret, 2014). There is ongoing research worldwide exploring the unique traits of microorganisms in nutrient conversion, improving soil texture and secreting extracellular substances, such as hormones and secondary metabolites which promote plant growth (Backer *et al.*, 2018).

Use of plant growth promoting rhizobacteria (PGPR) has been studied and developed because of their plant growth stimulating effects and subsequent

potential for enabling simultaneous decreases in chemical inputs. There are many PGPR bioproducts which have different terms yet similar mechanisms; for example, plant growth promoting rhizobacteria/bacteria (PGPR/PBPB), biostimulators, bioactivators, plant hormone/phytohormone inducers, plant growth regulator (Calvo *et al*., 2014; Pokluda *et al*., 2018).

Plant growth promoting rhizobacteria are bacteria which inhabit the plant rhizosphere and directly or indirectly, accelerate plant growth and development by secreting various compounds/metabolites (Vessey, 2003). Previous research has suggested that PGPR improve plant growth and yield by releasing phytohormones (auxin, cytokinin and gibberellin), enhancing nutrient availability, assisting nutrient uptake and reducing heavy metal toxicity in the plants (Helaly, 2017). Table 1.1 describes the various plant growth promoting compounds (and traits) released (and imposed) by various types of rhizobacteria. Different types of PGPR synthesize the phytohormone auxin (Indole Acetic Acid/IAA) and Patten and Glick (1996) concluded that 80% of microorganisms isolated from the rhizosphere potentially secreted auxins as secondary metabolites.

Zaidi *et al.* (2009) identified the main precursor for IAA formation was tryptophan and Spaepen and Vanderleyden (2010) found the enhancement of IAA production in most of the rhizobacteria when supplemented with tryptophan.

Glick (2012) studied the mechanism of PGPR in soil and found that rhizobacteria produced IAA and changed the plant auxin pool, which eventually disrupted the physiological processes of the plants. This metabolite extends the root area and length which open the way to gaining more soil nutrients. IAA also loosens the plant cell walls and facilitates root exudation which provides additional nutrients to support the growth of the rhizosphere bacteria.

Table 1.1. The various growth promoting substances released by PGPR

PGPR	Growth promoting traits	References
Rhizobium, Bradyrhizobium	P-solubilization	Abd-Alla (1994)
Rhizobium leguminosarum	Cytokinin	Noel *et al.* (1996)
Bradyrhizobium japonicum	Siderophore	Wittenberg *et al.* (1996)
Kluyvera ascorbata	ACC deaminase, siderophores, metal resistance	Burd *et al.* (1998)
Bradyrhizobium, Rhizobium	IAA	Antoun *et al.* (1998)
Azotobacter chroococcum	Gibberellin, kinetin, IAA	Verma *et al.* (2001)
Bacillus, Pseudomonas, Azotobacter	P-solubilization and IAA	Tank and Saraf (2003)
Pseudomonas fluorescens	IAA, phosphate solubilization	Jeon *et al.* (2003)
Pseudomonas fluorescens	IAA, siderophores, antifungal activity	Dey *et al.* (2004)
Bacillus, Azospirillum sp.	IAA, P-solubilization	Yasmin *et al.* (2004)
Bacillus sp., *Rhodococcus* sp., *Cellulomonas* sp., *Pseudomonas* sp.	IAA	Tsavkelova *et al.* (2005)
Variovorax paradoxus, *Rhodococcus* sp., *Flavobacterium*,	IAA and siderophores	Belimov *et al.* (2005)
Pseudomonas putida	Siderophores, Pb and Cd resistence	Tripathi *et al.* (2005)
Bacillus sp.	P-solubilization	Canbolat *et al.* (2006)
Bacillus subtilis	IAA, phosphate solubilization	Zaidi *et al.* (2006)
Bradyrhizobium japonicum	IAA	Shaharoona *et al.* (2006)
Xanthomonas sp. RJ3, *Azomonas* sp. RJ4, *Pseudomonas* sp. RJ10, *Bacillus* sp. RJ31	IAA	Sheng and Xia (2006)
Bravobacterium sp.	Siderophore	Noordman *et al.* (2006)
Pseudomonas putida	Antifungal activity, sidephore, HCN, phosphate solubilization	Pandey *et al.* (2006)
Pseudomonas sp., *Bacillus* sp.	IAA, siderophore, phosphate solubilization	Rajkumar *et al.* (2006)
Brevibacillus spp.	Zn resistance, IAA	Vivas *et al.* (2006)
Gluconacetobacter diazotrophicus	Zinc solubilization	Saravanan *et al.* (2007)
Bacillus subtilis	Antifungal activity	Cazorla *et al.* (2007)
Pseudomonas fluorescens	Induced systemic resistance, antifungal activity	Saravanakumar *et al.* (2007)
Klebsiella oxytoca	IAA, phosphate solubilization, nitrogenase activity	Jha and Kumar (2007)
Mesorhizobium cicero, Azotobacter chroococcum, Pseudomonas, Bacillus	IAA, siderophores, phosphate solubilization,	Wani *et al.* (2007)
Bradyrhizobium sp.	IAA, siderophores, HCN, ammonia	Wani *et al.* (2007a)
Azotobacter sp., *Mesorhizobium* sp., *Pseudomonas* sp., *Bacillus* sp.	IAA, siderophore, antifungal activity, ammonia production, HCN	Ahmad *et al.* (2008)
Pseudomonas sp.	ACC deaminase, IAA, siderophore, heavt metal solubilization, phosphate solubilization	Rajkumar and Freitas (2008)
Burkholderia	ACC deaminase, IAA, siderophore, heavy metal solubilization, phosphate solubilization	Jiang *et al.* (2008)
Enterobacter sp.	ACC deaminase, IAA, siderophore, phosphate solubilization	Kumar *et al.* (2008)
Serratia marcescens	IAA, siderophore, HCN	Selvakumar *et al.* (2008)
Pseudomonas aeruginosa	IAA, siderophores, HCN, ammonia, exo-polysaccharides, phosphate solubilization	Ahemad and Khan (2009)
Bacillus species PSB10	IAA, siderophores, HCN, ammonia	Wani and Khan (2010)
Bradyrhizobium sp. 750, *Pseudomonas* sp., *Ochrabactrum cytisi*	Heavy metal mobilization	Dary *et al.* (2010)
Rhizobium sp. (lentil)	IAA, siderophores, HCN, ammonia, exo-polysaccharides	Ahemad and Khan (2011)
Pseudomonas sp. A3R3	IAA, siderophores	Ma *et al.* (2011)
Acinetobacter spp.	IAA, phosphate solubilization, siderophores	Zamin *et al.* (2011)
Pseudomonas putida	IAA, siderophores, HCN, ammonia, exo-polysaccharides, phosphate solubilization	Ahemad and Khan (2012)
Tricoderma spp.	Auxin, gibberellin	Doni *et al.* (2014)
Enterobacter sp. UPMR18	ACC deaminase	Habib *et al.* (2016)

The direct mechanisms by which plant growth promoting rhizobacteria expedite plant growth are by assisting plant nutrient acquisition (e.g. nitrogen, phosphorus, potassium and essential micronutrients) or by regulating plant hormone levels; whereas an indirect method is by supressing growth of pathogens by acting as biocontrol agents (Glick, 2012). Saengsanga (2018) summarized various PGPR mechanisms for improving plant productivity, including increased nitrogen fixation, phosphate solubilisation, potassium solubilisation, siderophore production, vitamin, antibiotic, amino acid and phytohormone production (particularly indole acetic acid).

Calvo *et al*. (2014) recommend use of growth promoting rhizobacteria as biostimulators to achieve maximum yields and their environmentally friendly credentials support sustainable agriculture (Pokluda *et al.*, 2018).

According to Doni *et al.* (2014), PGPBs can enhance seed germination (success and speed) and seedling vigour. In addition, plant growth promoting rhizobacteria increase plant health and productivity, under either normal or stressed conditions (Ahemad and Kibret, 2014). Rubin *et al.* (2017) also added that PGPR function frequently enhance the stimulation of growth under stressful conditions, such as under drought stress.

Table 1.2 presents the result of PGPR introduction to various types of crops, such as soybean, mungbean, rice, peanut, *Brassica juncea*, mustard, *Brassica napus*, *Trifolium repens*, pea, cotton, bean, chickpea, tomato, okra, indian spinach, pumpkin, *Triticum aestivum*, *Lupinus luteus*, *Alyssym serpyllifolium*, wheat, artichoke, cucumber, Indian brassia, and kale.

Gupta *et al.* (2002) found that *Pseudomonas* sp. encouraged the growth of soybean, mungbean and wheat in pots. Two years after inoculation, a consortium of bacteria (*Azospirillum brasilense*, *Bacillus pantothenticus*) enhanced rice productivity up to 76.9 % (Thakuria *et al.*, 2004). Beside promoting the growth of plants, PGPR also decreases Pb and Cd uptake (Tripathi *et al.*, 2005); stimulates root elongation (Belimov *et al.*, 2005); preserves plants from metal toxicity (Wu *et al.*, 2006); increases nutrition of plants (Vivas *et al.*, 2006); promotes plant height (5%) and enhances microbial populations in soil up to 41% (Anjum *et al.*, 2007).

Despite many advantages of the utilization of PGPR, its disadvantages should also be considered as they result in restrictions in the development of PGPR for agricultural use. The performance of PGPR in crop production varies and is inconsistent under laboratory, greenhouse and field trials. This variability might be due to unfavourable environmental factors, such as climate, soil conditions, cultivation method, etc., which affect plant growth and proliferation (Zaidi *et al.*, 2009; Gouda *et al.*, 2018).

Martínez-Viveros *et al.* (2010) described the challenge PGPR face in achieving success and whether they could survive in soil, be compatible with the crop and indigenous microflora in soil, and other environmental factors. Finding the best mode of action of PGPR and the right rhizobacteria were the solutions offered by Dey *et al.* (2004) and Choudhary *et al.* (2011).

Table 1.2. The effect of PGPR addition to the growth of plants

PGPR	Plant	Conditions	Results of addition of bacteria to plants	References
Pseudomonas sp.	Soybean, mungbean, wheat	Pots	Stimulated plant growth	Gupta *et al.* (2002)
Azospirillum brasilense, Bacillus pantothenticus	Rice (*Oryza sativa*)	Micro-plots	Enhanced rice grain yield up to 76.9%	Thakuria *et al.* (2004)

PGPR	Plant	Conditions	Results of addition of bacteria to plants	References
Pseudomonas fluorescens PGPR1, PGPR2, PGPR4	Peanut (*Arachis hypogaea* L.)	Pots, fields	Significantly improved pod yield, haulm yield and nodule dry weight	Dey *et al.* (2004)
Pseudomonas fluorescens	Soybean	Greenhouse	Promoted plant growth	Gupta *et al.* (2005)
Pseudomonas putida KNP9	Mung bean	Greenhouse	Enhanced the plant growth, decreased Pb and Cd uptake	Tripathi *et al.* (2005)
Variovox paradoxus, Rhodococcus sp., *Flavobacterium*	*Brassica juncea*	In vitro	Stimulated root elongation	Belimov *et al.* (2005)
Rhizobacterial strains A3 and S32	*Brassica juncea*	Pots	Increased the plant growth under chromium stress	Rajkumar *et al.* (2005)
Bacillus subtilis SJ-101	*Brassica juncea*	Growth chamber	Facilitated Ni accumulation	Zaidi *et al.* (2006)
Azotobacter chroococcum HKN-5, *Bacillus megaterium*	*Brassica juncea*	Greenhouse	Protected plant from metal toxicity, improved plant growth	Wu *et al.* (2006)
Pseudomonas sp., *Bacillus* sp.	Mustard	Pots	Enhanced plant growth and decreased Cr (IV) content	Rajkumar *et al.* (2006)
Xanthomonas sp. RJ3, Azomonas sp. RJ4	*Brassica napus*	Pots	Promoted plant growth and increased cadmium accumulation	Sheng and Xia (2006)
Brevibacillus	*Trifolium repens*	Pots	Stimulated plant growth and nutrition of plants and reduced zinc	Vivas *et al.* (2006)
Rhizobium sp. RP5	Pea (*Pisum sativum*)	Pots	Improved the dry matter, nodule, numbers, root N and shoot N	Wani *et al.* (2007)
Azotobacter chroococcum, Azospirillum lipoferum	Cotton (*Gossypium hirsutum* L.)	Fields	Enhanced seed yield (21%), plant height (5%) and microbial population in soil (41%)	Anjum *et al*. (2007)
Azospirillum brasilense Sp245	Common bean (*Phaseolus vulgaris* L.)	Greenhouse	Stimulated root growth	Remans *et al.* (2007)
Pseudomonas jessenii PS06, *Mesorhizobium cicero* C-2/2	*Cicer arietinum* (chickpea)	Greenhouse, fields	Increased seed yield (52%) and nodule fresh weight	Valverde *et al*. (2007)
Bacillus subtilis, Pseudomonas aeruginosa	*Solanum lycopersicum* L. (tomato), *Abelmoschus esculentus* (okra), *Amaranthus* sp. (African spinach)	Greenhouse	Enhanced dry biomass 31% for tomato, 36% for okra and 83% for African spinach	Adesemoye *et al.* (2008)
Bacillus sp., *Paenibacillus* sp.	Rice	Pots	Significantly stimulated the root and shoot growth	Beneduzi *et al. (*2008)
Pseudomonas aeruginosa	Indian mustard and pumpkin	Pots	Increased plant growth, reduced Cd uptake	Sinha and Mukherjee (2008)
Mesorhizobium sp. RC3	Chickpea (Cicer arietinum)	Pots	Improved the dry matter accumulation, number of nodules, seed yield and grain protein	Wani *et al.* (2008)
Klebsiella pneumonia	*Triticum aestivum*	Pots	Significantly promoted the root length and shoot length	Sachdev *et al.* (2009)
Bradyrhizobium sp. 750, *Pseudomonas* sp., *Ochrobactrum cytisi*	*Lupinus luteus*	Fields	Enhanced both biomass, nitrogen content, accumulation of metals, increased phytostabilisation potential)	Dary *et al.* (2010)
Pseudomonas sp. A3R3	*Alyssum serpyllifolium, Brassica juncea*	Pots	Significantly improved the biomass (*B. juncea*) and Ni content (*A. serpyllifolium*)	Ma *et al.* (2011)

PGPR	Plant	Conditions	Results of addition of bacteria to plants	References
Pseudomonas sp.	Soybean, wheat	Fields	Increased soil enzyme activities, total productivity, and nutrient uptake	Sharma *et al.* (2011)
Pseudomonas putida, Azospirilium, Azotobacter	Artichoke (*Cynara scolymus*)	In vitro	The combination of phosphate solubilizing and nitrogen fixing bacteria led to significantly promoted radicle and shoot length, shoot weight, coefficient of velocity of germination, seedling vigority index, and reduced mean time of germination	Jahanian *et al.* (2012)
Burkholdera cepacian SE4, *Promicromonospora* sp. SE18 and *Acinetobacter calcoaceticus* SE370	Cucumber (*Cucumis sativus*)	Greenhouse	Sgnificantly enhanced biomass and chlorophyll contents under salinity and drought stress	Kang *et al.* (2014)
Trichoderma spp.	Rice	In vitro	Promoted seed germination and vigour	Doni *et al.* (2014)
Bacillus subtilis	Indian bassia	Pots	Stimulated the growth and fitness under salt stress	Abeer *et al.* (2015)
Enterobacter sp. UPMR18	Okra	In vitro	Increased germination percentage, growth parameters, and chlorophyll content	Habib *et al.* (2016)
Serratia marcescens, Pseudomonas poae, Plantibacter flavus and *Bacillus amyloliquefaciens* subsp. *plantarum*	Kale	Pots	Improved vegetative growth and yield productivity and quality	Helaly (2017)
Enterobacter sp. NRRU-N13, NRRU-N20, NRRU-N21, NRRU-D47, and *Bacillus* sp. NRRU-D40	Rice	Pots	Promoted the growth of rice seedlings	Saengsanga (2018)

One of the amino acids produced by plant growth promoting rhizobacteria is 5-aminolevulinic acid (ALA), which is safe for organisms and nature because it is biodegradable (Sasikala *et al*., 1994; Nishikawa and Murooka, 2001; Sasaki *et al.*, 2002; Qin *et al.*, 2006). ALA, a nonprotein amino acid, is a common precursor of tetrapyrrole compounds (e.g. chlorophyll, heme and vitamin B12) widely found in the cells of plants, algae, animals and bacteria (Rebeiz *et al.*, 1984; Carmichael, 1992; Sasikala *et al*., 1994; Sasaki *et al.*, 2002). The harmless effect of ALA is one of the characteristics that makes it popular as a plant growth promoter (Akram and Ashraf, 2013).

ALA is a plant growth promoting hormone as it regulates growth and development of higher plants because it is a precursor of chlorophyll (Smoleń *et al*., 2010; Pokluda *et al*., 2018; Wu *et al*., 2018), which is essential for photosynthesis and contributes to plant growth and productivity (Pessarakli, 2011).

1.2 The biosynthesis of ALA

Jordan and Shemin (1972) stated the mechanism of ALA production in animal tissue, bacteria, and yeast is through the reaction of succinyl CoA and glycine, catalysed by pyridoxal and ALA synthetase; this results in α-amino-β-ketoadipic acid production and subsequent transformation into ALA, the final product. Moreover, ALA as the intermediate in the biosynthesis pathway of tetrapyrrole compounds is formed in two different ways; the C5 pathway with glutamate as the precursor and the Shemin (C4) pathway, formed by ALA synthase (ALAS) from succinyl CoA and glycine (Figure 1.2; Nishikawa and Murooka, 2001).

The Shemin (C4) pathway occurs in photosynthetic bacteria (Choorit *et al.*, 2011). The enzyme, ALA synthase, is involved in ALA formation in the C4 pathway and speeds up the reaction of pyridoxal phosphate, a contingent condensation of succinyl-CoA and glycine. On the other hand, the formation of ALA in the C5 pathway occurs in three stages including tRNA ligation for glutamate, glutamyl-tRNA reduction for generating glutamate l-semialdehyde (GSA), and GSA transamination for ALA production (Pessarakli, 2011). Furthermore, ALA in algae, most bacteria, and higher plants, is catalysed using glutamate (Sasaki *et al.*, 2002).

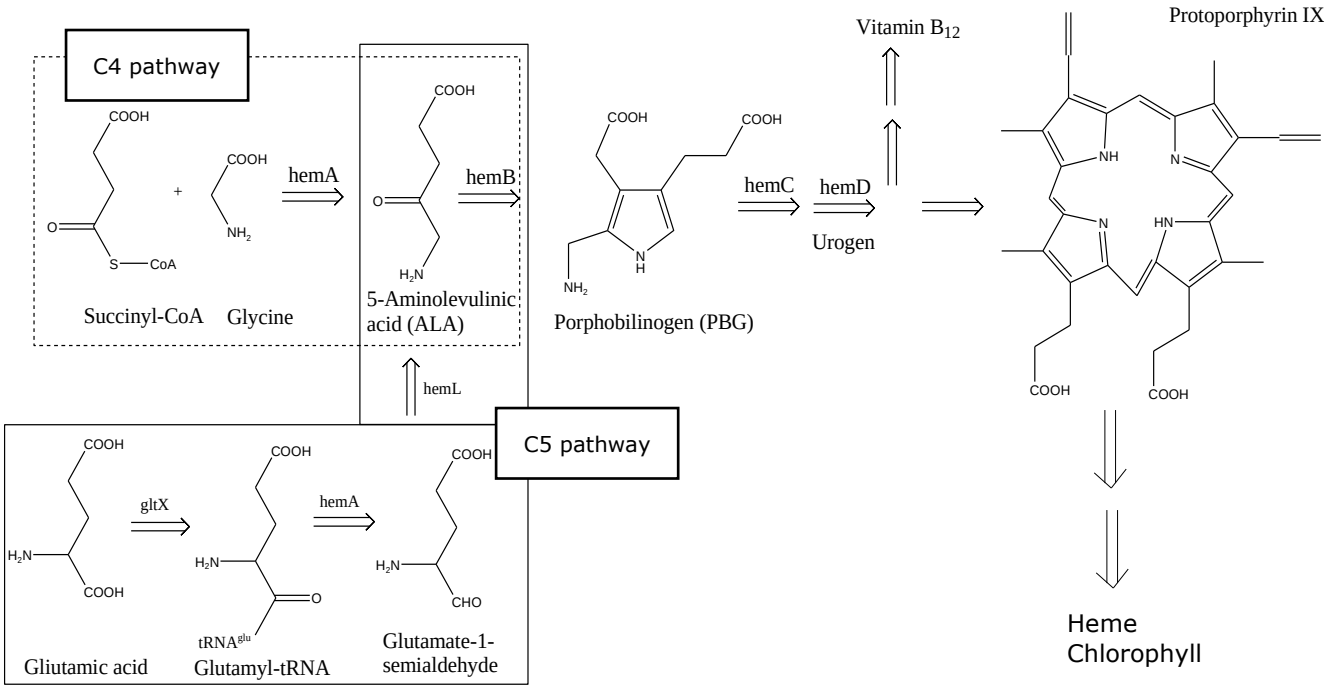

Figure 1.2. Alternative ALA biosynthesis pathways and synthetic pathway of tetrapyrrole derivative from ALA (Nishikawa and Murooka, 2001).

1.3 Microbial production of ALA

During the past 30 years, researchers have chosen to study ALA production in photosynthetic bacteria (e.g. *Rhodopseudomonas palustris*, *Rhodopseudomonas sphaeroides*, *Rhodobacter sphaeroides*, *Rhodovulum* spp.) and algae (e.g. *Chlorella vulgaris*, *Chlorella regularis*) (Table 1.1). Various substrates have been added to support ALA production by microorganisms, such as acetate, glutamate, glycine, glucose, succinate and levulinic acid (LA). The studies also demonstrated that the substrates should be compatible with the microbes to maximise ALA concentration.

Experiments designed to enhance ALA biosynthesis began with the aerobic bacterium, *Propionibacterium shermanii*, using succinate and glutamate as precursors, but the resulting ALA concentration was low, only 0.04 mM (Menon

and Shemin, 1967). Various photosynthetic bacteria were subsequently explored, although most work was conducted on *Rhodobacter sphaeroides*. A variety of substrates were tested and ALA production increased from 0.16 mM to 20 mM through addition of glucose compared to succinate (Ishii *et al*., 1990; Nishikawa *et al.*, 1999). Watanabe *et al*. (1996) initiated the production of a mutant photosynthetic bacterium and obtain an ALA yield of 14.3 mM. In the same year, Van Der Werf and Zeikus (1996) began to consider utilizing recombinant *Escherichia coli* as an ALA producer. Lin *et al*. (2009) reported the highest ALA production of 56.0 mM by recombinant *Escherichia coli* Rosetta (DE3) using succinate, glycine, glucose and xylose as precursors. It is worth noting that the highest ALA concentration (664 mM) was obtained from *Rhodopseudomonas palustris* mutant KG31 (Choorit *et al.*, 2011).

Table 1.3. Production of ALA by different species of microorganisms.

Microorganisms	C and N source	ALA (mM)	Reference
Photosynthetic bacteria			
Anabaena variabilis	Glutamate	0.019×10^{-3}	Avissar (1980)
Anacystis nidulans	Glutamate	0.38×10^{-3}	Andersen *et al*. (1983)
Chlorobium limicola	Glutamate	3.95×10^{-3}	Andersen *et al.* (1983)
Chloroflexus auranticus	Glutamate	0.58×10^{-3}	Andersen *et al. (*1983)
Rhodopseudomonas palustris	Succinate & glycine	0.75×10^{-3}	Andersen *et al.* (1983)
Rhodobacter sphaeroides	Succinate and glycine	2.0	Sasaki *et al.* (1987)
R. sphaeroides	Acetate, glycine & propionate	4.2	Sasaki *et al.* (1990)
R. sphaeroides	Succinate, glycine & Levulinic acid (LA)	0.16	Ishii *et al.* (1990)
R. sphaeroides CR520	Glucose & glycine	14.3	Watanabe *et al.* (1996)
R. sphaeroides	Succinate & glycine	16.0	Sasaki *et al.* (1998)
R. sphaeroides	Glucose, glycine & LA	20.0	Nishikawa *et al.* (1999)
R. sphaeroides CR720	Glucose & glycine	27.5	Kamiyama *et al.* (2000)
Rhodovulum sp.	Glutamate & glycine	2	Noparatnaraporn *et al.* (2000)
Rp. palustris KG31	Glutamate & acetate	0.18	Saikeur *et al.* (2009)
Rp. palustris KG31	Succinate, glycine & LA	0.664×10^{-3}	Choorit *et al.* (2011)
R. sphaeroides ATCC17023	Succinate, glycine & glucose	3.55 g L^{-1}	Liu *et al.* (2016)
Algae			
Chlorella vulgaris	CO_2	1.4	Beale (1970)
Cyanidium caldarium	Glutamate	0.483×10^{-3}	Jurgenson *et al.* (1976)
Agmenellum quadruplicatum	Glutamate	0.225×10^{-3}	Kipe-Nolt and Stevens (1980)
Chlorella sp.	Glutamate & LA	1.9	Sasaki *et al.* (1995)

Microorganisms	C and N source	ALA (mM)	Reference
C. regularis YA-603	Glucose & glycine	3.7	Ano *et al.* (1999)
C. regularis YA-603	Glucose, glycine & LA	4.0	Ano *et al.* (1999)
Aerobes			
Propionibacterium shermanii	Succinate & glutamate	0.04	Menon and Shemin (1967)
Pseudomonas riboflavina	L-Alanine	0.2	Rhee *et al.* (1987)
Eschericia coli	Succinate & glycine	2.25	Van Der Werf and Zeikus (1996)
Recombinant *E. coli* BL21 (DE3)	Succinate, glycine & LA	20.0	Choi *et al.* (1999)
Recombinant *E. coli* BL21 (DE3)	Glucose	3.8 g L^{-1}	Lee *et al.* (2003)
Recombinant *E. coli* MG1655 (hemA)	Succinate & glycine	30.6	Xie, *et al.* (2003a)
Recombinant *E. coli* MG1655 (hemA)	Succinate & glycine	39.0	Xie *et al.* (2003b)
Recombinant *E. coli* BL21	Glycine, succinic acid & glucose	32.0	Lee *et al.* (2004)
Recombinant *E. coli*	Glycine, succinic acid & LA	1.3 g L^{-1}	Chung *et al.* (2005)
Recombinant *E. coli* BL21 (DE3)	Glycine & succinic acid	23.0	Lee *et al.* (2005)
Recombinant *E. coli* BL21 (DE3)	Glycine, succinic acid & glucose	3.01 g L^{-1}	Qin *et al.* (2006)
Bacillus cereus 1-1	Glucose, acetic acid & glutamic acid	2.0	Ahn (2007)
Recombinant *E. coli* BL21	Succinate, glycine & glucose	39.3	Choi *et al. (*2008)
Recombinant *E. coli* Rosetta (DE3)	Succinate, glycine & glucose	6.6 g L^{-1}	Fu *et al.* (2008)
Recombinant *E. coli* Rosetta (DE3)	Succinate, glycine, glucose & xylose	56.0	Lin *et al.* (2009)
Recombinant *E. coli* BL21 (DE3)	Succinate, glycine & glucose	23.7	Liu *et al.* (2010)
Recombinant *E. coli* Rosetta (DE3)	Succinate, glycine & glucose	50.0	Fu *et al.* (2010)
Recombinant *E. coli* QZ1111	Glucose	3.3	Kang *et al.* (2011[a])
Recombinant *E. coli* DH5a	Glucose	31.6	Kang *et al.* (2011[b])
Propionibacterium acidipropionici TISTR442	Glycine	18 g L^{-1}	Kiatpapan *et al.* (2011)
Recombinant *Corynebacterium glutamicum*	Glycine, succinate & glucose	14.7 g L^{-1}	Yang *et al.* (2016)
Anaerobes			
Clostridium thermoaceticum	Glucose & L-cysteine	0.155	Koesnandar *et al.* (1989)
Methanosarcina bakeri	Methanol & 2-oxoglutamate	0.40 x 10^{-3}	Lin, *et al.* (1989)
Methanobacterium thermoautotrophicum	H_2 & CO_2	0.20 x 10^{-3}	Lin *et al.* (1989)

1.4 Commercial production of ALA

Currently, Pentakeep™ is the only commercial product based on ALA and is marketed as a plant biostimulator. The product also contains NPK (8-5-3(3)) in

addition to boron, copper, Dtpa-iron, Mn, Mo and Zn. Its unique selling point is the ALA content (Pokluda *et al*., 2018). Pentakeep enhances photosynthesis, water and fertilizer uptake and maintains plant growth even under abiotic stress (Al-afifi and Al-shrouf, 2010).

One of the Pentakeep products, Pentakeep-V, is a conditioning fertilizer which enhanced flower production by *Begonia* sp. (Kisvarga *et al*., 2015) and decreased sodium uptake thereby increases plant tolerance to salt (Watanabe *et al.*, 2000).

Cosmo Oil Co. Ltd. has a worldwide patent on ALA related to agricultural application. This includes the process for producing 5-aminolevulinic acid (Takeya et al., 1995), 5-aminolevulinic acid-producing microorganisms (Watanabe *et al.*, 1998), production of fertilizers containing 5-aminolevulinic acid (Funada and Iwai, 2013), and the method for producing salts of 5-aminolevulinic acid (Saito *et al*., 2016). However, Cosmo Oil Co. Ltd. has signed a patent licensing agreement with SBI Pharmaceuticals Co. Ltd. to further develop their ALA business (Cosmo Energy Holdings; Review of Operations).

1.5 The application of ALA

With its natural and environmentally friendly characteristics, ALA has potential for wide application in agricultural production (Akram and Ashraf, 2013). At present, ALA is acknowledged as a new type of growth regulator in plant cultivation (Watanabe *et al.*, 2000; Wu *et al.*, 2018). The positive effects of ALA are concentration dependent. At low concentrations it plays a role as a chlorophyll precursor and in heme biosynthesis which improves plant photosynthesis and increases plant growth; while at higher concentrations, it acts as a potential

herbicide because accumulation of ALA increases levels of reactive oxygen species (ROS) and induces oxidative stress in plants (Balestrasse *et al.*, 2010).

Table 1.4. The effects of ALA by foliar application in vegetables.

ALA concentration	Plant species	Effect	References
180 µM (0.18 mM)	Radish (*Raphanus sativus*)	ALA application enhanced plant growth and yield up to 63%	Hotta *et al.* (1997[a])
600 µM (0.6 mM)	Kidney beans (*Phaseolus vulgalis*) Potato (*Solanum tuberosum*)		
0.06 µM	Horseradish (*Armoracia rusticana*)	ALA increased plant growth, chlorophyll synthesis and photosynthesis	Hotta *et al.* (1997[b])
298.35 µM (50 mg L^{-1})	Pakchoi (*Brassica campestris*)	The content of total chlorophyll, photosynthetic rate, and activities of POD, CAT and SOD enzymes were increased.	Memon *et al.* (2009)
23.87 µM (0.04%)	Tomato (*Solanum lycopersicum*)	Application of ALA increased yield, macro- and micro-nutrient content and uptake for fruit, stem and roots.	Al-afifi and Al-shrouf (2010)
11.93 µM (0.02%)	Spinach (*Spinacia oleracea*)	The concentration of Ca, K, Mg, Na, P, S, B, Cu, Fe, Mn and Zn unaffected.	Smoleń and Sady (2010)
$2x10^6$ µM (2 mg L^{-1})	Zucchini squash (*Cucurbita pepo*)	ALA application increased the number of fruits.	Pokluda *et al.* (2018)

Funada and Iwai (2013) reported that ALA increased seed germination, promoted root formation, increased leaf greenness, improved seedling health, increased the number of tillers, increased photosynthetic activity, supressed respiration,

enhanced chlorophyll content and increased yield. Similar results were obtained with a range of species, e.g. radish, kidney beans, potato, tomato, and spinach (Table 1.2; Hotta *et al.*, 1997[a]; Al-afifi and Al-shrouf, 2010; Smoleń and Sady, 2010; Xu *et al*., 2012; Pokluda *et al*., 2018). Tanaka (1992) and Hotta *et al*., (1997[b]) found that ALA application to barley, garlic, potato and rice improved growth rates and photosynthesis. Hotta *et al.* (1997[b]) further suggested that ALA enhances chlorophyll biosynthesis, efficiency of photosynthetic activity, raises nitrate reductase activity, degrades nitrates and also suppresses respiration.

The positive effect of the commercial product containing ALA, Pentakeep-V, was reported for sweet pepper since application increased yield (weight) and number of fruits by 8% and 6% respectively (Eveleens, 2005). In the presence of sufficient soil N, foliar application of Pentakeep-V increased foliar Ca and Fe concentrations (Smoleń and Sady, 2010) in spinach. In the same year, Al-afifi and Al-shrouf (2010) also demonstrated a significant yield increase of tomato (by up to 146%) following application of Pentakeep-V. The identical product also enhanced the shoot biomass of rooted cuttings of *Prunus mahaleb* L. (Szabó *et al.*, 2014).

1.6 Effect of ALA under stress conditions

ALA also plays an important role in alleviating abiotic stresses, such as chilling, dark, drought and salinity. The exogenous application of ALA has been reported to modulate some physiological and biochemical processes, including photosynthesis under both optimal and stress conditions (Tanaka *et al*., 1993; Kanto *et al.*, 2015). Morales-Payan and Stall (2005) demonstrated that ALA could

confer salt and suboptimal temperature tolerance in plants, and also simultaneously enhance vegetative growth and increase photosynthetic activity.

The effectiveness of exogenous ALA treatment on the growth of lettuce (Härtel *et al.*, 1996; Aksakal *et al.*, 2017), spinach (Nishihara *et al.*, 2001, 2003), cucumber (Wu *et al.*, 2018), pakchoi (Wang *et al.*, 2005), potato (Zhang *et al.*, 2006), cauliflower (Ahmad *et al.*, 2017) and pepper (Korkmaz and Korkmaz, 2009) under stress conditions varied with the different concentrations and mode of application (Table 1.3). The growth of potato microtubers *in vitro* increased by 73% after exogenous application of ALA at 0.3 mg L^{-1} under 0.5% NaCl stress conditions (Zhang *et al.*, 2006). Seed treatment of ALA on pepper also enhanced the germination rate, prevented crop losses and improved seedling growth (Korkmaz and Korkmaz, 2009; Korkmaz *et al*., 2010). Aksakal *et al.* (2017) reported ALA pre-treatment worked in mitigating UV-B stress on lettuce seedlings. Foliar applied ALA also reduced the oxidative stress caused by Cr toxicity in cauliflower (Ahmad *et al.*, 2017). ALA also improved salt tolerance of pakchoi, spinach and potato (Nishihara *et al.*, 2001, 2003; Wang *et al.*, 2005; Zhang *et al.*, 2006) and increased heat tolerance of cucumber leaves (Zhang *et al.*, 2012).

Table 1.5. The effects of ALA by foliar application in vegetables under different abiotic stress.

Type of stress	Mode of ALA application	ALA concentration	Vegetables	Effect	References
Dark conditions	Pre-soaking	10 mM	Lettuce (*Lactuca sativa*)	ALA enhanced photosensitizing effect triggered by protochlorophyllide (PChlide) in response to dark treatment.	Härtel *et al.* (1996)

Type of stress	Mode of ALA application	ALA concentration	Vegetables	Effect	References
Salt stress	Foliar spray	0.60 mM 1.8 mM	Spinach (*Spinacia oleracea*)	ALA enhanced rate of photosynthesis, H_2O_2, glutathione content, catalase, ascorbate peroxidase activity and glutathione reductase.	Nishihara *et al.* (2001)
		0.60 mM & 1.8 mM	Spinach (*Spinacia oleracea*)	Salinity tolerance improved by increasing activities of antioxidant enzymes and activating ascorbate-gluathione cycle and rate of photosynthesis.	Nishihara *et al.* (2003)
		0.15 mM	Cucumber (*Cucumis sativus*)	Exogenous application of ALA alleviated salinity stress and increased chlorophyll biosynthesis.	Wu *et al.* (2018)
	Pre-soaking	0.60 mM	Pakchoi (*Brassica campestris*)	ALA application enhanced seed germination by increasing respiration rate.	Wang *et al.* (2005)
	Rooting medium	0.18 mM	Potato (*Solanum tuberosum*)	Enhanced microtuber formation by increasing the number, diameter, and fresh weight of tubers under saline conditions.	Zhang *et al.* (2006)
Metal stress	Foliar	0.90 mM	Cauliflower (*Brassica oleracea botrytis*)	ALA enhanced photosynthetic pigments at lower Cr (10 and 100 µM) stress but at higher Cr stress (200 µM) no additional increase. Minimizing Cr uptake and its toxic effects in cauliflower.	Ahmad *et al.* (2017)

Type of stress	Mode of ALA application	ALA concentration	Vegetables	Effect	References
Ultraviolet-B radiation (UV-B)	Foliar	0.15 mM	Lettuce (*Lactuca sativa*)	ALA alleviated tnegative effect of UV-B through lowering reactive oxygen, while enhanced PAL and γ-TMT gene expression, antioxidant enzyme activities, Chl a and b content, total phenolics, antioxidant capacity and soluble sugars concentration.	Aksakal *et al.* (2017)
Low temperature	Seed soaking	149 mM	Pepper (*Capsicum annuum*)	ALA prevented crop losses in pepper due to chilling stress. Pepper seeds were stored for 1 month at 4°C or 25°C and improved germination at 15°C.	Korkmaz and Korkmaz (2009)
	Seed soaking and foliar sprays	149 mM		Exogenous application enhanced seedling growth, plant biomass, chlorophyll, and stomatal conductance.	Korkmaz et al. (2010)
Heat stress	Presoaking	0.003 mM	Cucumber (*Cucumis sativus*)	The level of heat-induced MDA, O_2^-, and H_2O_2 were reduced and simultaneously proline, soluble sugar contents, and plant growth were improved.	Zhang *et al.* (2006)

1.7 Research rationale

Many researchers have investigated the microbial production of ALA as a means of promoting plant growth (Koh and Song, 2007). However, most have focused

on photosynthetic microorganisms; common rhizosphere bacteria such as *Bacillus* spp. have largely been ignored. However, using glutamic acid as a precursor, (Ahn, 2007) enabled *Bacillus cereus* 1-1 strain to produce 2 mM of ALA. *Bacillus* spp. are thought to act as plant-growth promoters; for example, Noha and Shixue (2018) demonstrated enhanced pea growth under glasshouse conditions following inoculation with *Bacillus* species. The mechanisms which led to promotion of pea plant growth were not elucidated, but could include secretion of metabolites, production of vitamins or amino acids.

Therefore, the rationale for the current study was to determine if a *Bacillus* sp. previously demonstrated to improve plant growth, produces ALA and whether any plant growth promotion effects could be attributed to ALA production. If so, there would be good reason to establish a protocol for enhancing ALA production by the bacterium, which could be used to inoculate plants for enhancement of germination and growth. To complement this, the effects of exogenously applied ALA on plant growth were also quantified. This was carried out to (i) determine if the published effects relating to the commercial Pentakeep (which includes macro- and micro-nutrients) were due to the ALA within the product (the unique selling point) and (ii) an understanding of the effects of ALA on plant growth is necessary prior to encouraging ALA production by a rhizosphere bacterium.

1.8 Research aims, objectives and hypotheses

The **key aims** of this investigation were to (i) establish whether exogenously applied ALA enhanced plant growth and (ii) determine the potential for a soil bacterium to increase plant biomass through natural production of ALA.

These key aims were addressed by the following **specific objectives**:

1) Identify a bacterial isolate with previously demonstrated plant-growth-promoting effects (on rice) and quantify its ability to produce ALA.
2) Establish if bacterial inoculation of plants with the known isolate increased growth.
3) Quantify the effects of foliar and root application of commercial ALA on plant growth.

The **main hypotheses** addressed in this project were:

1) Bacterial ALA production will enhance growth and biomass of cress and kale plants.
2) Application of exogenous ALA production will increase growth of cress, kale and radish plants.

2 BACTERIA: IDENTIFICATION AND QUANTIFICATION OF ALA PRODUCTION

2.1 Introduction

ALA has been used in agriculture as an herbicide, insecticide, growth-promoting factor and for alleviating plant stress (Sasikala, Ramana and Rao, 1994; Nishikawa and Murooka, 2001; Sasaki *et al.*, 2005). However, commercial use of ALA has generally been limited due to high costs associated with chemical synthesis and concomitant low yields (Nishikawa and Murooka, 2001). Therefore, microbial production of ALA has recently been considered as an environmentally friendly, sustainable and renewable methodology (Kang *et al.*, 2012).

It is known that ALA is a precursor of tetrapyrrole biosynthesis, including chlorophyll, heme and vitamin B12 which extensively exists in plants, algae, animals and bacteria (Rebeiz *et al.*, 1984; Beale, 1990; Carmichael, 1992; Sasikala, Ramana and Rao, 1994; Sasaki *et al.*, 2002). However, the synthesis pathway of ALA in most bacteria and animals is similar to that in plants, which is *via* the C5 pathway and uses glutamate as a precursor (Yonezawa et al., 2015).

A precursor is a substance which has a role in inducing metabolite production in living organisms including plants and microorganisms (Demain, 1998; Nissar *et al.*, 2015). Avissar (1980) inisiating the use of a precursor in ALA production and Sasaki, *et al.* (1995) demonstrated that addition of glutamate and levulinic acid increased the ALA concentrations in *Chlorella regularis* up to 1.9 mM. Four years later, Ano *et al.* (1999) reported that *Chlorella regularis* performed better with the addition of glycine which enhanced the specific growth rate and ALA production by up to 3.72 mM.

In contrast, inhibitors delay metabolic processes (Schonborn and Gwinnutt, 2010). Lee *et al.* (2005) discovered that glucose inhibited degradation of ALA by ALA dehydratase (ALAD) and resulted in increased ALA yield. Furthermore, Saikeur *et al.* (2009) showed the positive effect of levulinic acid (LA) as an inhibitor of ALAD, and enhanced ALA production of *Rhodopseudomonas palustris* KG31.

Many studies have reported the production of ALA by photosynthetic bacteria, rhizobacteria and recombinant *Escherichia coli*. Nevertheless, a few have observed ALA synthesis from *Bacillus* species. Ahn (2007) reported ALA production (up to 2 mM) in *Bacillus cereus* 1-1 following addition of glucose, acetic acid and glutamic acid as precursors. The effect of *Bacillus* species on plant growth was described by (Koh and Song, 2007); bacteria are known to produce metabolites such as amino acids, organic acids and growth factors which enhance plant growth.

Since ALA is considered as a potential plant stimulating compound produced by bacteria, the aim of this Chapter was to determine if a bacterium previously isolated from peat soil and which showed plant growth promoting properties when applied to rice produced ALA.

The **specific objectives** of this Chapter were to:

1) Identify a bacterium previously isolated from peat.
2) Quantify ALA production by the bacterium.
3) Optimise ALA production by determining the most suitable time during bacterial growth to add a precursor to, and inhibitor of, ALA dehydratase.

2.2 Materials and Methods

2.2.1 Bacterial identification

The bacterial isolate was maintained on Nutrient Agar (Oxoid) and regularly sub-cultured as required. DNA was extracted by suspending cells from a 24 h old colony into 100 mL of sterile 1 × TE buffer (10 mM Tris-Cl, 1 mM EDTA buffer, pH 7.6) and heating to 100°C for 30 minutes to rupture the cells (Eppendorf Thermomixer Comfort, Germany). After heating, the solution was centrifuged (Heraeus Pico17 centrifuge, UK) at 13,000 × g for 15 mins. The extracted total DNA was transferred into sterile microcentrifuge tubes. For PCR, 2 μL of DNA was used in a 25 μL reaction mixture containing 12.5 μL of DreamTaq™ Green PCR master mix (2 ×) (ThermoFisher Scientific, UK), 8.5 μL of nuclease-free water and 1 μL (10 μM) of each primer (8F and 805R universal primers). The DreamTaq Green PCR master mix contained DreamTaq DNA polymerase, optimised DreamTaq Green Buffer, 0.4 mM of dNTPs and 4 mM $MgCl_2$. The quality and concentration of DNA was tested using nanodrop and a qubit fluorometer respectively. DNA was sequenced by Eurofins Scientific (UK) and the sequence compared with the NCBI reference database.

2.2.2 Establishing the bacterial growth curve and ALA concentrations

Bacterial cultures were grown in Luria Bertani (LB) broth at 35°C in a 50 mL sterile Falcon Tube. After 24 h, 10 mL of the bacterial suspension were added to a 250 mL flask and incubated at 35°C. Every 2 h over a 24 h period, an aliquot was removed from the flask and the optical density (absorbance) was determined

at 600 nm using an ultraviolet-visible spectrophotometer (Cecil Instruments, UK) and the ALA concentration of the medium quantified.

ALA measurement was carried out by taking a 100 µL aliquot of the medium and dispensing it into a 10 mL glass tube to which were added 2 mL of acetate buffer containing 1% acetylacetone (76 g sodium acetate, 800 mL ultra-pure water, 70 mL acetic acid, adjusted pH to 4.5-4.6; then 10 mL acetylacetone added and adjusted to final volume of 1 L with ultra-pure water). The solution was mixed by vortexing for 5 seconds.

After mixing, the solution was heated in a water bath at 90°C for 15 minutes, together with a set of ALA standards (0-1000 µm ALA (Sigma-Aldrich) in acetate buffer). After 15 mins, the samples and standards were removed from the water bath and cooled on ice for 10 minutes. The solutions were mixed and 3.5 mL of Ehrlich's reagent (4 g of 4-(dimethylamino) benzaldehyde, 46 mL of ultra-pure water, 120 mL of acetic acid and 34 mL of 70% perchloric acid) were added to each tube, mixed, and left at room temperature for 15 mins. Solutions were vortex mixed and the absorbance measured at 553 nm. The blank used was acetate buffer/acetylacetone solution. The ALA concentrations at each time point were calculated against the standards. The aim was to determine whether there was a relationship between cell number (expressed as OD) and ALA concentration.

2.2.3 Determining the effect on bacterial ALA production of adding a precursor and an inhibitor

A means of attempting to increase bacterial ALA production in this experiment was through addition of a precursor of ALA formation (glutamate) and an inhibitor of ALA dehydratase to prevent its degradation (levulinic acid). For these experiments, bacterial cultures were established as described above (section 2.2.2) and the glutamate solution (9.36 g glutamate made to 100 mL with sterile deionized water) and levulinic acid solution (2.56 mL levulinic acid made up to 50 mL with deionized water) were added at specific time points during the bacterial incubation as follows: (i) precursor (glutamate) addition at 3 h incubation (P3); (ii) precursor (glutamate) addition at 4 h incubation (P4); (iii) precursor (glutamate) addition at 3 h incubation and inhibitor (levulinic acid) addition at 10 h incubation (P3I10); (iv) precursor (glutamate) addition at 4 h incubation and inhibitor (levulinic acid) addition at 10 h incubation (P4I10); (v) uninoculated medium. The concentration of ALA in cultures was measured after 24 h of incubation (followed the method in 2.2.2). All treatments were replicated (×3). Three replicates were used for logistical reasons.

This experiment was subsequently repeated using a wider range of time points (2, 4, 8 hours for the glutamate amendment and 4, 8, 10 hours for the levulinic acid addition). The combination of each amendment was omitted. Prior to measuring ALA concentrations, the bacterial growth medium was centrifuged to remove cells and 1 mL of the supernatant used in the assay.

2.2.4 Statistical analysis

A one-way analysis of variance was conducted on ALA concentrations following amendment of the incubation medium with the precursor and inhibitor solutions (factors) for the repeated precursor/inhibitor tests. Data were analysed by GenStat version 19 (VSN International, Hemel Hempstead, UK). Post hoc comparisons between means were based on the Tukey test at the 0.05 probability level.

2.3 Results

2.3.1 Bacterium identification

The 16S DNA sequence of the bacterium genes were compared to the NCBI (National Centre for Biotechnology Information) reference database and submitted to the NCBI Gene Bank. The bacterium was identified by PCR-sequencing as *Bacillus* sp. with 99% similarity, but as *Bacillus paramycoides* with 100% similarity.

2.3.2 Bacterial growth curve and ALA concentrations

The bacterial growth curve over the 24 h incubation period (Figure 2.1) shows a lag phase between 0-2 h after inoculation with the cells entering the exponential (log) phase from 3 h up to 15 h incubation. Cell numbers (expressed as absorbance) increased 10-fold from the point of inoculation to the start of the stationary phase after 16-18 h.

The ALA concentration (Figure 2.1) also increased over the incubation period, with maximum concentrations observed when the cells reached the end of the exponential phase and were entering the stationary phase. The final ALA concentration was 102 μM and the starting concentration at time zero was 56 μM. ALA synthesis thus appeared to increase as cells numbers increased. This test was very time intensive and ideally should be repeated/replicated. This is addressed in the following experiments.

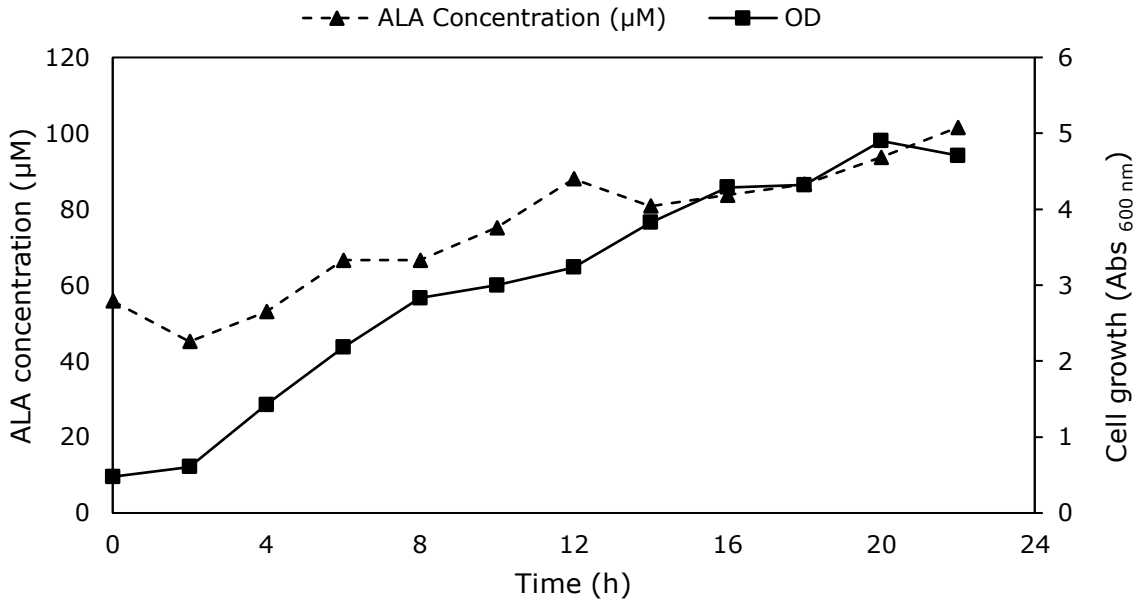

Figure 2.1. Bacterial growth expressed as absorbance at 600 nm and ALA concentrations measured over a 24-hour incubation period. Bacteria were cultured in LB broth at 35°C.

A further set of bacterial cells were incubated for 8 h in order to confirm the optimum time during the cell growth curve to add the precursor (glutamate) and inhibitor (levulinic acid). Ideally the glutamate should be added during the exponential phase and the levulinic acid near to the end of the phase. This time the OD was measured every hour after inoculation for 8 h (Figure 2.2). Determination of the optimum time to introduce the inhibitor was calculated by

regression. The exponential phase started 5 h after inoculation which is in line with the previous test (Figure 2.1) and the calculated time to add the inhibitor was after 9 h and 45 minutes of incubation. For the next experiment the time for adding the inhibitor was rounded to 10 h after inoculation.

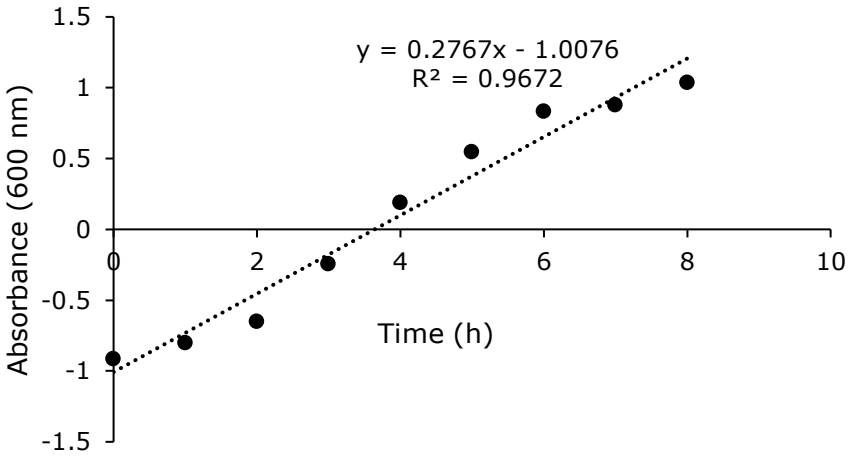

Figure 2.2. Bacterial growth over an 8 h period in LB broth at 35°C. Data are absorbance values at 660 nm.

2.3.3 The effect on bacterial ALA production of adding a precursor and an inhibitor to the incubation medium

ALA concentrations within the bacterial culture medium were calculated against the standards using regression. In the first of the two experiments (Figure 2.3), glutamate addition at 4 h after inoculation (P4) resulted in the highest ALA concentration (almost 200 μM). The combination of adding glutamate and levulinic acid (P3I10 and P4I10) decreased ALA production by the bacteria (123

μM). However, ALA measurement within the uninoculated medium was quite high (above 150 μM).

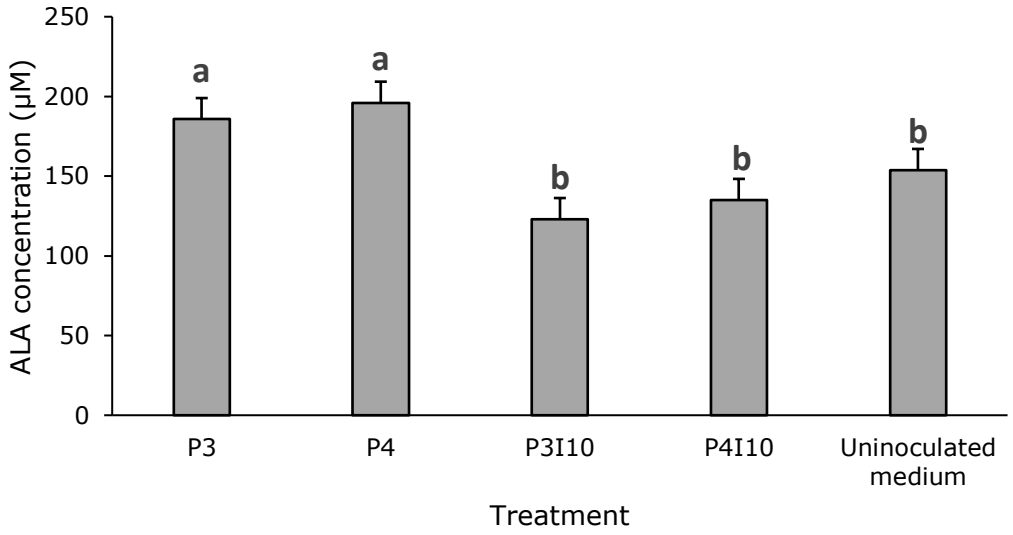

Figure 2.3. ALA concentrations of the bacterial culture medium following amendments. P3, glutamate addition at 3 h; P4, glutamate addition at 4 h; P3I10, glutamate addition at 3 h and levulinic acid amendment at 10 h; P4I10, glutamate addition at 4 h and levulinic acid amendment at 10 h. Columns similarly superscripted are not significantly different (Tukey post hoc test). There was a significant treatment effect $p=0.005$. Individual error bars are based on the pooled variance estimate from the ANOVA with 10 degrees of freedom.

In the second of the two experiments (where the medium was centrifuged before analysis, a higher concentration of medium was taken for ALA analysis and amendments were made at more time points); the amendments resulted in ALA concentrations within a lower range as previously measured, but of a similar order of magnitude. However, in this case, the values for the uninoculated medium were much lower (Figure 2.4).

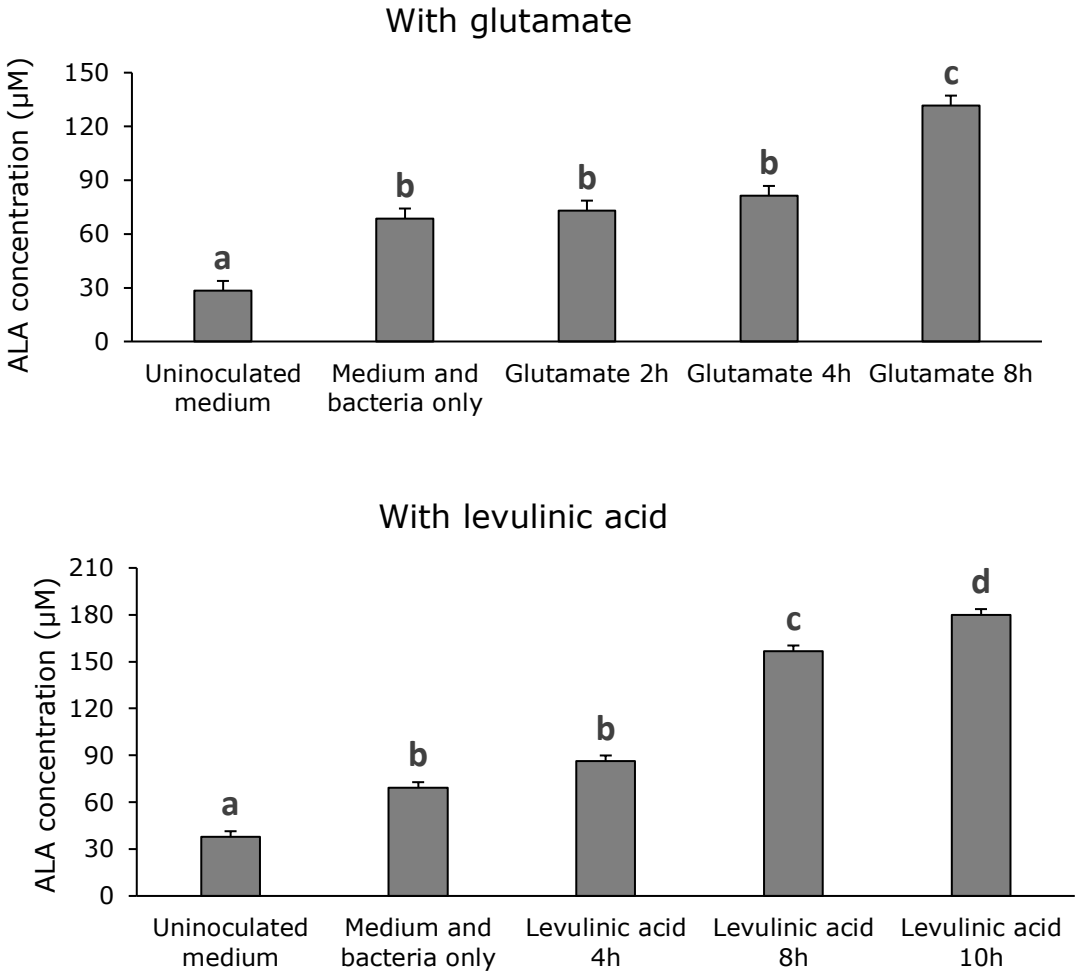

Figure 2.4. ALA concentrations in the bacterial culture medium following amendments after 2, 4 and 8 h of glutamate (top graph) and 4, 8 and 10 h of levulinic acid (bottom graph). Columns similarly superscripted are not significantly different (Tukey post hoc test). There was a significant treatment effect $p<0.001$ for both sets of data. Individual error bars are based on the pooled variance estimate from the ANOVA with 10 degrees of freedom.

2.4 Discussion

2.4.1 Bacteria identification

Based on PCR and sequencing, the bacterial isolate is a *Bacillus* species and likely to be *Bacillus paramycoides* based on the percentage match with the database. According to Noha and Shixue (2018), *Bacillus paramycoides* is a plant growth

promoting rhizobacterium which improved the growth of pea plants (*Pisum sativum*). The mechanisms involved in promoting pea growth and biomass were stated as phosphate solubilisation and siderophore and IAA production. Previous pilot tests indicated that this bacterium promoted growth of rice (unpublished data) and therefore is worth of further study.

2.4.2 Bacterial growth curve and ALA concentrations

The data for bacterial growth (absorbance) and ALA production (Figure 2.1), suggest that ALA concentrations within the medium increased with increasing bacterial growth. ALA is excreted by bacterial cells into the surrounding medium. Sasaki *et al.* (1987) reported that ALA production in recombinant strains was concomitant with the bacterial growth.

The lag phase observed here is normal and is due to physiological adaptation of cells to the new culture environment during the early stages (Yates and Smotzer, 2007). It was noted that ALA (56 μM) was detected at the first point, suggesting that ALA was produced in the preculture stage. The exponential (log) phase was initiated when the bacteria adapted to the environment; at this point, cells increase in number as well as size and metabolite production (in this case ALA) is enhanced. The enhanced ALA concentration in the middle of the exponential stage is contrary to observations in a previous study which demonstrated that maximum ALA production occurred at the end of the exponential phase (Lee *et al.*, 2004). As the cells reached the stationary phase, ALA synthesis decreased, which corroborates observations in an earlier study which showed that ALA synthase activity slowed at the onset of the stationary stage (Foley and Beale,

1982). The maximum ALA production reached 102 μM, which is consistent with the work of Foley and Beale (1982), who measured values in the range of 100 μM. Ishii *et al.* (1990) and Saikeur *et al.* (2009) found that *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* KG31 produced ALA up to 160-180 μM. The apparent increase in ALA concentration after 22 hours suggests that ALA was a component of secondary metabolism, since according to (Singh *et al.*, 2017), secondary metabolites are organic compounds that are indirectly connected with the growth, development and reproduction of microorganisms and are usually formed towards the end of the stationary stage. This contradicts the comment by Ruiz *et al.* (2010) that formation of microbial secondary metabolites usually occurs during the late log stage.

The optimum time for adding the precursor (glutamate) to the bacterial culture was determined from the first growth curve (Figure 2.1) by observing the point where the exponential phase started (3-4 h after inoculation). Theoretically, adding a precursor at the early exponential phase should support ALA synthesis by the bacteria (Saikeur *et al.*, 2009). Furthermore, Cihak *et al.* (2017) found that secondary metabolites are usually synthesized at the early phase of microbial growth, contradicting the statements made earlier by other authors. Weber and Kim (2016) pointed out that for optimal production of secondary metabolites, the metabolic engineering should be performed at the early stage of the secondary metabolite production.

The second curve (Figure 2.2) became the base for determining the optimum time for adding the inhibitor (levulinic acid). From the calculation using regression formulae, the best time for adding the inhibitor was after 9 h and 45 mins, or rounded to 10 h after inoculation. The utilisation of an inhibitor was an attempt

to enhance the ALA production from the bacteria. Fu et al. (2008) stated the function of an inhibitor was to prevent degradation of ALA during fermentation which is caused by the activity of ALA dehydratase.

2.4.3 The effect on bacterial ALA production of adding a precursor and an inhibitor

In the present study, addition of glutamate as a precursor after 4 h of incubation, enhanced the ALA content in the bacterial growth medium. This finding suggests that the precursor supported the bacteria in producing/excreting ALA. As mentioned in the literature review, photosynthetic bacteria, such as *Anabaena variabilis*, *Anacystis nidulans*, *Chlorobium limicola*, *Chloroflexus auranticus,* and algae, such as *Cyanidium caldarium* and *Agmenellum quadruplicatum* utilised glutamate as a sole precursor to increase the ALA production (Jurgenson et al., 1976; Avissar, 1980; Kipe-Nolt and Stevens, 1980; Andersen *et al.*, 1983). The findings in the current investigation using *Bacillus paramycoides* confirmed the work of (Ahn, 2007), who synthesised 2.0 mM of ALA from *Bacillus cereus* using glutamic acid, glucose and acetic acid. The results from the second experiment yielded lower concentrations of ALA from the growth medium than in the first experiment. This might be because cells were removed during the centrifugation step here and any ALA that might have been generated by lysis of cells in the first experiment would not be present. Glutamate addition was more effective after addition at 8 hours in contrast to the earlier experiment. Nevertheless, both experiments demonstrate that glutamate is an effective precursor for enhancing bacterial ALA production.

Lee *et al.* (2003) proposed the use of an inhibitor in order to limit formation of ALA dehydratase during fermentation as a means of improving ALA yield. However, in the first experiment here, levulinic acid reduced ALA production. The reason for this is unknown, but it is worth noting that levulinic acid was added to media which had also previously been amended with glutamate. It is possible that a form of metabolic feedback occurred, preventing ALA production or excretion, or perhaps an excess of ALA itself acted as a precursor for other metabolites since the pathways involved are complex. In contrast, in the second experiment when levulinic acid was added after 8 and 10 h of incubation (in the absence of glutamate), ALA concentrations exceeded those observed in the cultures which has been amended with glutamate. These data support the proposition by (Lee *et al.*, 2003).

2.5 Conclusion

Bacterial production of ALA in high concentrations is commercially important because it is expensive to produce and has numerous uses in the field of medicine as well as other areas. ALA enhances plant growth and alleviates plant stress and therefore has potential for use in agriculture. The question is whether it is feasible to utilise a soil bacterium as a means of delivering ALA to plant roots where is might act as a growth promotor. The results presented in the current Chapter show that the bacterium, *Bacillus paramycoides*, which was originally isolated from peat, produced ALA as a metabolite. By adding a precursor to ALA production, or an inhibitor of the ALA degradative enzyme, ALA dehydratase, an increased concentration of ALA was measured in the culture medium. The challenge will be to establish if the bacterium (and its ALA metabolite), can enhance plant growth *in vitro* and *in vivo.*

3 EFFECT OF *BACILLUS PARAMYCOIDES* INOCULATION ON GROWTH OF CRESS (*LEPIDIUM SATIVUM*) AND KALE (*BRASSICA OLERACEA* VAR. *ACEPHALA*)

3.1 Introduction

Germination and seedling growth are complex processes marked by the emerging radicle and are critical phases in the plant life cycle (Finch-Savage and Leubner-Metzger, 2006; Bentsink and Koornneef, 2008; Sanz *et al.*, 2015). During germination, plants are sensitive to injury, disease and environmental stress (Doni *et al.*, 2014), all of which affect growth and quantity and quality of crop yield (Almansouri et al., 2001).

Plant can synthesise phytohormones, but they can also utilise exogenous sources such as those produced by microorganisms which can contribute to plant growth promotion (Glick *et al*., 1998; Ahemad and Kibret, 2014). Seed inoculation with beneficial bacteria through seed priming, has become an acceptable method of green technology for sustainable agriculture. As a pre-germination seed treatment, seed priming may enhance seedling quality and vigour, ultimately enhancing plant productivity and protecting the plant from diseases (Farooq *et al.*, 2010; Anwar *et al.*, 2013; Doni *et al.*, 2014; Mahmood *et al.*, 2016). Biopriming seeds with plant growth promoting bacteria (PGPB) can improve plant growth due to increased nutrient uptake, biocontrol of plant pathogens, hormone production and increased stress tolerance (Glick *et al*., 1995; Glick *et al.*, 1999; Glick, 2010). For example, soaking rice seeds with a 1:10 dilution of *Rhodopseudomonas palustris* TN 1 14 cells significantly improved yield; this was attributed to bacterial ALA production (Nunkaew *et al.*, 2014). According to (Sarawaneeyaruk *et al.*, 2019), inoculating plant roots with PGPR is beneficial

and (Helaly *et al*., 2018) reported that treating collard cabbage roots with *Bacillus cereus* (strain AP-51) enhanced vegetative growth, pigment and phytochemical production, yield and mineral content. A year before, (Helaly, 2017) observed improved vegetative growth, yield and elemental composition of kale following inoculation with *Bacillus amyloliquefaciens* subsp. *plantarum* (strain AP-303).

Bacterial promotion of plant growth is through indirect stimulation of plant metabolite production or due to the direct effects of microbial metabolites. The classification of metabolites is built upon the source, whether intracellular or extracellular and upon their function, whether primary or secondary (Horak *et al.*, 2019). During the growth of microorganisms, primary and secondary components are produced and secreted into the surrounding environment in detectable amounts (Pinu *et al*., 2017).

According to Granucci *et al.* (2015), intracellular metabolites are secreted inside the cell as the outcome of intracellular metabolism. In contrast, extracellular metabolites, also known as exometabolome, are the compounds excreted outside the microbial cell and include metabolites present in broth media in which bacteria are cultured (Mashego *et al.*, 2007; Pinu *et al*., 2017). Intracellular metabolites are called metabolic fingerprinting, while extracellular metabolites are named metabolic footprinting (Pinu, Villas-Boas and Aggio, 2017). Extracting intracellular metabolites for study is more complex than separating extracellular metabolites; the latter can be done by centrifugation and filtration (Tredwell *et al.*, 2011). Bacteria footprints in the outside environment are a consequence of nutrient uptake and metabolite secretion during growth (Villas-Boas *et al.*, 2007; Behrends *et al.*, 2013).

There are contradictory arguments in the literature as to whether ALA is produced as an intracellular or an extracellular metabolite. Sasaki *et al.* (1987) found that ALA was formed extracellularly by *Rhodobacter sphaeroides* in the presence of levulinic acid which inhibits ALA dehydratase, but Tangprasittipap *et al.* (2007) demonstrated intracellular ALA production by a similar photosynthetic bacterium when using the same inhibitor.

As a common precursor of many tetrapyrroles *in vivo*, including chlorophyll, heme and vitamin B12, ALA plays an important part in the formation of porphyrin for seed germination and seedling growth (Wang *et al.*, 2005; Naeem *et al.*, 2011). According to Hotta *et al.* (1997[b]) and Fu *et al.* (2014), ALA increases seed germination and yield of various plant species in both optimum and adverse environments. Han *et al.* (2018) also found that ALA application increased the length of the radicle and resulted in improved water absorption. The seedling length affected the vigour of seed (Gholamalizadeh *et al*., 2017). The index of vigour determines the seedling health, growth and the plant yield (McDonald and Copeland, 1997).

However, only a few studies have investigated the potential of bacterial ALA production to enhance seed germination or plant growth. The current study evaluated the bacterial effects on the early growth stage of cress by observing percentage germination, radicle length, and biomass when grown in Petri dishes containing a range of dilution of the culture medium. From this cress experiment, the 1:500 dilution of the bacterial medium was selected and used in a pot trial aimed at evaluating the effect of the bacterial metabolites on the vegetative growth of kale grown in a peat-based potting compost.

This Chapter therefore addresses two separate experiments. Cress was selected for the Petri dish experiments because it germinates quickly, and the data obtained from those tests informed the kale/soil experiment. Kale was chosen because it is a valuable nutrient-rich leafy vegetable which is easily grown.

The **specific objectives** of this Chapter were:

1) To evaluate the effects of various dilutions of bacterial growth medium on seed germination and seedling growth of cress.
2) To quantify any effect of the bacterial growth medium on growth of kale following soil amendment.

The key hypotheses were:

1) Seedling growth of both cress and kale will be enhanced following addition of bacterial growth medium because it will contain ALA produced as a result of bacterial metabolism.
2) Higher dilutions of the bacterial growth medium will be more effective in promoting seedling growth than lower dilutions because ALA concentrations may be too high in the more concentrated solutions.

3.2 Materials and Methods

3.2.1 Experiment 1: Cress germination and growth

Cress seeds were grown in Petri dishes with the addition of a range of dilutions of supernatant derived from bacterial (*Bacillus paramycoides*) culture media. Cress seeds were germinated under controlled conditions in a growth room at

20°C. Germination took place in the dark, after which Petri dishes were subjected to a 16-hour day and an 8-hour night until the seedlings were harvested.

3.2.1.1 Preparation of the bacterium

A colony of *Bacillus paramycoides* was used to inoculate Luria Bertani (LB) broth (Sigma) and this was cultured at 35°C for 24 hours in an orbital incubator at 110 rpm. After 24 h, the replicate cultures (× 3) were centrifuged for 5 minutes at 3000 rpm and the supernatant was used for subsequent dilutions depending on the experiment. The diluted medium was used for the cress germination tests.

3.2.1.2 Seed preparation

Cress seeds were surface sterilised with 90% ethanol and washed twice with sterile deionised water. After surface sterilising, 10 seeds were placed on filter paper lining the bottom of a 9 cm Petri dish and 5 mL of the test solutions were gently pipetted onto the filter paper.

3.2.1.3 Experiment 1.1: Application of various dilutions of bacterial culture medium on cress germination and growth

The bacterium was cultured in full strength LB broth as described in section 3.2.2.1 and after 24 h, culture tubes were centrifuged for 5 minutes at 3000 rpm to remove bacterial cells. The supernatant was diluted to give the following dilutions: 1:10, 1:100, 1:500 and 1:1000. 'Clean' medium that was not used for

bacterial growth as similarly diluted. The cress germination/growth test treatments were: (i) Sterile water; (ii) LB medium, 1:10 dilution; (iii) LB medium, 1:100 dilution; (iv) LB medium, 1:500 dilution; (v) LB medium, 1:1000 dilution; (vi) LB medium + bacterial metabolites, 1:10 dilution; (vii) LB medium + bacterial metabolites, 1:100 dilution; (viii) LB medium + bacterial metabolites, 1:500 dilution; (ix) LB medium + bacterial metabolites, 1:1000 dilution.

3.2.1.4 Experiment 1.2: Effect of culturing the bacterium in different concentrations of LB broth and consequences for cress growth

Bacteria were cultured in LB broth as described in sections 3.2.2.2 and 3.2.2.3, except that in addition to culturing *Bacillus paramycoides* in full strength broth according the manufacturer's instructions, the bacterium was also cultured in ½ strength and a third strength LB broth. After 24 h of bacterial growth, each medium was centrifuged as before and 1:100 and 1:1000 dilutions of the full strength, the ½ strength and the third strength media were made. These solutions were used to dampen the filter paper lining three replicate Petri dishes in preparation for the cress germination and growth tests. A sterile water control and equivalent dilutions of each concentration of medium minus bacterial cultures were also used to test cress germination and growth.

3.2.1.5 Cress harvesting and sample processing

Germination of cress seeds was quantified four days after set-up and expressed as a percentage of the 10 seeds 'sown' in each replicate Petri dish. For measuring

radicle length, the sprouted seeds were carefully removed from each Petri dish, the seed coat was removed if still adhering and the seedling was then blotted dry. All the germinated seeds on one Petri dish were lined up against a ruler and photographed. The photographs were then transferred to ImageJ (ImageJ 1.52a, National Institute of Health, USA) for root length measurement. After photographing, the seedlings were dried at 35°C until constant weight to give the dry biomass. Here, plant dry weight includes the shoot dry weight plus the root dry weight. All analyses represent the relative percentage compared to control grown with sterile water.

Germination rate (seedling emergence) was calculated using the following formula:

$$\% \text{ germination} = \frac{\text{number of seeds that germinated}}{\text{number of seeds in the Petri dish}} \times 100 \qquad \textit{Equation } 3.1$$

Germination Index (GI) was measured according to the formula proposed by Abul-Baki & Anderson (1973):

$$GI = (\text{relative germination } (\%) \times \text{relative root length } (\%))/100 \qquad \textit{Equation 3.2}$$

3.2.2 Experiment 2: Effect of bacterial metabolites and ALA on kale growth

3.2.2.1 Experimental approach

Kale seeds were sown in a peat-based compost in 9 cm diameter pots and maintained in a controlled temperature growth room. Prior to sowing, kale seeds

were soaked in a solution of bacterial broth, a solution of bacterium-free broth, chemical ALA, or sterile water. Half the plants derived from water-soaked seeds were later (at the two-true leaf stage) treated with bacterial culture, or ALA by injecting into the soil. The remaining plants germinated from water-soaked seeds were maintained as controls.

3.2.2.2 Kale seed preparation

Prior to sowing and pre-treating, seeds were surface sterilised as described in section 3.2.1.2. Following sterilisation, seeds were soaked in a range of solutions before to sowing.

For ALA treatment, kale seeds were soaked for 24 h in aqueous solutions of ALA (5-aminolevulinic acid hydrochloride 98%, Sigma) at concentrations of 0.0012 mM or 0.12 mM. For seed soaking with bacterial broth, kale seeds were soaked for 22 h in sterile water and then for 2 h in a diluted bacterial suspension (1:500; 1:1000) where the cell count in the original suspension was 10^7 cells mL^{-1}. A parallel set of seeds were soaked in dilutions of uninoculated broth.

For the control treatment and for plants that were going to be treated later by injecting solutions into the soil, the seeds were soaked in sterile water for 24 hours. All seeds were sown in pots containing Levington M3 pot and bedding compost which contain mostly peat with a small amount of coir. The pH of this compost was 5.7.

The soil injection treatments were applied at the two true-leaf growth stage to half the plants which had had their seeds soaked in sterile water. Three mL of diluted bacterial broth solution (1:500; 1:1000) where the cell count in the

original suspension was 10^7 cells mL^{-1} were injected into the soil as near to the rooting zone as was possible to estimate.

3.2.2.3 Experimental set-up

Three seeds were sown in each pot and grown in an automated day-night temperature-controlled growth room with 16 h light and 6 h dark with 1 h of dusk and 1 h of dawn with a daytime temperature of 20°C and a night temperature of 17°C. One week after sowing, seedlings were thinned to one per pot. Pots were arranged in a fully randomised design and treatments were: two ALA dilutions, two seed soaking treatments with bacteria culture dilutions, two seed soaking treatments with uninoculated broth dilutions, two soil injection bacteria culture treatments and a control. Each treatment was replicated (× 3) to give a total of 27 pots.

Soil and Plant Analyzer Development (SPAD) readings were conducted using a portable chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan) prior to harvest. From each plant, using the first expanded leaf from the top of the plant, two leaves of the same colour were selected for measurements made under diffuse lighting. Every treatment measurement was an average of 6 SPAD-502 readings.

Kale plants were harvested at 27 days after sowing. Before harvesting, the number of leaves were counted. Then all shoots were removed and the roots extracted from the soil, washed thoroughly with tap water and dried at 35°C until constant weight was reached.

3.2.3 Statistical analyses

Statistical analyses were performed using GenStat version 19.1 (VSN International, Hemel Hempstead, UK).

For the cress (Petri dish) experiments, an analysis of variance was conducted on % germination, root length, germination index and dry weight. In the first cress experiment, dilution (10, 100, 500, 1000) and bacteria (yes and no) were the factors; in the second cress experiment, LB broth (full strength, ½ strength, third strength), dilution (100, 1000), and bacteria (yes and no) were the factors.

For the kale (soil) experiment, a two-way analysis of variance was conducted on SPAD data, number of leaves and root and shoot biomass. Factors were treatment type (sterile water, ALA, uninoculated medium, seed soaking and root injecting) and broth dilution (1:500; 1:1000). After a complete analysis, due to the complexity of the comparisons and the requirement to conduct an ANOVA for unbalanced designs, the data relating to the ALA treatments were separated from the bacterial data and analysed by one way ANOVA in which the water treatment and the two ALA treatments were compared.

For both sets of experiments, normality was tested by plotting residuals against expected normal quantities and post hoc comparisons between means were based on least significant differences (LSD) at the 0.05 probability level where appropriate.

3.3 Results

3.3.1 The vegetative characteristics of cress following addition of various dilutions of bacterial supernatant

The key findings of the first cress experiment were that dilution of LB broth as a single factor generally reduced percentage root length and germination index, except for the 1:100 metabolite-free treatment. The data are expressed as a percentage of the sterile water control value (Table 3.1). The presence of bacterial metabolites (i.e. LB broth in which bacteria had been cultured and cells removed prior to testing) further significantly reduced percentage root length and germination index relative to the equivalent metabolite-free dilution for the 1:10 and 1:100 treatments, but the metabolite-related reductions were not significantly lower for the 1:500 and 1:1000 dilutions. Interestingly, a dilution × bacterial metabolite interaction ($p=0.007$; Table 3.2) indicated that the presence of bacterial metabolites in the 1:500 dilution broth treatment resulted in a significantly greater percentage biomass than the equivalent metabolite-free dilution and also resulted in marginally (but not significantly) higher biomass than the sterile water treatment. Percentage germination followed a similar trend.

Table 3.1. Effects of a range of dilutions of fresh LB broth and of LB broth containing bacterial metabolites on vegetative growth parameters of cress.

Treatment	Root length (%)	Germination (%)	Germination index (%)	Total biomass (%)
Sterile water	100.0	100.0	100.0	100.0
Medium, 1:10	56.9	108.8	61.1	129.9
Medium + BM, 1:10	21.9	94.1	20.7	104.1
Medium, 1:100	104.7	100.0	104.4	109.8
Medium + BM, 1:100	62.5	97.1	60.6	97.3
Medium, 1:500	82.5	100.0	81.9	96.1
Medium + BM, 1:500	68.3	111.8	76.6	114.6
Medium, 1:1000	64.3	97.1	63.2	98.3
Medium + BM, 1:1000	66.1	105.9	69.9	103.8

BM = Bacterial Metabolites

Table 3.2. Summary of two-way ANOVA output for vegetative growth of cress. Factors were LB broth dilution and presence or absence of bacterial metabolites. See Table 3.1 for corresponding data.

Variate	Factor	DF	F	P VALUE	LSD (95%)
Root length	Dilution	3	7.74	<0.001	20.13
	BM	1	10.53	0.003	14.24
	Interaction	3	2.10	0.126	28.47
	Residual	24			
Germination percentage	Dilution	3	0.84	0.487	9.68
	BM	1	0.05	0.826	6.84
	Interaction	3	3.33	0.037	13.69
	Residual	24			
Germination index	Dilution	3	6.73	0.002	21.27
	BM	1	8.04	0.009	15.04
	Interaction	3	2.99	0.051	30.08
	Residual	24			
Biomass	Dilution	3	2.72	0.067	12.52
	BM	1	0.70	0.412	8.85
	Interaction	3	5.18	0.007	17.71
	Residual	24			

BM = Bacterial Metabolites

3.3.2 The plant properties of cress following the application of bacterial supernatant cultivated in various LB concentrations

To attempt to minimise the effects of LB broth on the growth of cress and enhance those of any metabolites, the bacterium was cultured in full strength LB broth following the manufacturer's instructions and also in two dilutions of the LB broth (half strength and one third strength).

The bacterium grew well in all three concentrations of LB broth, with cell numbers in the 10^7 range after 24 hours of incubation for the full strength broth and 10^6 for both the half and third strength broths. Cell numbers were calculated from OD_{600} measurements of bacteria cultured in each broth dilution (Figure 3.1).

The only effects of the bacteria/LB broth dilutions were on root length (metabolite presence $\times$ LB strength $\times$ dilution interaction, $p=0.024$) and germination index

(LB strength × dilution × bacterial metabolites interaction, p=0.044) (Figure 3.2, Table 3.3). The significant root length effect was primarily because of a lower value for the one third LB/1:100 treatment and is likely to be a Type I error. Germination index was unaffected by any of the one third LB strength treatments, but the 1:1000 dilution of the ½ strength LB broth containing bacterial metabolites significantly reduced GI relative to the other treatments (Figure 3.2). The grand mean percentage values (relative to sterile water) were either just below 100% (root length, 97%; biomass, 97%) or just above (germination index, 107%; percentage germination, 104%).

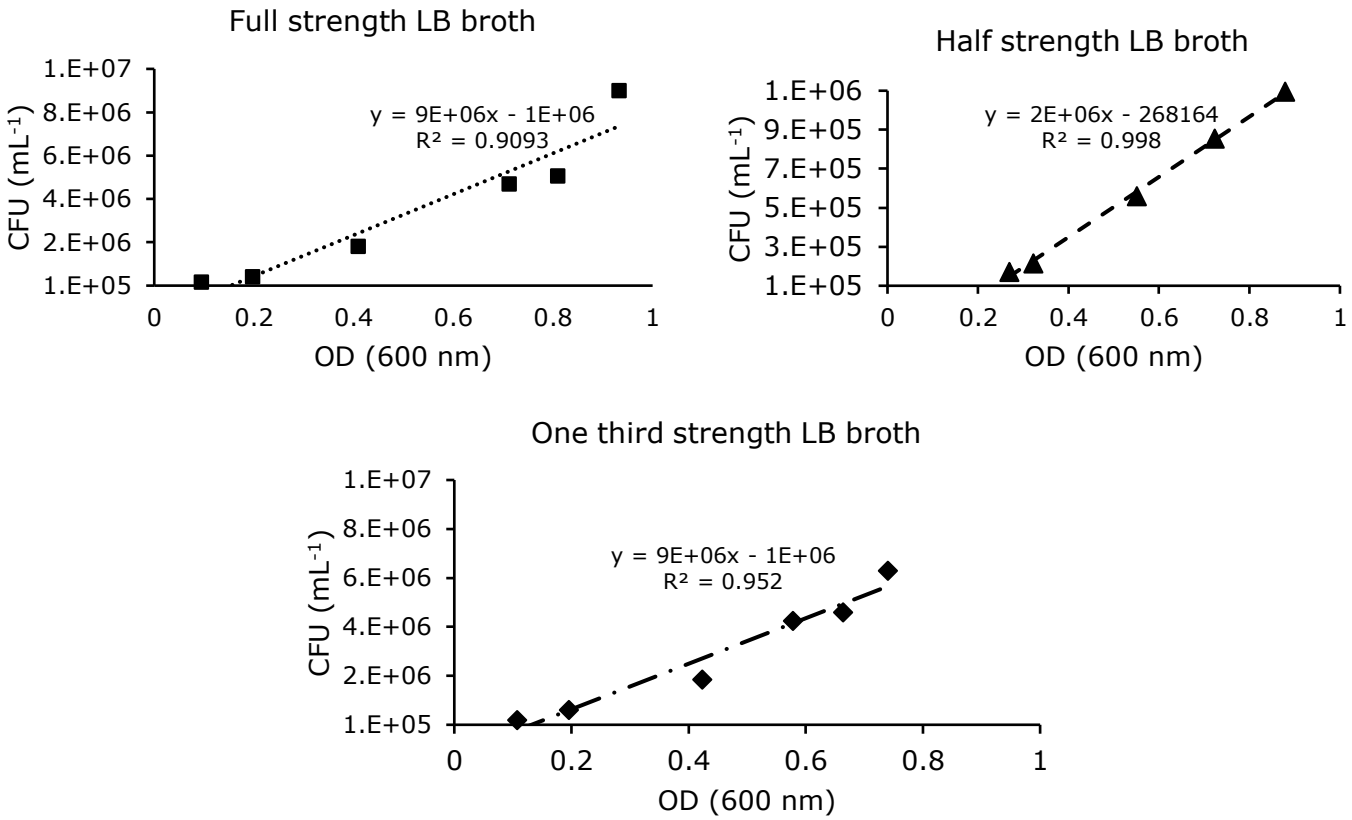

Figure 3.1. Bacteria cell numbers versus the OD_{600}. Original cell numbers after 24 h growth in full, half and third strength LB broth were diluted (10-fold dilution series) to enable future calculations of cell number for *Bacillus paramycoides* of based on OD measurement.

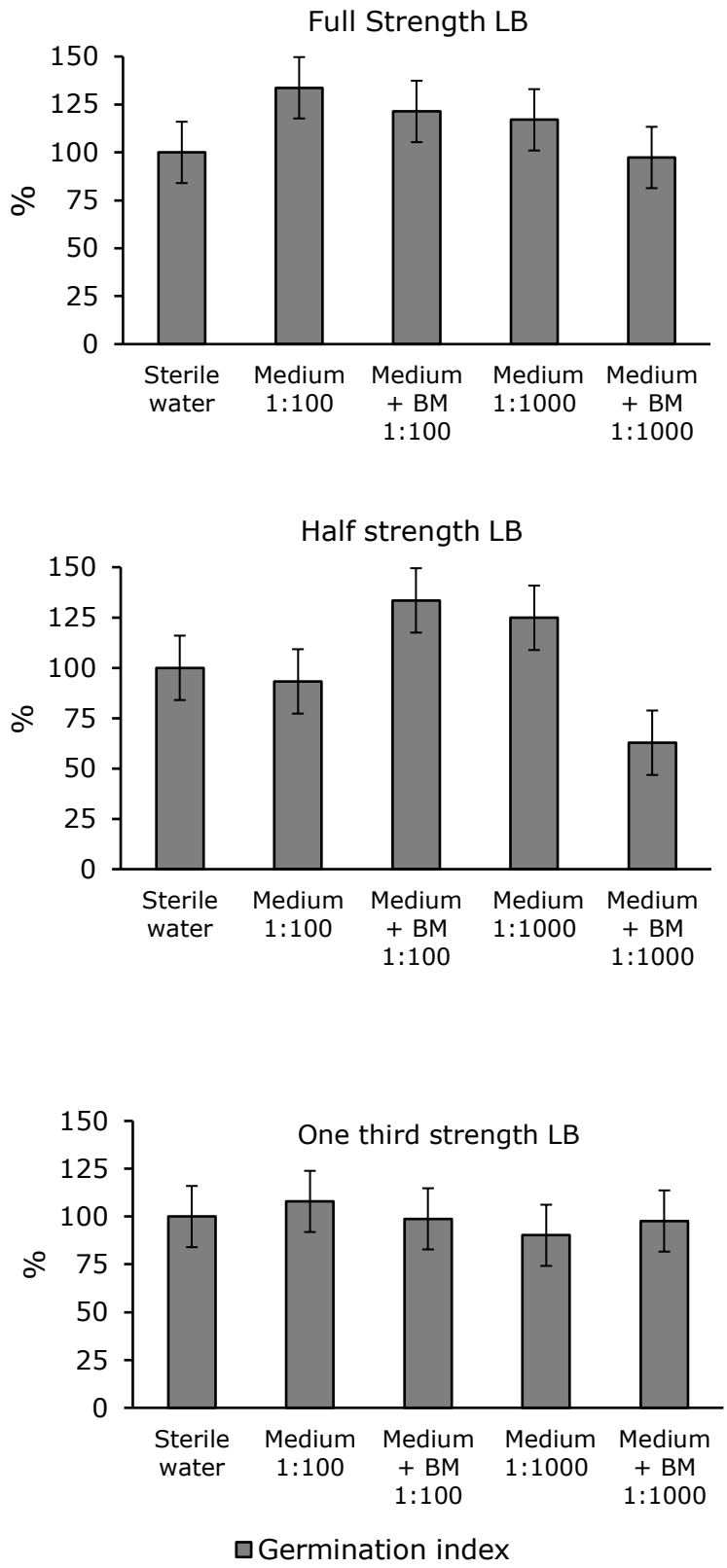

Figure 3.2. The effects of application of bacterial (cell-free) supernatant derived from *Bacillus paramycoides* cultured in LB broth on the germination index of cress. The individual error bars are based on pooled variance estimate from the ANOVA with 24 degrees of freedom; LSD = 48.50; p=0.044 (LB strength × dilution × bacterial metabolites interaction).

Table 3.3. Summary of 2-factor ANOVA for growth parameters of cress.

Variate	Factor	DF	F	P VALUE	LSD (95%)
Root length	Bacteria	1	0.69	0.413	16.05
	Dilution	1	3.21	0.086	16.05
	LB medium	2	0.82	0.453	19.66
	Bacteria.Dilution	1	5.02	0.035	22.70
	Bacteria.LB medium	2	0.19	0.824	27.80
	Dilution.LB medium	2	0.22	0.804	27.80
	Bacteria.Dilution.LB medium	2	4.37	0.024	39.32
	Residual	24			
Germination	Bacteria	1	0.41	0.528	5.98
	Dilution	1	0.41	0.528	5.98
	LB medium	2	2.23	0.130	7.32
	Bacteria.Dilution	1	1.14	0.297	8.45
	Bacteria.LB medium	2	0.14	0.873	10.35
	Dilution.LB medium	2	0.95	0.399	10.35
	Bacteria.Dilution.LB medium	2	0.32	0.730	14.64
	Residual	24			
Germination index	Bacteria	1	0.93	0.345	19.80
	Dilution	1	2.94	0.099	19.80
	LB medium	2	1.36	0.276	24.25
	Bacteria.Dilution	1	2.61	0.119	28.00
	Bacteria.LB medium	2	0.22	0.807	34.29
	Dilution.LB medium	2	0.13	0.875	34.29
	Bacteria.Dilution.LB medium	2	3.58	0.044	48.50
	Residual	24			
Biomass	Bacteria	1	1.96	0.175	13.12
	Dilution	1	2.24	0.148	13.12
	LB medium	2	2.97	0.070	16.07
	Bacteria.Dilution	1	1.64	0.213	18.55
	Bacteria.LB medium	2	2.69	0.089	22.72
	Dilution.LB medium	2	2.93	0.073	22.72
	Bacteria.Dilution.LB medium	2	0.20	0.823	32.14
	Residual	24			

3.3.3 The vegetative characteristics of kale following addition of various dilutions of bacteria supernatant

Neither soaking the seeds prior to sowing, nor injecting bacterial culture into soil had any significant effect on the plant parameters measured. The most notable effect was with percentage germination, which shows a trend towards being lower

in the presence of bacteria. The uninoculated medium appeared to increase germination (114%) relative to the sterile water control treatment (100%), although not significantly. There was no bacterial effect on any other parameter.

Table 3.1. Summary of vegetative growth of kale following the uninoculated medium and bacteria treatments.

Treatment	Germination (%)	Number of leaves	SPAD reading	Root biomass (g)	Shoot biomass (g)
Uninoculated medium, 1:500	114 ± 14.3	8.33 ± 0.33	75.90 ± 1.02	0.26 ± 0.03	1.349 ± 0.07
Uninoculated medium, 1:1000	114 ± 14.3	8.33 ± 0.33	74.85 ± 1.02	0.30 ± 0.03	1.627 ± 0.07
Bacteria, 1:500	78.60 ± 13.17	8.33 ± 0.32	76.14 ± 0.65	0.24 ± 0.04	1.26 ± 0.16
Bacteria, 1:1000	92.90 ± 13.17	8.17 ± 0.32	72.73 ± 0.65	0.26 ± 0.04	1.32 ± 0.16
Bacteria root injection	78.60 ± 13.17	8.00 ± 0.30	73.50 ± 0.78	0.24 ± 0.04	1.23 ± 0.16
Bacteria seed soaked	92.90 ± 13.17	8.5 ± 0.30	75.38 ± 0.78	0.26 ± 0.04	1.35 ± 0.16

The means and standard errors derived from one-way ANOVA

3.3.4 The vegetative characteristics of kale following addition of ALA

Soaking kale seeds in ALA solutions prior to sowing, resulted in two distinct trends which were visible after nearly 4 weeks from sowing; firstly, that of the lower ALA concentration having a beneficial effect on germination (albeit not significant, $p=0.17$) and on SPAD readings ($p=0.002$) (Figure 3.3, A and B). Secondly, the

higher concentration of ALA had a detrimental effect on shoot and root biomass (Figure 3.3, C and D).

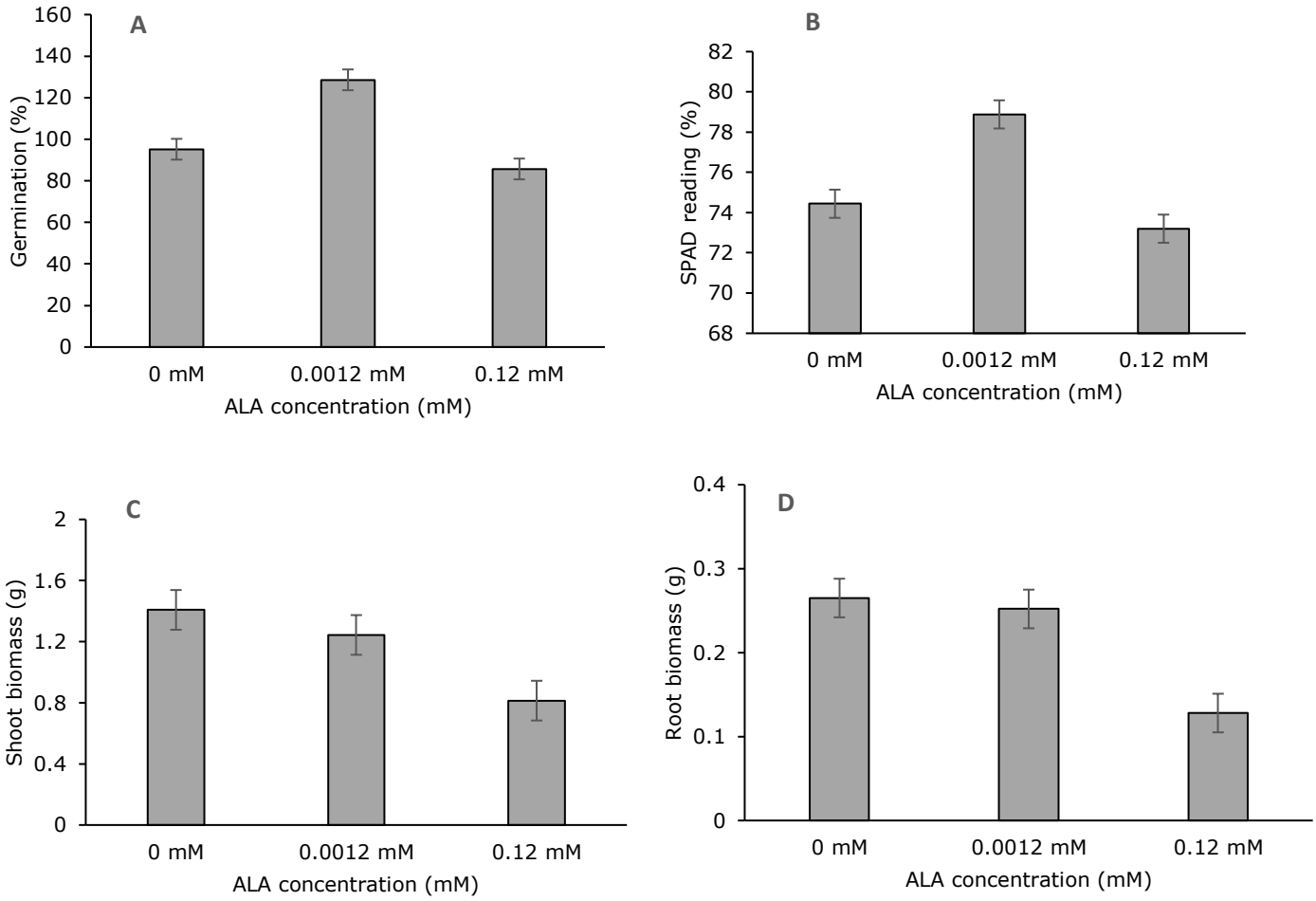

Figure 3.3. The effects of ALA seed priming treatments on: (A) per cent germination relative to the control (NS), (B) SPAD readings (p=0.002), (C) shoot biomass (p=0.039), (D) root biomass (p=0.023). Individual error bars are based on the pooled variance estimate from the one-way ANOVA with 6 degrees of freedom.

There was no significant effect of ALA on leaf number, although there were fewer leaves on the plants treated with the higher concentration (7 leaves vs 9 for the control and the low ALA concentration, p=0.07).

3.4 Discussion

3.4.1 *The effects of various dilutions of bacteria supernatant on vegetative characteristics of cress*

In the first cress experiment, only the 1:500 and 1:1000 dilutions of the culture medium containing bacterial metabolites resulted in an increase in plant biomass and percentage germination relative to the corresponding 'clean' medium of the same dilution. At higher concentrations, bacterial metabolites appeared to have a negative effect on cress growth relative to the corresponding dilution of 'clean' medium. This finding suggests that the bacterial metabolites in high concentrations hinder cress growth. Furthermore, even at lower concentrations, the bacterial medium also stunted the radicle length and percentage germination. This result is similar to that obtained by Kennedy and Stubbs (2007) who showed that 23-90% of the root of jointed goat grass (*Aegilops cylindrica*) was inhibited by inoculation with bacteria, although they were considering the effects of pseudomonads and not *Bacillus* spp. However, Banowetz *et al.* (2009) reported that *Pseudomonas fluorescens* supressed germination and plant growth and referred to the extracellular compound produced by the bacterium as 'Germination Arrest Factor' (GAF). Co-cultivation with bacteria reduced root length of *Arabidopsis thaliana* (Weise et al., 2013) and (Bender and Fink, 1998) also observed that secondary metabolites produced by bacteria have phyto-inhibitory effects.

Non-fluorescent *Pseudomonas* strains also produce toxic metabolites and inhibit wheat root growth (Fredrickson and Elliott, 1985). Kennedy and Stubbs (2007) proposed the idea that plant-suppressive bacteria may severely inhibit the growth and development of plants at crucial growth phases. It would appear that the

metabolites produced by the bacterial isolate used in the current study (*Bacillus paramycoides*) had some suppressive characteristics at higher concentrations. In contrast, the addition of low concentrations (1:500 and 1:1000 dilutions) improved seed germination and biomass of cress compared to the control. These results corroborate the concept proposed by Koh and Song (2007) that enhancement of seed germination is supported by bacterial metabolism, probably in connection with the mechanism of plant growth promotion.

According to Pfeilmeier et al., (2016) bacteria can act against plants by producing phytotoxic molecules. In addition, beneficial bacteria have a role in the growth enhancement of plants by producing phytohormones (Lynch, 1985) which are the chemical messengers that play a role in a wide spectrum of physiological and biochemical processes at very low concentrations (Han *et al.*, 2018). The metabolites generated here were not measured and whilst it can be surmised that ALA was produced by the bacterium (see Chapter 2), other compounds may also have been present. Nevertheless, ALA is known to be phytotoxic at high concentrations (>2 mM) and suppresses growth, whilst at low concentrations it can enhance growth (Hotta *et al.*, 1997b; Koh and Song, 2007). There is therefore a fine line between the beneficial effects of ALA and the phytotoxic properties of the compound.

In the second cress experiment, the effect of growing the bacterium in three different concentrations of LB broth was studied in order to explore how changes in nutrient availability affect production of secondary metabolites. The nutrient stress strategy was employed by Buitelaar and Tramper (1992) by changing the strength of the culture medium; it is known that nutrient concentrations in the culture medium affect synthesis of secondary metabolites (Ruiz *et al.*, 2010).

Furthermore, Pinu *et al*. (2017) found that environmental factors, like temperature, pH and nutrient levels influenced the cycle of uptake and secretion of metabolites in the culture medium.

In the current experiment, LB (Sigma) broth, which consist of tryptone (10 g L^{-1}), sodium chloride (5 g L^{-1}) and yeast extract (5 g L^{-1}) was used. The enhancement of germination index with addition of uninoculated LB in 1:100 and 1:1000 dilutions might be attributed to the yeast extract present in the medium; this is equally relevant to the findings in the first cress experiment. It has been previously shown that yeast extract improves seedling growth and soil fertility (Xi *et al.*, 2019). Whilst yeast extract is nutrient rich, the precursor of indole acetic acid is also present in yeast extract and plays an important role in plant development (Xi *et al.*, 2019). Mady (2009) reported that a foliar spray of yeast extract (50 mL L^{-1} yeast extract with 75 mL L^{-1} zinc) improved growth and yield of faba bean and Darwesh (2016) found that yeast extract increased the root length and number of leaves of date palm (*Phoenix dactylifera*). Furthermore, Nassar *et al*. (2016) demonstrated that *Leucaena* sp. was able to survive salinity stress following foliar spray of yeast extract.

In this study, the germination index after addition of the 1:100 dilution of bacterial growth medium originating from the half strength broth increased relative to the sterile water control and to the 'clean' broth control. The possible explanation for this might be that bacteria require a medium with lower nutrient concentrations to enforce metabolite production which gratuitously also assists seed germination. Seshasayee *et al.* (2006) reported that in regulating amino acid synthetic pathways, microorganisms grown with high nutrient concentrations prefer to import essential metabolites from the environment rather than expend

energy producing them. Since yeast extract consists of essential water-soluble vitamins, peptides, carbohydrates and amino acids necessary for growth, the full strength LA broth is highly nutritious.

The number of bacterial cells in the one third strength LB broth after 24 hours was greater than that in the ½ strength broth (Figure 3.1). Low nutrient concentrations might be only able to support bacterial growth and not metabolite production. Thus, bacteria cells can grow rapidly and produce the macromolecules required (e.g. proteins, lipids and carbohydrates) for proliferation (Li et al., 2014). Therefore, ALA production may have been minimal in the one third strength broth, was optimal in the ½ strength and too high in the full strength broth. ALA was not measured here, but the study bacterium, *Bacillus paramycoides*, is known to produce it (see Chapter 2) and it is known that high concentrations are phytotoxic. Furthermore, ALA was previously measured in the same uninoculated broth used here (Chapter 2) at a concentration of around 0.03 mM. That concentration is 4 times less than the higher ALA concentration applied as a seed dressing in water and 25 times more concentrated than the lower seed dressing used here. It is therefore difficult to separate out the effects of the medium from those of the bacterium.

3.4.2 *The effects of various dilutions of bacteria supernatant on vegetative characteristics of kale*

In this study, bacterial cells were allowed to remain in the medium and were used as a seed dressing prior to germination, or as a soil dressing where a small volume was injected into the soil as near to the root zone as possible. It the

bacterium has any potential as a plant growth promoting species, it should benefit plants growing in a soil not too dissimilar from the one from which it was isolated.

The treatment of soaking the seeds in 1:500 and 1:1000 dilutions of uninoculated medium enhanced the percentage germination relative to the control; the plants with the greatest shoot biomass were those treated with uninoculated 1:1000 broth dilution. The role of yeast extract in the medium might be the reason for this. Sampedro *et al.* (2004) demonstrated that application of 5% yeast extract on soybean improved initiation of the shoot, and increased shoot length and fresh and dry weight. Shafeek *et al.* (2015) found that spraying yeast extract on turnip leaves stimulated the plant length, chlorophyll content, number of leaves and total biomass. This corroborated Mady (2009) earlier work that showed that yeast extract has a role in chlorophyll formation in seedlings.

Priming the kale seeds with bacteria had no effect on any of the parameters measured. According to McQuilken *et al*. (1998) and Abuamsha *et al*. (2011), seed priming in some period builds favourable conditions for bacteria to imbibe and colonize the seed. Soaking the seed with *B. pumilus* INR7 improved the growth and number of leaves of cucumber (Wei *et al*., 1996). The improvement in root and shoot length, fresh and dry weight of root and shoot and leaf number of tea plants was the effect of seed priming with a consortium of four bacterial strains (Dutta and Thakur, 2017). It is likely that a consortium would be more beneficial than just one species. The bacterium used here (*Bacillus paramycoides*) may not have been suited to the soil in the experimental pots, or kale may not benefit from the bacterium. However, Turan *et al.* (2014) found that PRPR improved vegetative traits of another brassica, that of cabbage, including root and shoot fresh and dry weight, stem diameter, seedling length, chlorophyll

values and leaf area. Abeer *et al.* (2015), primed seeds with *Bacillus subtilis* which played a role in root and shoot development, in total lipid content, the phospholipid fraction and photosynthetic pigments (chlorophyll and carotenoid contents). The increase of chlorophyll content indirectly affects the ability of plants in alleviating stress by improving photosynthetic rate, actively converting carbon dioxide and water to glucose, which drives metabolism and ultimately enhances plant growth in stressful environment (Kang *et al.*, 2014).

The bacterial application method used (seed priming or soil injection) made no difference to the plant parameters measured. In contrast, Madhaiyan *et al.* (2013) confirmed that seeds soaked with *Enterobacter* sp. R4-368 increased the agronomic benefits of Jatropha, possibly because pre-soaking enables bacteria to colonise the seed, thereby benefiting growth. In addition, bacteria produce metabolites, such as amino acids and organic acids which have a role as a nutrient supplement for plants, stimulating beneficial indigenous bacteria and eventually promoting plant growth (Koh and Song, 2007). Inoculation with bacteria having the ability to produce 5-aminolevulinic acid (ALA) resulted in growth improvements in different plant species (Hotta *et al.*, 1997[b]). The bacterium used in this current experiment was capable of producing ALA under laboratory conditions, although this was not demonstrated or measured during the growth experiments. What is not known, is whether the bacterium is ineffective, or whether it produces sufficiently high concentrations of ALA to be phytotoxic, or at least not beneficial. Furthermore, the commercial ALA sold as a growth promotor (Pentakeep) also contains macro- and micro-nutrients, therefore it is unclear whether the benefits attributed to ALA are solely due to the actions of that compound. The remainder of this thesis therefore focuses on ALA, since before pushing forward with developing a bacterium that produces ALA in the

rhizosphere, it would be beneficial to understand whether this is, in reality, a useful strategy.

3.4.3 *The effects of ALA as a seed dressing on the vegetative characteristics of kale*

ALA application at the lower concentration (0.0012 mM) significantly increased the SPAD readings obtained, suggesting that chlorophyll in the leaves of the growing plant was positively affected by the seed dressing. This corroborates the work of Wu *et al.* (2018) who found that ALA enhanced chlorophyll biosynthesis in cucumber. ALA improved seed germination of pakchoi at low concentrations (Wang *et al.*, 2005) and whilst there was an apparent increase in percentage germination with the 0.0012 mM ALA seed dressing in this experiment, it was not significant. The high concentration of ALA had detrimental effects on shoot and root biomass, possibly because of oxidative stress (Balestrasse *et al.*, 2010). The effects of ALA on pant species are considered in more detail throughout the rest of the thesis.

3.5 Conclusion

The lack of response of either cress or kale to the bacterial metabolites or to the bacterium is disappointing, but perhaps not unsurprising. Whilst the bacterium produced ALA under controlled culture conditions (Chapter 2), more stressful environments (e.g. in Petri dishes or soil) may not be conducive to ALA production. Furthermore, it is clear that the benefits or otherwise of ALA need to be understood in more detail before one should utilise ALA producing bacteria in the field.

4 THE RESPONSE OF CRESS (*LEPIDIUM SATIVUM*), KALE (*BRASSICA OLERACEA* VAR*. ACEPHALA*) AND RADISH (*RAPHANUS SATIVUS*) TO DIFFERENT APPLICATION METHODS AND CONCENTRATIONS OF ALA

4.1 Introduction

Besides promoting seed germination, ALA also affects plant growth (Wu *et al.*, 2018); according to Shao *et al.* (2009) and Liu *et al.* (2018), ALA is a precursor of chlorophyll biosynthesis and is directly involved in photosynthesis and enhances accumulation and transformation of organic matter. So far, published studies using ALA as a fertiliser (either as a pure compound or as Pentakeep), have use various modes of application, such as foliar spray, pre-soaking seeds, or as a rooting medium (Tanaka *et al.*, 2005; Babik and Babik, 2007; Akram and Ashraf, 2013).

According to Memon *et al.* (2009), foliar application affected the yield of pakchoi through enhanced chloroplast pigments, photosynthesis and antioxidant actions. Others also reported that foliar application of ALA increased the concentration of photosynthetic pigments in leaves and furthered photosynthetic activity (Smoleń et al., 2010). Foliar spray of ALA in kudzu plants significantly improved plant biomass, chlorophyll contents, leaf nitrogen, soluble sugar, phosphorus, potassium, calcium, starch and vitamin C contents (Xu *et al.*, 2010). Akram and Ashraf (2013) suggested that ALA treatment as a foliar spray is better applied at initial growth stages because chlorophyll formation is more active during the seedling phase.

In contrast to foliar application, Roy and Vivekanandan (1998) demonstrated that pre-soaking seeds with 10 mg L^{-1} of ALA increase plant growth, dry matter

accumulation and chlorophyll content of three *Vigna* species (*Vigna catjung*, *V. mungo* and *V. radiata*). Seed soaking with ALA also increased seed germination, plant growth and yield and improved antioxidant metabolism under both abiotic stressed and non-stressed conditions (Wang *et al.*, 2005; Korkmaz and Korkmaz, 2009; Korkmaz *et al*., 2010).

A third method of applying ALA is directly to the growing medium. Soil application or rooting medium application has been reported to enhance growth, accumulation of chlorophyll and increased photosystem I and II activities of *Spirulina platensis* under normal growth conditions (Sasaki *et a*l., 1995). However, addition of ALA to the plant growing medium might not economically feasible due to the large quantities needed (Akram and Ashraf, 2013).

Previous research findings showed that the promotive or inhibitory effect of ALA was greatly dependent on the concentration applied (Hotta *et al.*, 1997[a]). Chon (2003) stated that a low level of ALA promoted plant growth, but significantly inhibited it when higher concentrations were applied. The study by Kuk *et al. (*2003) showed that ALA promoted growth of barley at low concentrations (0.05-0.5 mM) but damaged the plants at relatively high ALA concentrations (5, 10 mM).

ALA solutions become more acidic as the concentration of ALA added to water increases (personal observation). None of the papers published to date mentioned any pH adjustment made to ALA solutions in order to differentiate between the effects of pH and those of the ALA. The fact that ALA is a water-soluble compound does not lessen the importance of knowing the effective pH of ALA for large-scale application (Akram and Ashraf, 2013). Moreover, no previous study has directly compared ALA application method on different plant species.

Therefore, the present study was conducted to compare ALA solutions (± an adjustment to pH 7) at a range of concentrations on the vegetative growth of cress, kale and radish plants. Cress (*Lepidium sativum*), radish (*Raphanus sativus*) and kale (*Brassica oleracea*) are all members of the *Brassicaceae* and are fast growing crops usually harvested 25-30 days after sowing.

The specific objectives of this Chapter were:

i) To evaluate the effect of different concentrations of ALA solutions and pH on early stage growth of cress and radish.

ii) To compare seed soaking and foliar spraying application methods of ALA solutions on vegetative growth and biomass production of kale, radish and cress.

The key hypotheses were:

1) ALA solutions will enhance plant growth.
2) Foliar spray application will be more effective in enhancing growth than the seed dressing.

4.2 Materials and Methods

4.2.1 Experimental approach

Two experiments were carried out; the first, was a Petri dish-based trial to test a range of ALA concentrations (0.00012 mM, 0.0012 mM, 0.012 mM, 0.12 mM, 1.2 mM) at the 'natural' solution pH and at pH 7.0 on cress and radish seedling growth. This was designed to determine the optimum ALA concentrations and pH to use in the second experiment, which was a pot trial using ALA (0.0012 mM, 0.012 mM, and 0.12 mM) either applied as a seed soak or as a foliar spray to

kale, radish and cress plants. The ALA used was 5-aminolevulinic acid hydrochloride (Merck) which necessitated the pH test.

4.2.2 Seed preparation for germination and growth in Petri dishes

Cress and radish seeds were disinfected with 90% ethanol and washed twice with sterile deionised water. After this, 10 cress seeds and six radish seeds were placed on filter paper in Petri dish and 5 mL of one of the following solutions was used to impregnate the filter paper: (i) Ultrapure (MilliQ) water; (ii) ALA, 0.00012 mM; (iii) ALA, 0.0012 mM; (iv) ALA, 0.012 mM; (v) ALA, 0.12 mM; (vi) ALA, 1.2 mM. These ALA dilutions were then divided into two sets of solutions. One set was pH adjusted to pH 7.0 using NaOH, whilst the other set remained unadjusted. The pH of each solution was measured using a pH meter (model Hanna pH-209 with a combined glass electrode (Ag/AgCl; PHE 1400) calibrated against pH 4.01 and 7.0 buffer solutions.

There were three replicate Petri dishes for cress (each Petri dish containing 10 seeds) and five replicate Petri dishes for radish (with five seeds per dish). Petri dishes were sealed with Parafilm tape to prevent evaporation and kept in a dark growth room for 2 days at 20°C until germination began and were then moved to a growth room with 16/8 hour day/night regime at the same temperature. Seedlings were harvested once the plumule was visible and quite advanced with cotyledons. Seedlings were processed as described in Section 3.2.1.5.

4.2.3 The effects of ALA on the growth of kale, radish and cress

4.2.3.1 Seed and foliar ALA treatments

ALA was applied by either seed soaking or foliar spraying treatments. For the pre-soaking seed treatment, seeds of kale, radish and cress were soaked for 24 h in aqueous solutions of ALA (5-aminolevulinic acid hydrochloride 98%, Sigma) at a concentration of 0.0012 mM, 0.012 mM or 0.12 mM. No pH adjustments were made to these solutions. Seeds for both the control treatment and those which would later receive a foliar application of ALA, were soaked in deionized water.

For plants receiving the foliar spray treatment, ALA aqueous solutions of 0, 0.0012 mM, 0.012 mM, 0.12 mM were sprayed at the 3-4 leaf stage (15 days after sowing). This was done by gently spraying the upper surfaces of all leaves until a light complete coverage was achieved using an airbrush (Timbertech). No pH adjustment was made to these solutions.

4.2.3.2 Experimental set-up

Three seeds of each treatment (described in Section 4.2.3.1) were sown into replicate ($\times$ 6) 9 cm plant pot containing Levington's M3 pot and bedding compost, which is peat based with a small amount of coir. The pH of this compost was 5.7. Plants were grown in automated day-night temperature-controlled growth room with 16 h light and 6 h dark with 1 h of dusk and 1 h of dawn at a day/night temperature of 20°C and 17°C respectively.

Pots were arranged in a completely randomised design. On the tenth day after sowing, over 90% germination was observed. The plants were then thinned to one germinated plant per pot.

Plants were harvested 28 days after sowing. Before harvesting, number of leaves and petioles were counted and SPAD measurements were taken (as described in Section 3.2.2.3) on days 18, 22 and 25 days for plants subjected to foliar treatment and 18 days after sowing for those whose seeds were soaked in ALA solutions prior to sowing. After harvesting, all shoots were removed and put into paper bags for drying. The roots were extracted from the soil, washed thoroughly with tap water and put into paper bags. Plant material was dried at 35°C until constant weight was recorded.

4.2.4 Statistical Analyses

Statistical analyses were performed using GenStat version 19.1 (VSN International, Hemel Hempstead, UK). In the first experiment, an unbalanced ANOVA (because of the different number of replicate Petri dishes, three for cress and five for radish) was conducted on percentage germination, root length, germination index and dry weight with plant (cress or radish), ALA dilution (0.00012 mM; 0.0012 mM; 0.012 mM; 0.12 mM; 1.2 mM), acid treatment (adjusted to 7 or not adjusted) as the factors.

For the second experiment, a repeated measures ANOVA was conducted on SPAD readings taken at 18, 22, 25 after foliar treatment, with plant species (kale, radish, cress) and ALA dilution (0, 0.0012 mM; 0.012 mM; 0.12 mM) as the main factors. A two-way ANOVA was conducted on the SPAD readings taken from the

three plant species 18 days after sowing where plant species (kale, radish, cress) and ALA dilution (0, 0.0012 mM; 0.012 mM; 0.12 mM) were the main factors.

Root and shoot biomass and number of leaves were analysed by ANOVA where plant species (kale, radish or cress), ALA treatment/application type (foliar or seed), ALA dilution (0.0012 mM; 0.012 mM; 0.12 mM) were the main factors. Normality was tested by plotting residuals against expected normal quantities and post hoc comparisons between means were based on least significant differences (LSD) at the 0.05 probability level where appropriate.

4.3 Results

4.3.1 The growth response of cress and radish to a range of ALA concentrations; Petri dish experiment

Data for seedling root length, percentage germination and dry weight of cress and radish (radical and plumule) were expressed as a percentage of the control (sterile water treatment) values (Table 4.1). With regard to cress, higher values for each parameter were generally obtained when seeds were grown with the lower ALA concentrations in which pH was unadjusted. The effects of ALA on radish were less marked than on cress, although the lower concentrations of ALA (unadjusted pH) increased root length relative to the pH amended solutions or the control.

Plant species × pH and plant species × ALA concentration were significant interaction effects for each parameter measured (Table 4.2), except for % germination where ALA addition had no effect.

The germination index (GI) was increased in radish by low concentrations of ALA (unadjusted pH) and reflect the increased root length observed in those treatments (Figure 4.1). The cress GI spiked with the 0.012 mM ALA treatment (unadjusted pH), likely due to the seedling root length (Table 4.1).

Table 4.1. Effects of ALA solutions on three plant growth indicators.

Plant	Dilution (mM)	pH	Root length (%)	Germination (%)	Total biomass (%)
Cress	0.00012	7	82.8	100.0	90.8
	0.0012	7	87.9	96.6	85.4
	0.012	7	82.9	89.7	88.6
	0.12	7	43.1	100.0	95.0
	1.2	7	23.8	100.0	69.2
	0.00012	5.5	112.8	111.5	100.5
	0.0012	4.79	95.5	111.5	115.4
	0.012	4.4	150.8	111.5	124.1
	0.12	3.69	83.6	100.0	87.6
	1.2	3.24	66.2	115.4	93.0
Radish	0.00012	7	116.6	107.1	94.1
	0.0012	7	103.2	103.6	91.9
	0.012	7	93.8	103.6	88.6
	0.12	7	63.8	103.6	84.4
	1.2	7	12.6	107.1	65.8
	0.00012	5.5	119.1	96.7	97.7
	0.0012	4.79	133.1	96.7	101.5
	0.012	4.4	110.8	96.7	97.3
	0.12	3.69	70.0	100.0	86.3
	1.2	3.24	28.8	96.7	67.8

N.B. These data are expressed as a percentage of the control plants (i.e. those grown with deionised water) which is why some values are greater than 100%.

Different ALA dosages enhanced the GI and root length of cress and radish. Based on root length, germination index and biomass, ALA solutions at the concentration of 0.012 mM significantly promoted cress growth, while radish grew better when amended with 0.0012 mM (unadjusted pH).

Overall, the results indicated that the unadjusted solution pH values were preferable to those of the pH adjusted solutions in promoting plant growth. Thus, the best concentration of ALA (unadjusted pH) for radish was 0.0012 mM and for

cress, 0.012 mM. Therefore, these ALA concentrations were selected for the following pot experiment. However, to allow for possible ALA adsorption within the soil the higher concentration was also used (0.12 mM).

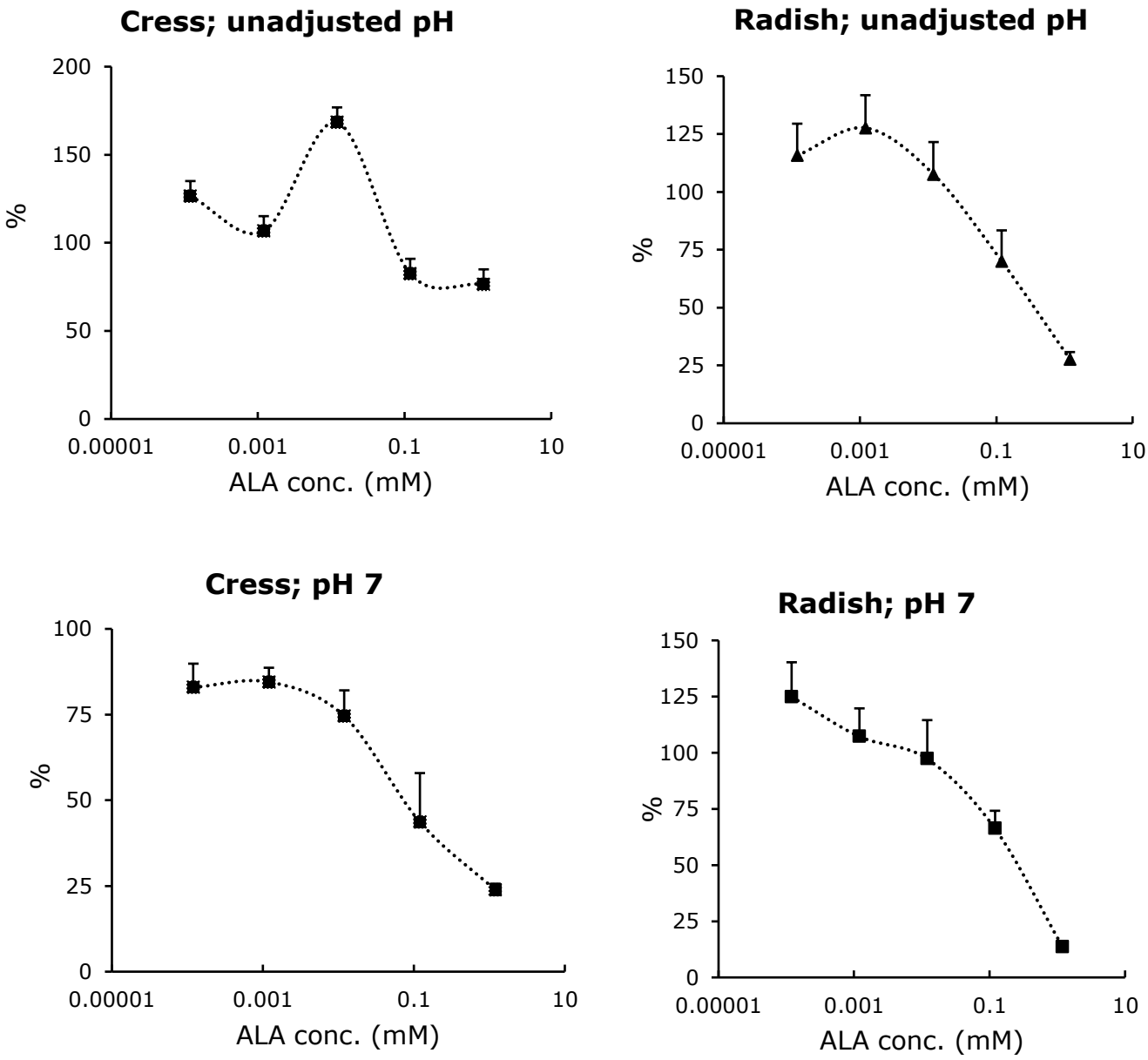

Figure 4.2. The effects of a range of ALA concentrations with or without solution pH amendment on the germination index of cress and radish. LSD for cress = 31.80 and radish = 24.63. The individual error bars are based on pooled variance estimate from the ANOVA with 60 degrees of freedom.

Table 4.2. ANOVA output for the plant growth parameters measured for cress and radish when grown in Petri dishes with ALA solutions (pH amended or unamended).

Variate	Factor	DF	F	P VALUE	*LSD (95%)
Percentage germination	Plant	1	2.38	0.128	3.18
	ALA conc	4	0.84	0.506	4.87
	Acid	1	0.00	0.996	3.08
	Plant.ALA conc.	4	0.73	0.572	7.95 (min) 7.11 (max-min) 6.16 (max)
	Plant.pH	1	41.22	<0.001	5.03 (min) 4.50 (max-min) 3.90 (max)
	ALA conc.pH	4	0.58	0.681	6.89
	Plant.ALA conc.pH	4	1.92	0.119	11.25 (min) 10.06 (max-min) 8.71 (max)
	Residual	60			
Root length	Plant	1	0.30	0.584	8.1§
	ALA conc	4	65.38	<0.001	12.52
	Acid	1	34.16	<0.001	7.92
	Plant.ALA conc.	4	5.66	<0.001	20.44 (min) 18.28 (max-min) 15.83 (max)
	Plant.pH	1	8.12	0.006	12.93 (min) 11.56 (max-min) 10.01 (max)
	ALA conc.pH	4	0.96	0.434	17.70
	Plant.ALA conc.pH	4	2.24	0.076	28.91 (min) 25.86(max-min) 22.39 (max)
	Residual	60			
Germination Index	Plant	1	0.07	0.794	8.99
	ALA conc	4	55.34	<0.001	13.77
	Acid	1	29.79	<0.001	8.71
	Plant.ALA conc.	4	4.87	0.002	22.49 (min) 20.11 (max-min) 17.42 (max)

Variate	Factor	DF	F	P VALUE	*LSD (95%)
	Plant.pH	1	22.03	<0.001	14.22 (min) 12.72 (max-min) 11.02 (max)
	ALA conc.pH	4	1.51	0.212	19.47
	Plant.ALA conc.pH	4	2.18	0.082	31.80 (min) 28.44 (max-min) 24.63 (max)
	Residual	60			
Dry weight	Plant	1	1.15	0.287	5.30
	ALA conc	4	14.99	<0.001	8.38
	pH	1	7.33	0.009	5.30
	Plant.ALA conc.	4	2.00	0.105	11.85
	Plant.pH	1	17.65	<0.001	7.50
	ALA conc.pH	4	4.29	0.004	11.85
	Plant.ALA conc.pH	4	0.79	0.536	16.76
	Residual	60			

*****LSDs – The min./max.-min./max. values relate to the LSD which should be used for comparisons of mean values (Table 4.1 and Figure 4.1). When comparing between means with the lowest number of replicates, the min. value should be used (i.e. when comparing between cress treatments, 3 replicates); when comparing between means with the highest number of replicates the max. value should be used (i.e. when comparing between radish treatments, 5 replicates); when comparing between radish and cress the max.-min. value should be used.

4.3.2 Growth parameters of kale, radish and cress following the addition of ALA solutions; soil grown

4.3.2.1 SPAD readings for kale, radish and cress

The ALA foliar treatments affected the plant species differently, with cress responding more positively than kale or radish (Figure 4.2). For clarity, the

ANOVA output corresponding to just the ALA dilution effect for each species on SPAD reading days is shown (Table 4.3). ALA (0.012 mM) increased the SPAD reading on day 22 (three days after treatment) relative to the control ($p=0.017$), but by day 25 this was no longer evident. Instead, the highest ALA concentration appeared to result in the highest SPAD value although this was only weakly significant ($p=0.059$). Despite the apparently negative effects of ALA foliar treatments on the radish, these were not significant. In contrast, the 0.0012 mM ALA treatment significantly increased the SPAD readings on days 22 ($p<0.001$) and 25 ($p=0.021$) relative to the water control treatment.

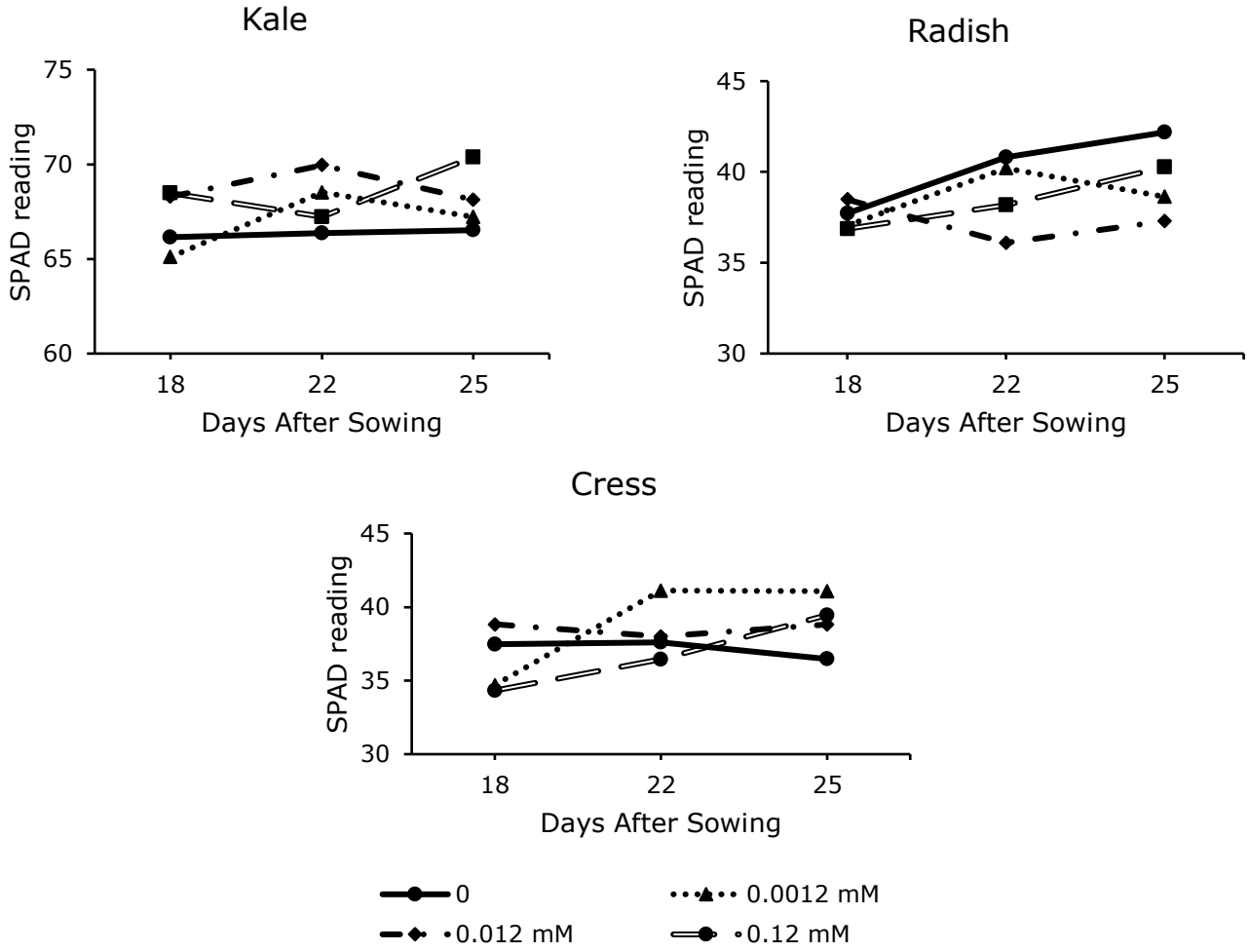

Figure 4.2. The SPAD readings for kale, radish and cress on days 18, 22 and 25 days after sowing. Foliar ALA was applied on day 15. Please refer to Table 4.2 for the corresponding LSD values.

SPAD readings were only taken on day 18 for plants subjected to the ALA seed-soaking treatments. As with the foliar treatments, only the cress plants significantly (p=0.020) responded to the ALA amendment where lower SPAD values were obtained from plants when seeds were treated with the highest ALA doses (Figure 4.3). The 0.012 mM seed treatment appeared to increase mean SPAD values for radish, although this was not significant (p=0.068).

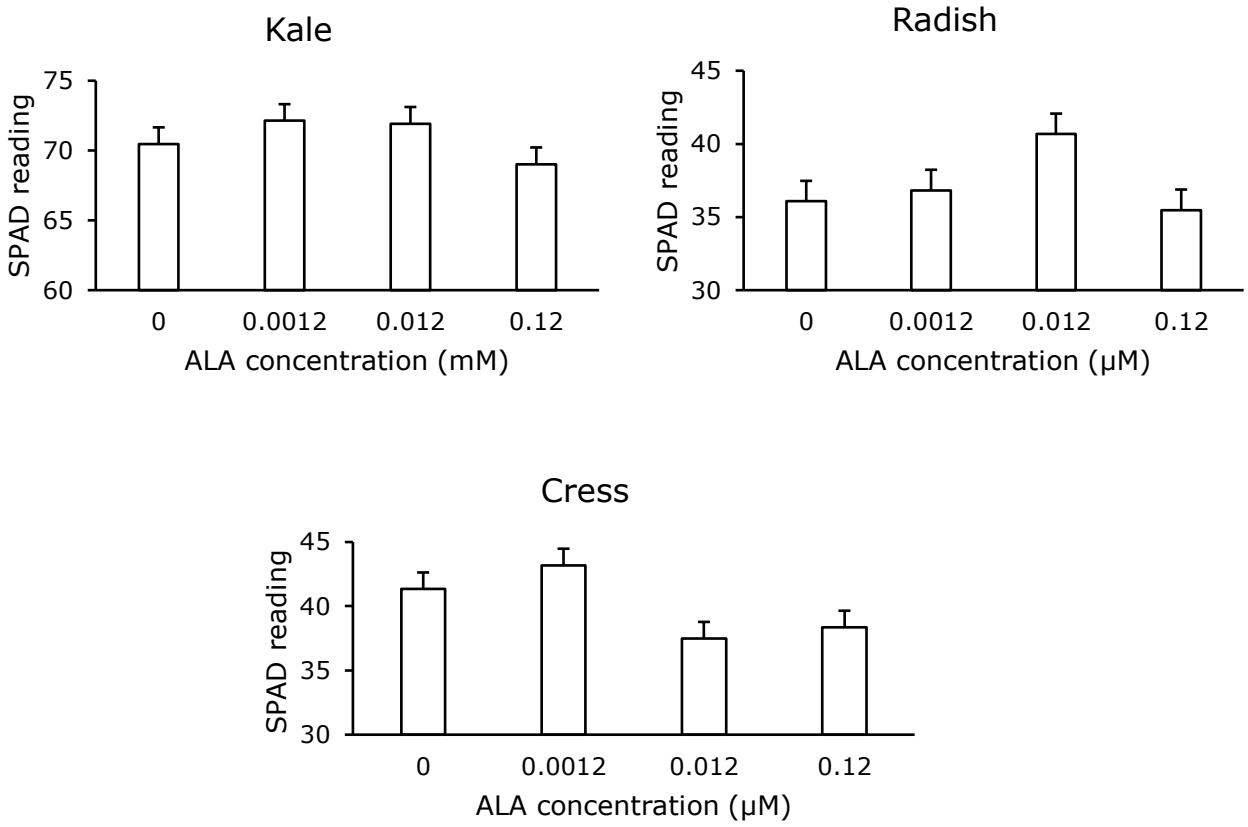

Figure 4.3. The SPAD readings for kale, radish and cress on day 18 days after sowing. ALA was applied as a seed dressing prior to sowing. Please refer to Table 4.2 for the corresponding LSD values.

Table 4.3. Summary of the ANOVA output corresponding to just the ALA dilution effect for each plant species on SPAD reading days.

Plant	Treatment	Days After Sowing	F	P value	LSD (95%)
Kale	**Foliar**	18	1.87	0.168	3.56
		22	4.27	0.017	2.23
		25	2.92	0.059	2.90
	Seed	18	1.47	0.252	3.51
Radish	**Foliar**	18	0.35	0.789	3.65
		22	2.00	0.146	4.45
		25	2.17	0.124	4.24
	Seed	18	2.78	0.068	4.13
Cress	**Foliar**	18	2.34	0.104	4.20
		22	11.18	<0.001	1.76
		25	4.07	0.021	2.80
	Seed	18	4.12	0.020	3.84

4.3.2.2 Vegetative growth of kale, radish and cress

The most notable treatment effect relating to plant root biomass was that of the ALA seed-soaking treatment resulting in reduced root growth relative to the foliar sprays (Figure 4.4). This effect was significant for kale, but not for radish and marginally for cress (Table 4.4). Nevertheless, this was a consistent trend across all three species. What is interesting is the difference between the seed and foliar control treatments since plants were treated similarly until day 15 when the foliar spray (water) was applied. This difference is consistent across all three plant species.

The foliar ALA treatments did not significantly affect root biomass. However, the highest concentration of ALA (0.12 mM) applied as a seed-dressing appeared to slightly lower kale root biomass and the 0.012 mM treatment increased radish root biomass relative to the corresponding control and the 0.0012 mM treatment.

Seed treatment did not affect cress root growth. Cress shoot biomass was similarly unaffected by either foliar or seed ALA amendments, although there was a significant reduction in shoot biomass in seed treated plants compared to those receiving foliar treatments. This trend was evident across all species, but this was ALA concentration-dependent for radish (Figure 4.5).

Foliar application of the two higher ALA doses significantly decreased kale shoot biomass relative to the control and the 0.0012 mM treatment whilst the highest dose (0.12 mM) applied as a seed-dressing lowered shoot biomass. In contrast, the two lower ALA concentrations increased kale shoot biomass (Figure 4.5; Table 4.4). The effects of ALA were greater on the kale shoot on the root.

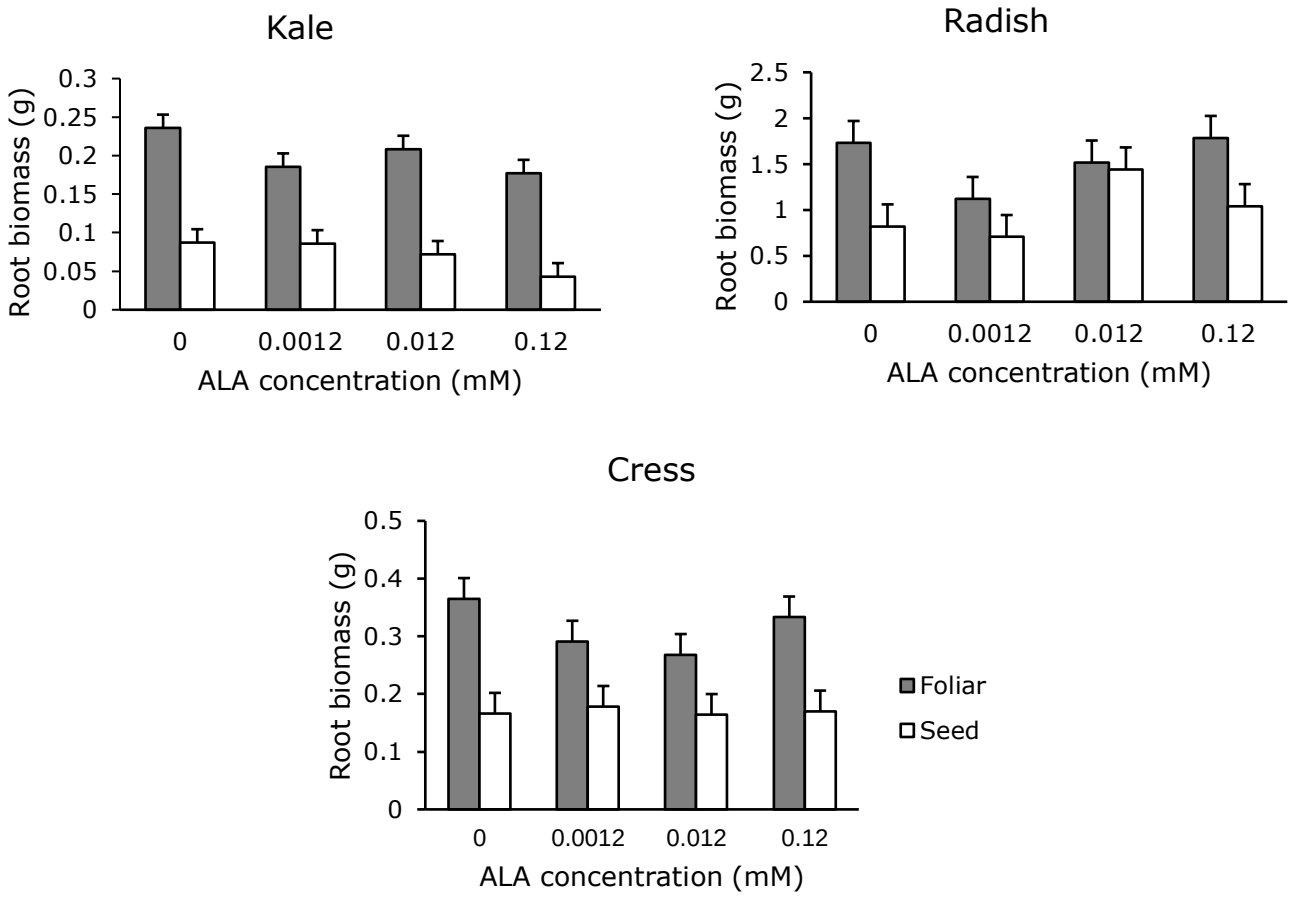

Figure 4.4. The root biomass of kale, radish and cress plants grown with a range of ALA amendments applied as a foliar spray or a seed dressing. The individual error bars are based on pooled variance estimate from the ANOVA with 48 degrees of freedom.

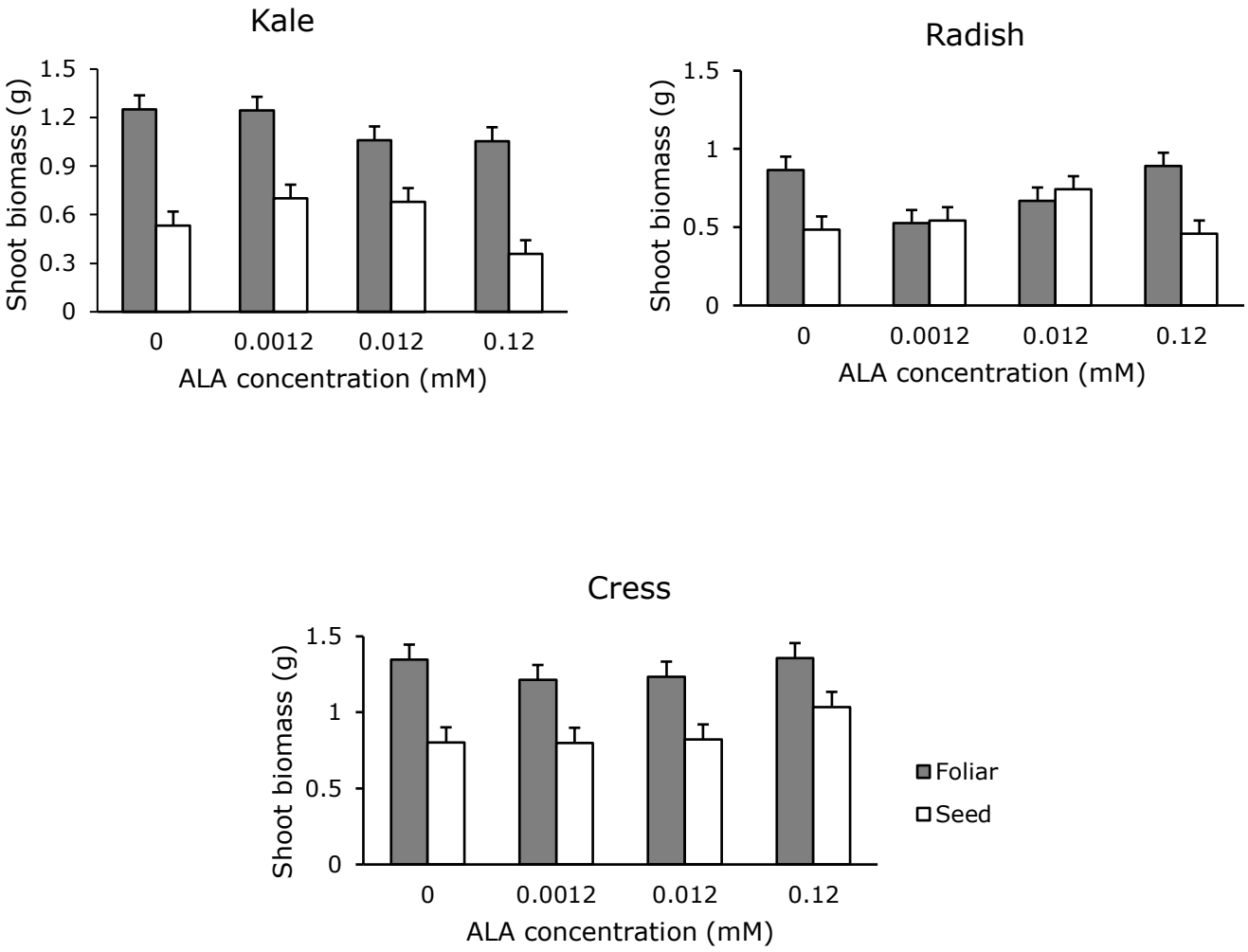

Figure 4.5. The shoot biomass of kale, radish and cress plants grown with a range of ALA amendments applied as a foliar spray or a seed dressing. The individual error bars are based on pooled variance estimate from the ANOVA with 48 degrees of freedom.

Leaf number was largely unaffected by ALA application, although for both kale and cress, fewer leaves were evident on plants subjected to the seed soaking treatment than the foliar spray (Figure 4.6; Table 4.4). Leaf number on kale plants subjected to the seed soaking treatment was increased in plants treated with the 0.0012 mM and the 0.012 mM ALA seed dressing.

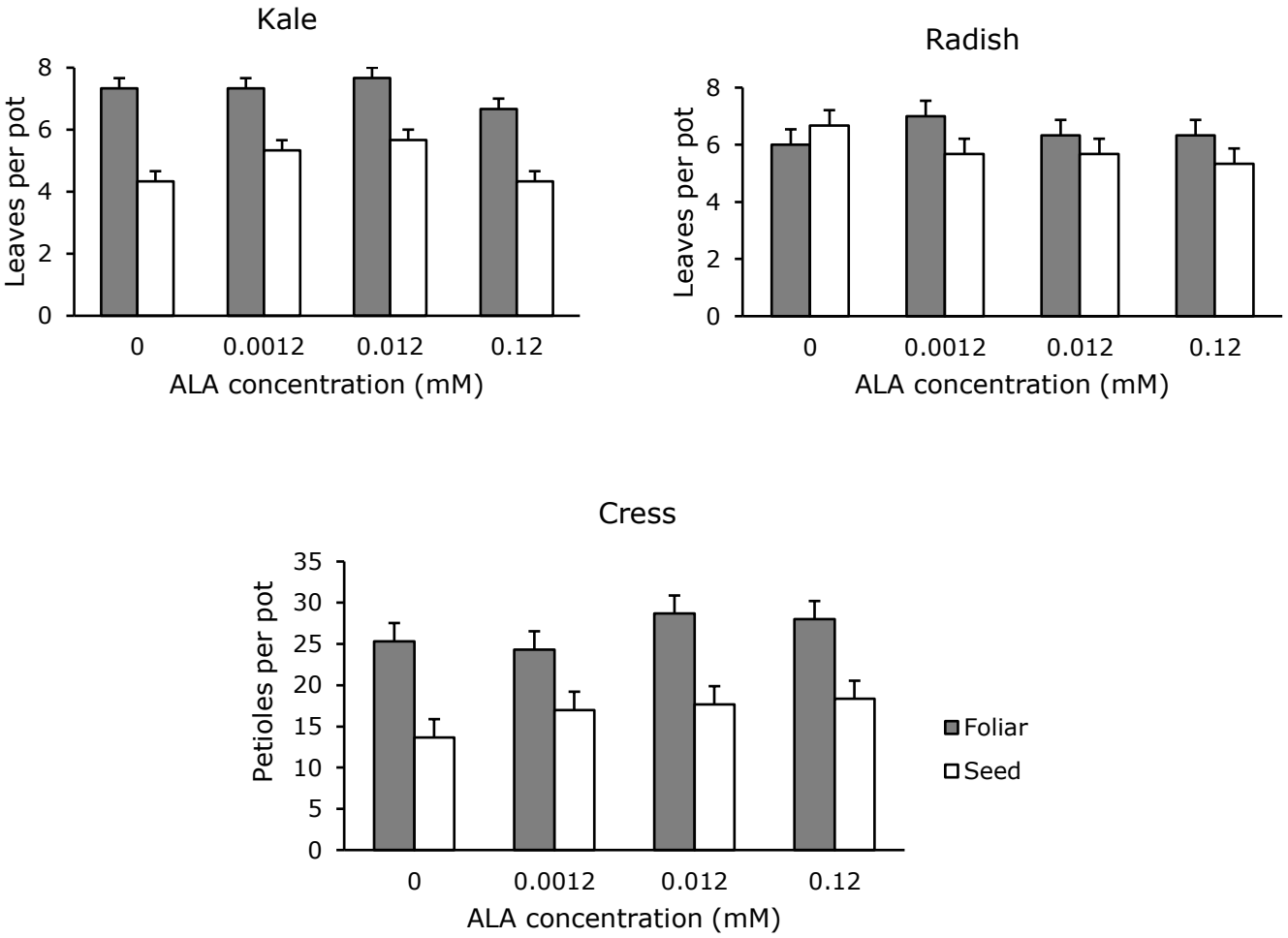

Figure 4.6. The number of leaves on kale and radish and number of petioles on cress plants when grown with a range of ALA amendments applied as a foliar spray or a seed dressing. The individual error bars are based on pooled variance estimate from the ANOVA with 48 degrees of freedom.

Table 4.4. Summary of 2-factor ANOVA of kale, radish and cress growth

Variate	Factor	DF	F	P VALUE	LSD (95%)
Root biomass	Plant	2	160.64	<0.001	0.140
	Treatment	1	22.32	<0.001	0.115
	Dilution	3	2.13	0.109	0.162
	Plant.Treatment	2	5.41	0.008	0.199
	Plant.Dilution	6	2.26	0.054	0.281
	Treatment.Dilution	3	1.51	0.225	0.230
	Plant.Treatment.Dilution	6	1.02	0.425	0.398
	Residual	48			
	Total	71			
Shoot biomass	Plant	2	45.63	<0.001	0.090
	Treatment	1	116.96	<0.001	0.074
	Dilution	3	0.25	0.861	0.104
	Plant.Treatment	2	10.23	<0.001	0.128
	Plant.Dilution	6	3.04	0.013	0.181
	Treatment.Dilution	3	3.84	0.015	0.147
	Plant.Treatment.Dilution	6	1.25	0.299	0.256
	Residual	48			
	Total	71			
Number of leaves	Plant	2	365.15	<0.001	1.333
	Treatment	1	62.41	<0.001	1.089
	Dilution	3	1.19	0.325	1.540
	Plant.Treatment	2	27.98	<0.001	1.886
	Plant.Dilution	6	1.53	0.190	2.667
	Treatment.Dilution	3	0.21	0.886	2.177
	Plant.Treatment.Dilution	6	0.55	0.765	3.771
	Residual	48			
	Total	71			

4.4 Discussion

4.4.1 The growth response of cress and radish to a range of ALA concentrations; Petri dish experiment

In the present study, application of ALA promoted % germination of cress and to a lesser extent of radish seeds. ALA accelerated seed germination and increased root length, which resulted in a higher germination index and biomass at the concentrations of 0.00012-0.012 mM for cress and 0.0012 mM for radish. These effects are in accordance with those of Kanto *et al.* (2015) and Sheteiwy *et al.*

(2017) who demonstrated increased seed germination and seedling growth after seed priming with ALA. Wu *et al.* (2018) reported that ALA, as a plant growth regulator, promoted seed germination in a dose-dependent manner. This growth promotion is related to the role of ALA as a biosynthetic precursor of chlorophyll; (Hotta *et al.*, 1997[a]) stated it increases CO_2 fixation in light and inhibits respiration of CO_2 in the dark.

Phour and Sindhu (2018) stated that ALA promotes plant growth at low concentrations (0.01-10 mg L^{-1}), whereas at high concentrations (>2 M), ALA suppresses plant growth. The data here support this since seedlings of both species were sensitive to the higher concentrations of ALA (0.12-1.2 mM). ALA application at the concentration of 1.2 mM retarded root growth and germination vigour of cress and radish seedlings by up to 80-90%, thus decreasing seedling biomass. These results showed that the treatment of high concentration of ALA stunted the plants. These data also corroborate those of Fu *et al.* (2014) who reported that germination was inhibited by 25 mg L^{-1} of ALA and of Chon (2003) who found that germination of Chinese cabbage (*Brassica rapa*) was inhibited by 0.5 mM ALA in the germination medium. The application of ALA at high concentrations changed the function of ALA in agricultural production from a plant growth promotor to a biodegradable photodynamic herbicide (Duke *et al.*, 1994).

Hotta *et al.* (1997[a] and 1997[b]) described the mechanism of photodynamic herbicidal activity of ALA whereby high concentrations cause increased endogenous ALA which leads to over-accumulation of several chlorophyll intermediates, such as protochlorophyllide and protoporphyrin IX. Following the treatments of ALA in darkness, ALA promotes the greening of etiolated plants and when the green plants are exposed to light, these excess chlorophyll

intermediates are photosynthesized and trigger photodynamic reactions which damage the plants and ultimately lead to plant death.

The pH of ALA was amended to pH 7.0 to determine if negative effects at higher concentrations were an indirect effect of solutions becoming increasingly acidic with larger amounts of ALA in solution. However, most of the growth of both plants was inhibited following the treatment of ALA at pH 7.0. The instability of ALA at alkaline pH might be the reason. According to Tewari and Eggleston (2018), ALA under neutral conditions is unstable. At alkaline pH, ALA undergoes dimerization to form 2,5-dicarboxyethyl-3,6-dihydroprazine (DHPY), which oxidises and forms 2,5-dicarboxyethylprazine (PY). In this study, cress and radish at the seedling stage were tolerant to low pH values of ALA which significantly improved the plant growth. This finding is consistent with that of Bunke *et al.* (2000) who found that ALA is more stable at low pH values in aqueous solutions (pH ≤ 5.5).

Seed germination directly affects plant growth and development, which in turn affect the yield. Crop yield is therefore influenced by seed quality, emergence percentage and time (Ellis, 1992). Plant growth promotion in this experiment was better when ALA solutions had not been adjusted to pH 7. Moreover, a significant relationship between ALA dilution and pH was noted for biomass, indicating that these factors affected the growth of cress and radish. The growth inhibition of cress and radish by the application of ALA in pH 7 impacted on the reduction of the biomass. The likely instability of ALA at neutral pH and higher concentrations of ALA disrupted the plant physiology and reduced plant biomass. These results are agreement with those of Bohnert et al. (1995), who suggested that under severe stress, a plant adapts its metabolism and alters its development.

The growth response curve for ALA on the early stages of cress and radish determined that the optimum dosage for cress was 0.012 mM and radish was 0.0012 mM and this information was used to inform the next experiment. However, because the ALA adsorption/buffering capacity of the soil used in the pot experiment was unknown, the higher concentration of ALA was also used.

4.4.2 Effects of ALA solutions on the growth parameters of kale, radish and cress

The effect of ALA on kale, radish and cress plants was evaluated by considering the plant growth parameters, including chlorophyll content, dry shoots and roots weight, number of leaves and petioles.

4.4.2.1 Effects of ALA solutions on SPAD values

Chlorophyll plays a key role in photosynthesis as it absorbs light (in many cases sunlight), and uses light energy, along with carbon dioxide and water to produce carbohydrates for the plant. It is well known that ALA has a role in chlorophyll biosynthesis and plant greening in higher plants (Beale, 1990) and ALA application increases chlorophyll accumulation (Kittsteiner et al., 1991). The accumulation of endogenous hormones in plants increased following ALA application, which effectively enhanced chlorophyll and nutrient concentrations (Memon *et al.*, 2009; Anwar *et al.*, 2018). Liu *et al.* (2018) demonstrated the relationship between increased chlorophyll content and enhanced photosynthesis following ALA application.

In the current study, SPAD values acted as a proxy for chlorophyll content. This proxy measurement of leaf chlorophyll content of kale, radish and cress plants was carried out using a Soil-Plant Analysis Development (SPAD) meter; a rapid, inexpensive and non-destructive device with a value range of 0 – 99. According to Xiong *et al.* (2015), there is a close link between SPAD readings and chlorophyll content per leaf area in both monocotyledonous and dicotyledonous plants. The bigger the SPAD value, the higher the concentration of chlorophyll of the plant, and therefore photosynthesis potential. As noted by (Kirschbaum, 2011), plant growth is determined by photosynthesis.

In this study, exogenous ALA application by foliar spray, of 0.012 mM significantly increased the chlorophyll content of kale at 22 days after sowing while after 25 days, the 0.12 mM application outperformed the control. In contrast, the seed soaking treatment at the same concentration did not enhance the chlorophyll content.

Pre-soaking the radish seeds with ALA at the level of 0.012 mM improved the SPAD readings, but this enhancement was not significant ($p=0.067$); in contrast the foliar spray treatment retarded the chlorophyll content. Foliar application of the lowest ALA concentration enhanced chlorophyll content of cress, but no significant improvement was observed following the seed treatment. Instead, seed application of ALA at the highest concentration reduced the SPAD readings was seen on both treatments on the same concentration of ALA, 0.0012 mM. These effects confirmed the study by Hotta *et al.* (1997[b]) who found that low concentration (0.06 – 0.6 µM = 0.00006-0.0006 mM) of ALA enhanced the chlorophyll levels of horseradish (*Armoracia rusticana*). ALA application increased the chlorophyll content of cabbage (*Brassica oleracea* var. *capitate*), tomato

(*Lycopersicon esculentum*) and pakchoi (*Brassica campestris*) as mentioned in the literature review (Chon, 2003; Memon *et al.*, 2009; Zhao *et al.*, 2014). In addition, ALA treatment promoted leaf chlorophyll content and improved nitrogen absorption in pakchoi (Wei *et al.*, 2012).

Nitrogen, as a structural component of chlorophyll affects chloroplast formation and chlorophyll accumulation (Evans, 1989; Daughtry *et al.*, 2000). Bojović and Marković (2009) confirmed a strong relationship between chlorophyll content and N content per leaf area and Xiong *et al.* (2015) emphasized the connection varied among species depending on environmental factors. Nevertheless, SPAD meter has been used for a few decades to monitor the leaf N status of several crops, such as cotton (Feibo et al., 1998), durum wheat (Debaeke et al., 2006), irrigated rice (Peng *et al.*, 1996; Huang *et al.*, 2008), maize (Bullock and Anderson, 2008), sorghum (Uchino *et al.*, 2013) and sweet pepper (Padilla *et al.*, 2018). Therefore, one could surmise that in the present study ALA increased leaf nitrogen content in kale, radish and cress with different concentrations between 0.0012 mM and 0.012 mM.

4.4.2.2 Effects of ALA solutions on growth of kale, radish and cress

In the present work, the appropriate concentration of ALA to enhance root and shoot biomass was different between three tested plants. The suitable application method, either through seed soaking or foliar spraying treatment, also determined the promotive effects of ALA. Seed treatment of ALA, particularly at the lower concentration (0.0012 mM), increased shoot biomass of kale, while foliar application of the same concentration did not have any effect. In accordance

with these outcomes, the seed treatment at the level of 0.012 mM and 0.0012 mM of ALA also significantly increase the number of kale leaves, while foliar application did not show any effect. A possible explanation for these findings might be that kale is cultivated for its edible leaves and accumulate nutrients such as carotenoids, vitamin A and C, calcium, iron, magnesium, and dietary fibre (Guerena, 2006). Enhance foliar nutrients may have interacted synergistically with the ALA to promote biomass. Indeed, the commercial product Pentakeep, is a formulation of ALA and macro- and micro-nutrients and the benefits of ALA may be predicated on supply of nutrients. However, the potting compost used here should have contained sufficient nutrients to support growth of the plants during this experiment.

Different from kale, radish was grown for its enlarged edible roots (Lavanya *et al.*, 2014). In this study, pre-soaking the seeds before sowing with 0.012 mM ALA was more effective in improving the yield than the foliar application. This outcome is contrary to that of (Shruthi et al., 2016) who showed that the method of plant growth regulators (GA_3) application by spraying at concentration 250 ppm contributed to the high yields of radish. However, Bawkar *et al.* (2011) found that seed soaking with 50 ppm of GA_3 increased the yield of carrot. It was also noted that ALA did not have any effect at all on the number of leaves.

Interestingly, ALA had little real effect on the growth of cress.

4.5 Conclusion

In first Petri dish experiment with cress and radish, response to ALA treatment demonstrated a dependency of plant response on the dilution of ALA applied. ALA

was demonstrated as having inhibitory and promotive effects depending on the concentration applied under soil-less conditions.

In the second soil-based experiment, increases in SPAD value and vegetative growth (root biomass, shoot biomass, number of leaves, and number of petioles) of kale, radish and cress also varied depending on the concentration of the ALA applied. However, the findings of the present study suggested that pre-treatment with ALA through seed soaking was more effective than foliar spraying. Furthermore, seed soaking might be the simplest and most convenient method in promoting the growth of plants. As for the plant comparison, kale was clearly more sensitive to ALA treatments rather than other plants due to its consistent performance.

5 EFFECTS OF ALA ON KALE (*BRASSICA OLERACEA* VAR*. ACEPHALA*) FOLIAR YIELD, NUTRIENT CONTENT AND BIOCHEMICAL DIFFERENCES MEASURED BY FTIR

5.1 Introduction

Kale (*Brassica oleracea* var. *acephala*), is a green leafy vegetable which originated from the eastern Mediterranean region. It is classed as one of the healthiest foods due to its rich nutrient composition. According to Migliozzi *et al. (*2015), kale contains several vitamins (A, K, C), essential minerals (potassium, calcium, magnesium), phytochemicals (folic acid, ascorbic acid, riboflavin, carotenes) and dietary fibre. In addition, kale is richer in protein, fibre and sulphur than is broccoli (Dias, 2012). As a member of the Brassicaceae, kale contains glucosinolates which are thought to protect against lung, prostate and breast cancer (Verhoeven *et al.*, 1996).

ALA is an essential precursor for chlorophyll biosynthesis and its treatment increased concentrations of macro- (N, P and S) and micro-nutrients (Mn, Zn, Fe and Cu) in the leaves and roots of *Brassica napus*, cucumber, tomato and watermelon seedlings (Al-afifi and Al-shrouf, 2010; Naeem *et al.*, 2010; Ali *et al.*, 2013; Chen *et al.*, 2017; Anwar *et al.*, 2018). But Smoleń et al. (2010) declared that ALA had no effect on Ca, K, Mg, Na, P, S, B, Cu, Fe, Mn and Zn in spinach and the content of Al, Ba, Cd, Co, Cr, Ga, Li, Ni, Pb, Sb, Sr, Ti, and V of lettuce were also affected.

Although extensive research has been carried out on ALA, no single study exists on the physiological effects of ALA on chlorophyll content, plant growth and total nutrient content of kale. Therefore, this present study was carried out to confirm

the results of the previous experiment and to expand that study into a more detailed assessment of ALA effects on kale.

The **specific objectives** of this Chapter were:

(i) To measure the effects of ALA application on foliar yield of kale.
(ii) To quantify the chemical properties and nutrient content of kale following the treatment of ALA.
(iii) To determine if metabolic changes in the foliage can be measured through FTIR analysis.

The key hypothesis was that ALA-related metabolic changes in the plants will be observed relative to the control (by FTIR), irrespective of yield or elemental differences.

5.2 Materials and Methods

5.2.1 Experimental set-up

Kale was grown in pots and subjected to ALA treatments (foliar or seed-dressing) as before. Kale was grown in larger pots than previously and in a loam rather than a peat soil. The use of different soil from that used previously was to determine whether the high organic content of the original peat soil inhibited the actions of ALA when applied as a seed dressing.

Following seed sterilisation (Section 3.2.2.2), ALA was applied by seed soaking prior to sowing or by foliar spraying after germination as previously described (Section 4.2.3.1). ALA solution pH was not adjusted and 0.0012 mM, 0.012 mM, 0.12 mM ALA concentrations were tested. Controls consisted of deionised water.

After 24 h of soaking in the appropriate solution, the seeds were sown in 15 cm diameter pots containing John Innes No. 3 which is a loam-based compost high in nutrients.

The kale seeds were sown in each pot and grown in automated day-night temperature-controlled environment growth room of 16 h light and 6 h dark with 1 h of dusk and 1 h of dawn at a day/night temperature of 20°C and 17°C respectively.

Pots (three replicates per treatment) were arranged in a completely randomised design. On the tenth day after sowing, over 90% germination was observed. The plants were then thinned to one germinated plant per pot. For the foliar spray treatment, ALA aqueous solutions of 0, 0.0012 mM, 0.012 mM and 0.12 mM were sprayed at the 3-4 leaf stage (15 days after sowing).

5.2.2 Plant harvesting and analysis

Before harvesting, the number of leaves was counted. Then all shoots were removed and put in individual paper bags. Roots were extracted from the soil, washed thoroughly with tap water and put into individual paper bags. Foliage was dried at 35°C until constant weight and biomass recorded. The dried shoots were ground with liquid nitrogen in preparation for Fourier Transform InfraRed Spectrophotometer (FTIR) analysis, and total elemental content. Fourier-transform infrared spectroscopy (FTIR) was performed with 4 cm^{-1} resolution measuring the absorbance from 4000 to 500 cm^{-1} with 120 scans per sample (Tensor 27; Bruker, USA). Samples were placed directly on a crystal and a flat tip powder press was used to achieve even distribution and contact. A correction was

made to spectra for the ATR to allow for differences in depth of beam penetration at different wavelengths in order to get composite spectral bands. The IR spectra of kale shoots were analysed using the optical spectroscopy software, SpectraGryph.

5.2.2.1 Acid digestion

The total concentrations of nutrients in shoot samples were determined by digesting the samples in nitric acid. The shoot samples were ground into powder using liquid nitrogen with a mortar and pestle and weighed in 50 mL 'DigiTUBEs' then digested using aqua regia. Three replicates consisting of 0.2 g of shoot sample were digested with 8.0 mL HNO_3 and 2 mL hydrogen peroxide in a 48-place Teflon-coated graphite block digester (Model A3, Analysco Ltd., UK) situated in a fume cupboard and left overnight. The samples were then heated at 95°C for 2 hours and then allowed to cool down for 10 minutes before making up to 50 mL with ultra-pure water. During the digestion, DigiTUBEs were covered with transparent tops to reduce evaporation. The multi-elemental analysis was then performed by ICP-MS after diluting the digest with ultra-pure water in the ratio of 1 mL sample and 9 mL water.

5.2.2.2 Multi-element analyses by ICP-MS

Multi-element analyses of diluted solutions (nitric acid digests) were undertaken by ICP-MS (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany). The instrument was run in three operational modes, including (i) a collision-cell (Q cell) using He with kinetic energy discrimination (He-cell) to

remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell is evacuated and (iii) hydrogen mode (H_2-cell) in which H_2 gas is used as the cell gas. Samples were introduced from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream on a separate line *via* the ASXpress unit and included Ge (10 µg L^{-1}), Rh (10 µg L^{-1}) and Ir (5 µg L^{-1}) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, TI, U, V and Zn, in the range 0-100 µg L^{-1} (0, 20, 40, 100 µg L^{-1}). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) was used to create Ca, Mg, Na and K standards in the range 0-30 mg L^{-1}. Phosphorus, boron and sulphur calibration utilized in-house standard solutions (KH_2PO_4, K_2SO_4 and H_3BO_3). In sample switching was used to measure B and P in STD mode, Se in H_2-cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required.

All elemental concentrations (µg L^{-1}) were converted to mg kg^{-1} following the equation below (Equation 5.1);

$$C_{shoot} = \frac{C_{sol} - C_{blank}}{M_{shoot}} \times Vol \qquad \text{Equation 5.1}$$

Where: C_{shoot} = the elemental concentration (mg kg^{-1}) in the shoot

C_{sol} and C_{blank} = the concentrations (µg L^{-1}) in the shoot and blank extract/digests respectively

Vol = the volume of the digest/extractant (10 mL)

M_{shoot} = the mass of shoot digested (0.2 g).

5.2.2.3 Extractable carbon and nitrogen

The total carbon (TC) and nitrogen (TN) were determined using a CN analyser (Thermo Scientific Flash EA 1112 Nitrogen and Carbon Analyser). Approximately, 0.05 – 0.06 g of powdered shoot samples were weighed on an analytical balance and placed into individual tin capsules. The capsules were then sealed, folded, and the exact weight recorded. Aspartic acid was used as the analytical standard. The capsules were transferred to the autosampler for analysis of total C and N through combustion, producing CO_2, N_2 and H_2O in high temperature reactor chambers.

5.2.3 Statistical analyses

Data were analysed using GenStat version 19.1 (VSN International, Hemel Hempsted, UK). A repeated measures analysis of variance was carried out on the SPAD values for the three reading dates using application method and ALA concentration as the main factors. Since application method was not significant, the analysis was repeated for date corresponding to ALA seed dressing and ALA foliar spray separately. Shoot elemental data were analysed by ANOVA using application method and ALA concentration as the main factors. Data were tested for normality by plotting residuals against expected normal quantiles and post hoc comparisons made between means were based on least significant differences (LSD) at the 0.05 probability level. FTIR spectra were analysed using SpectraGryph version 1.2.11 (Dr. Freidrich Menges Software, Entwicklung, Germany) after setting a simple baseline, normalising the peaks by setting the highest peak to a value of 1 and smoothing the spectra with a medium Savitsky-Golay algorithm. These options are built into the software.

5.3 Results

5.3.1 Effects of ALA treatment on SPAD values

SPAD values (Figure 5.1) for plants that had previously been sprayed with the foliar ALA treatment, showed an ALA concentration effect (concentration as a single factor, $p<0.001$) where the 0.0012 mM spray increased SPAD values relative to the other foliar concentrations. This concentration resulted in the highest SPAD readings on days 24 and 31 (time × concentration interaction, $p<0.001$; Figure 5.1). In contrast, there was no significant ALA concentration effect as a single factor for the seed dressing treatment, but a time × concentration interaction ($p<0.001$) showed a similar trend as the foliar treatment, with the 0.0012 mM ALA amendment increasing SPAD readings on days 27 and 34. The largest difference between the ALA and control treatments was on day 27. Kale treated with 0.0012 mM, either through seed soaking or foliar spraying resulted in the highest chlorophyll content compared to the control (Table 5.1).

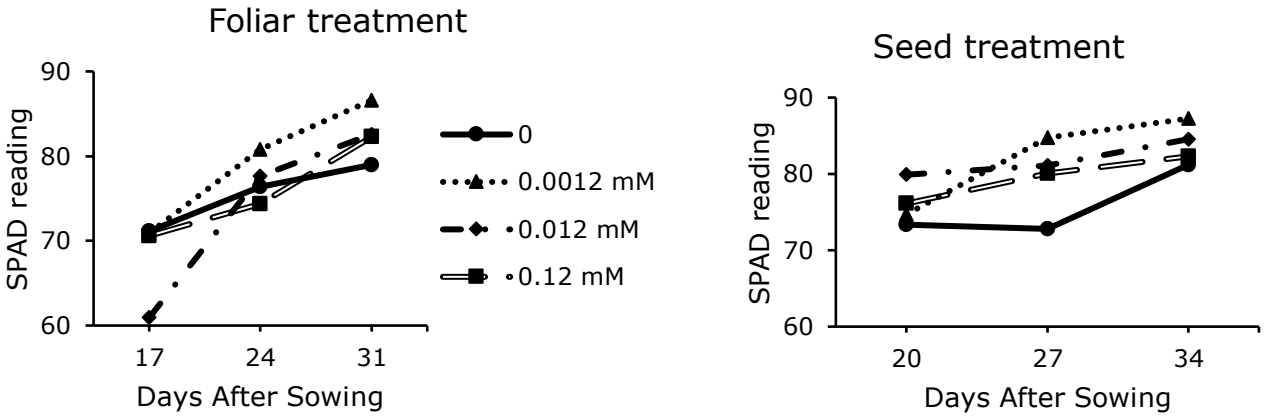

Figure 5.1. The SPAD readings for kale leaves following ALA treatments (4 ALA concentrations and two application methods).

Table 5.1. Summary of repeated measures ANOVA output for SPAD readings taken from kale. The foliar and seed treatments were analysed separately. An original repeated measures ANOVA showed there was no significant difference between the foliar and seed treatments.

		Foliar Spray		Seed Dressing	
	DF	**F**	**P**	**F**	**p**
Conc.	3	24.98	<0.001	1.77	0.185
Residual	20				
Time	2	263.6	<0.001	50.46	<0.001
Time × Conc.	6	15.74	<0.001	8.03	<0.001
Residual	40				
Total	71				

5.3.2 Effects of ALA treatment on biomass

The response of ALA application on the root and shoot yield are presented in Figure 5.2. Despite the apparent reduction in root biomass with increasing ALA concentration following foliar treatment, no effects were significant (Table 5.2).

There were no effects of treatment on leaf number, which ranged from 10-12.

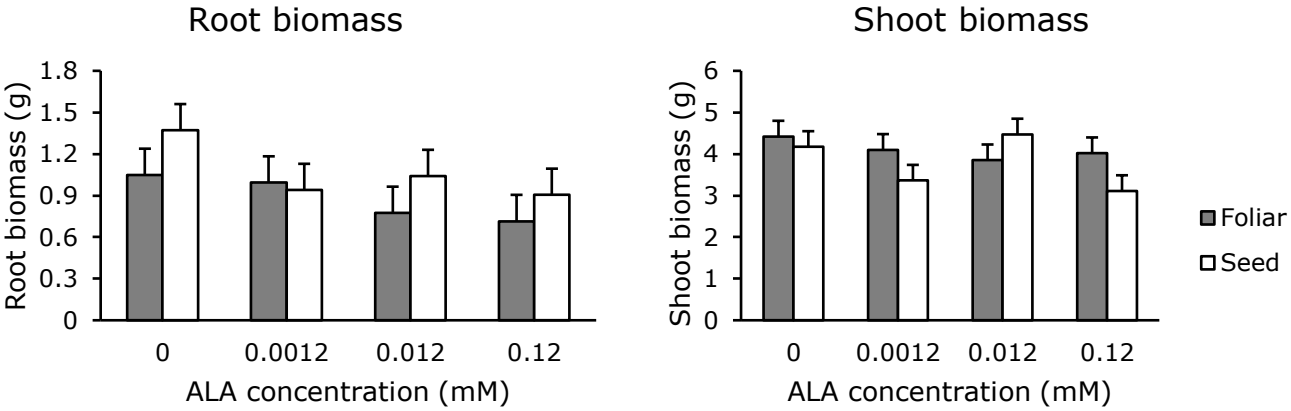

Figure 5.2. The biomass of root and shoot of kale. The individual error bars are based on pooled variance estimate from the ANOVA with 12 degrees of freedom.

Table 5.2. Summary of two-factor ANOVA for vegetative growth of kale.

Variate	Factor	DF	F	P VALUE	LSD (95%)
Root biomass	Treatment	1	1.81	0.198	0.285
	Dilution	2	1.60	0.228	0.403
	Interaction	2	0.38	0.769	0.570
	Residual	12			
Shoot biomass	Treatment	1	1.43	0.249	0.566
	Dilution	2	1.66	0.215	0.801
	Interaction	2	1.64	0.219	1.133
	Residual	12			
Number of leaves	Treatment	1	0.03	0.857	0.968
	Dilution	2	1.72	0.203	1.368
	Interaction	2	0.39	0.763	1.935
	Residual	12			

5.3.3 Effect of ALA treatment on the FTIR spectra of kale leaves

The FTIR spectrum was collected in the IR region between 400 and 4000 cm^{-1}. Since each functional group has its own absorption frequency within this range, any metabolic changes due to ALA treatment should be observed. The aim was to establish a 'fingerprint' for the shoots. The IR vibrational spectra derived from kale shoots treated with foliar or seed ALA solutions are shown in Figure 5.3 and assignment of peaks is in Tables 5.3 and 5.4.

The most noticeable differences between the spectra for plants subjected to the ALA foliar sprays compared to the seed treatments are the smaller peak heights associated with the high ALA concentrations when applied as a seed dressing (Figure 5.3a), compared to the equivalent peaks on the spectrum derived from the foliar treatment (Figure 5.3b). Higher ALA seed treatments appear to have 'dampened down' the response compared to the lowest ALA concentration in plants that had been subjected to seed treatment. The same degree of 'dampening down' was not observed for the peaks associated with the foliar

treatments. This is particularly evident in the regions assigned Nikalje *et al.* *(*2019) to carbohydrates (1200-1500 cm^{-1}), proteins (1500-1800 cm^{-1}) and lipids (2000-3000 cm^{-1}). Arguably, a similar effect is observed with the cell wall components (600-1000 cm^{-1}).

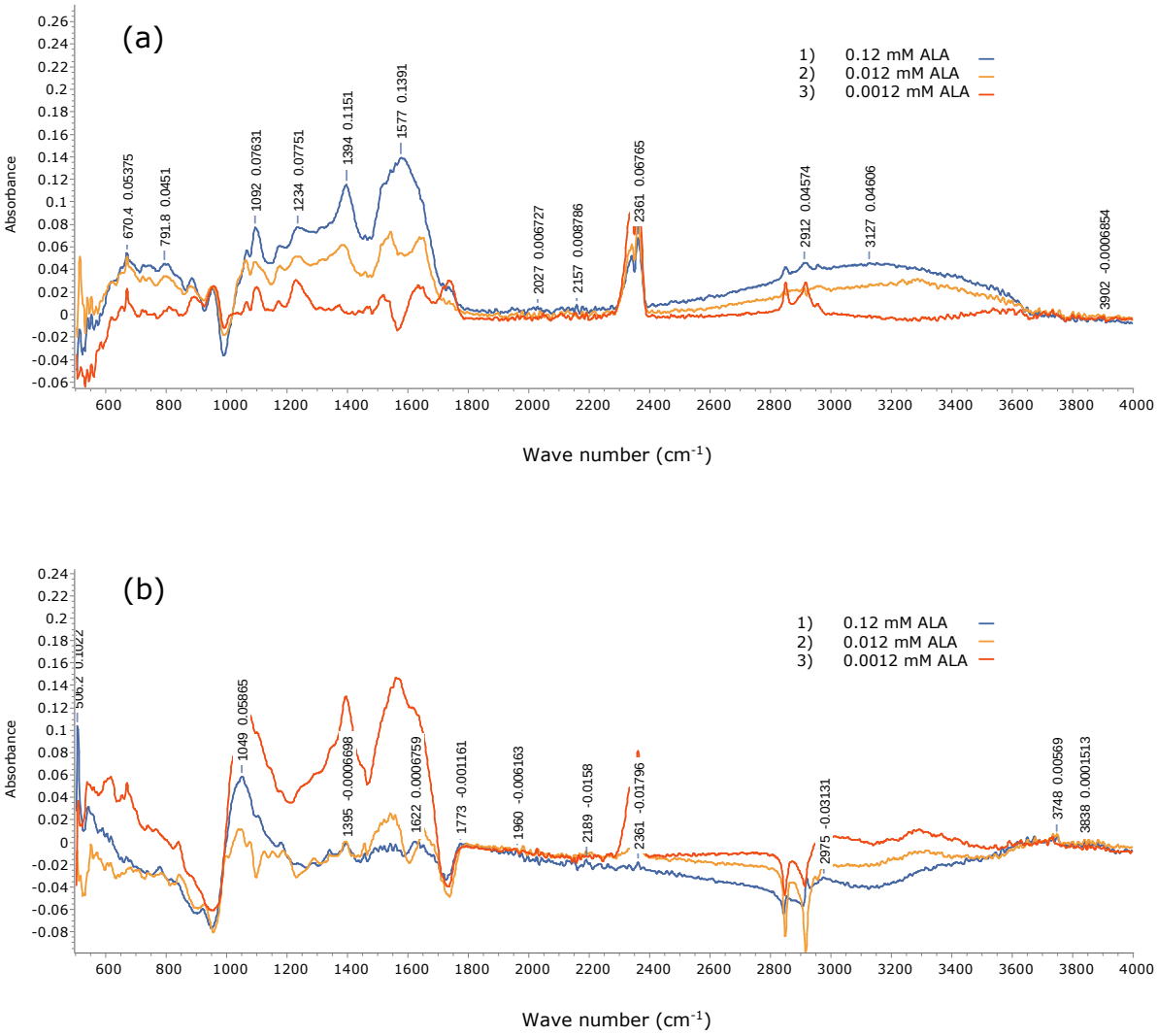

Figure 5.3. IR vibrational spectra derived from kale leaves subjected to **(a)** foliar ALA treatments and **(b)** seed dressing treatments. Test data were subtracted from the control spectra and then normalised using SpectraGryph software.

The specific details of spectra for shoots treated with the foliar spray (Figure 5.3a) are as follows: small peaks at 3902 cm^{-1} ascribed to stretching of molecular water in the sample. A broad absorption at 3127 cm^{-1} was attributed to stretching vibration O-H and N-H of the aromatic amino, carboxylic acid and phenolic groups, while the peaks at 2912 cm^{-1} indicated the stretching vibration of the aliphatic group of C-H. There were impurity compositions of silicon tetrafluoride at two peaks which were 2361 cm^{-1} and 2157 cm^{-1}. Weak bands centred at 2027 cm^{-1} were assigned to functional groups of the DMAP (4- dimethylaminopyridine) ligand. The characteristic strong band at 1577 cm^{-1} was attributed to C=O double bond (COOH) bending vibration. The peak absorbance at 1394 cm^{-1} represents O-H bending of the carboxylic and phenolic groups. Stretching vibrations of C-O-C in ethers and esters or top phenolic hydroxyl was present at the peak centred at 1234 cm^{-1}. The band peak at 1092 cm^{-1} indicated C-H bond in-plane /out-of-plane deformation. Finally, the small band at 791.8 cm^{-1} was assigned to quartz Si-O, while the weak band below 700 cm^{-1} was indicated as aromatic C-H bond out-of-plane bending of the aromatic C-H bond (Table 5.2).

The FTIR spectrum for kale leaves subjected to the ALA seed-soaking treatment is shown in Figure 5.3b. The first observation is the peak at 3838 cm^{-1} and 3748 cm^{-1} revealed the stretching of molecular water. The point of 2975 cm^{-1} indicated negative peak of functional group of C-H. The absorption values of 2361 cm^{-1} and 2189 cm^{-1} were marked the impurity compositions of silicon tetrafluoride. Small noises at 1960 cm^{-1} and 1773 cm^{-1} indicated stretching C-O bond and stretching vibration of C-O carboxyl respectively.

Table 5.2. Spectral positions of FTIR peaks, respective centre positions, and their corresponding assignments in kale leaves associated with the foliar ALA treatments (Figure 5.3a).

Peaks	Centre (cm^{-1})	Assignments	Reference
1	3902	Stretching of molecular water	Chuprov *et al.* (2006)
2	3127	Stretching vibration O-H and N-H of the aromatic amino, carboxylic acid and phenolic	Magarelli et al. (2010)
3	2912	Stretching vibration of aliphatic group of C-H	Centeno and Shamir, (2008); Tarangini and Mishra (2013)
4	2361	Impurity compositions of silicon tetrafluoride	Chuprov *et al.* (2006)
5	2157	Impurity compositions of silicon tetrafluoride	Chuprov *et al.* (2006)
6	2027	Functional groups of DMAP ligand	Xu *et al.* (2016)
7	1577	C=O double bond (COOH) bending vibration	Magarelli et al. (2010)
8	1394	O-H bending of the carboxylic and phenolic groups	Centeno and Shamir (2008)
9	1234	Stretching vibrations of COC in ethers and esters or top phenolic hydroxyl	Bykov (2008)
10	1092	C-H bond in-plane /out-of-plane deformation	Centeno and Shamir (2008)
11	791.8	Quartz Si-O	Sathya et al. (2012)
12	670.4	Aromatic C-H bond out-of-plane bending of the aromatic C-H bond	Magarelli et al. (2010)

Table 5.3. Spectral positions of FTIR peaks, respective centre positions, and their corresponding assignments in kale leaves associated with the seed ALA treatments (Figure 5.3b).

Peaks	Centre (cm^{-1})	Assignments	Reference
1	3838	Stretching of molecular water	Chuprov *et al.* (2006)
2	3748	Stretching of molecular water	Chuprov *et al.* (2006)
3	2975	Stretching vibration of aliphatic group of C-H	Centeno and Shamir (2008); Tarangini and Mishra (2013)
4	2361	Impurity compositions of silicon tetrafluoride	Chuprov *et al.* (2006)
5	2189	Impurity compositions of silicon tetrafluoride	Chuprov *et al.* (2006)
6	1960	Stretching C-O bond	Prasad et al. (2011)
7	1773	Stretching vibration of C-O carboxyl	Gupta et al. (2015)
8	1622	C=O double bond (COOH) bending vibration	Magarelli et al. (2010; Tarangini and Mishra (2013)
9	1395	O-H bending of the carboxylic and phenolic groups	Centeno and Shamir (2008)
10	1049	C-H bond in-plane /out-of-plane deformation	Centeno and Shamir (2008)
11	506.2	Stretching vibration of the phenyl rings and out-of plane deformation	Vahidzadeh et al. (2018)

The C=O double bond (COOH) bending vibration was present in the strong peak of 1622 cm^{-1}. The absorbance peak at 1395 cm^{-1} was identified as O-H bending of the carboxylic and phenolic groups. The peak centred at 1049 cm^{-1} indicates a C-H bond in-plane /out-of-plane deformation. The peak at 506.2 cm^{-1} was assigned to stretching vibration of the phenyl rings and out of plane deformation.

5.3.4 Effects of ALA treatment on the macro- and micro-nutrients in kale leaves

In the present study, application of ALA either as a foliar spray or as a seed dressing had little effect on foliar concentrations of macro- or micro-nutrients (Table 5.4). Nevertheless, some weak patterns were evident; for example, seed treatment with 0.0012 mM ALA resulted in higher concentrations of foliar Cs (0.06 mg kg^{-1}) than the other ALA treatments which had average concentrations of around 0.01 mg kg^{-1} (ANOVA: concentration × application method interaction, $p<0.001$). This trend was also observed for K, although the concentration × application method interaction was not significant ($p=0.075$; Table 5.4).

Foliar Pb concentrations were highest following treatment with the 0.012 mM ALA seed dressing (concentration × application method interaction, $p=0.056$) and Co and As were both generally higher in shoots when seeds were soaked compared to when leaves were sprayed, apart from when plants were subjected to the highest ALA concentration (Figure 5.4). However, seed application of ALA reduced foliar As compared to the control treatment and the foliar spray at low ALA concentrations (0.0012 mM and 0.012 mM) reduced shoot Co concentration.

Foliar carbon was unaffected by ALA treatments (grand mean = 40.4% C), nor were there any significant ALA effects on leaf N (grand mean = 3.4%). However, foliar N concentrations were higher in the plants treated with 0.0012 mM seed dressing (4.1% N) following the consistent (but in this case not significant) trend observed with other elements.

Table 5.4. The effect of two types of ALA application on the uptake of macro- and micro-nutrients (mg kg^{-1}) in the leaves of kale.

ALA concentration (mM)	Ca		K		Mg		P	
	Foliar	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**
0 mM	15067	16447	20205	21143	4145	4884	3554	4714
0.0012 mM	16479	19970	19512	28944	4809	5980	4005	4741
0.012 mM	15592	13642	24569	17877	4717	4377	3881	3082
0.12 mM	18881	16533	25029	21327	5968	4606	4476	3783

ANOVA	**Ca**	**K**	**Mg**	**P**
Conc.	0.159	0.612	0.346	0.194
Appl. Method	0.906	0.998	0.905	0.730
Conc. × Appln.	0.293	**0.075**	0.200	**0.063**
LSD (Conc. × Appln.)	5088	8949	610	1220

DF – Conc. 1; Appln. Method 1; Conc. × Appln. Method 3; Residual 16; Total 23

Table 5.4 continued

ALA concentration (mM)	Mn		S		Na	
	Foliar	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**
0 mM	93.3	107.9	3016	2566	882	899
0.0012 mM	86.9	93.7	3317	6145	804	1259
0.012 mM	68.3	68.6	4801	1927	1140	1232
0.12 mM	98.1	70.0	4422	4807	1225	953

ANOVA	Mn	S	Na
Conc.	**0.073**	0.303	0.224
Appl. Method	0.844	0.974	0.464
Conc. × Appln.	0.298	0.151	0.108
LSD (Conc. × Appln.)	34.09	3508	411.5

DF – Conc. 1; Appln. Method 1; Conc. × Appln. Method 3; Residual 16; Total 23

Table 5 .4 continued

ALA concentration (mM)	Ni		Al		Zn		Cr	
	Foliar	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**
0 mM	0.55	0.62	3.6	3.1	21.90	17.30	0.15	0.15
0.0012 mM	0.56	0.47	3.2	13.1	20.50	23.65	0.19	0.19
0.012 mM	0.87	0.68	1.4	11.0	21.66	19.84	0.18	0.15
0.12 mM	0.67	0.50	1.9	1.6	23.62	17.84	0.18	0.15

ANOVA	Ni	Al	Zn	Cr
Conc.	**0.061**	0.272	0.796	0.196
Appl. Method	0.176	**0.069**	0.211	0.621
Conc. × Appln.	0.482	0.252	0.305	0.335
LSD (Conc. × Appln.)	0.27	10.16	7.35	0.18

DF – Conc. 1; Appln. Method 1; Conc. × Appln. Method 3; Residual 16; Total 23

Table 5.4 continued

ALA concentration (mM)	Mo		Pb		Fe		Cd	
	Foliar	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**	**Foliar**	**Seed**
0 mM	59.2	55.3	0.06	0.01	67.3	92.1	0.13	0.14
0.0012 mM	56.7	61.0	0.04	0.03	72.1	100.5	0.08	0.11
0.012 mM	55.4	46.3	0.04	0.13	69.3	69.8	0.08	0.10
0.12 mM	58.4	62.2	0.02	0.02	83.2	67.1	0.13	0.09

ANOVA	**Mo**	**Pb**	**Fe**	**Cd**
Conc.	0.481	**0.052**	0.511	0.372
Appl. Method	0.784	0.622	0.253	0.252
Conc. × Appln.	0.681	**0.056**	0.197	0.762
LSD (Conc. ×Appln.)	19.08	0.07	33.64	0.06

DF – Conc. 1; Appln. Method 1; Conc. × Appln. Method 3; Residual 16; Total 23

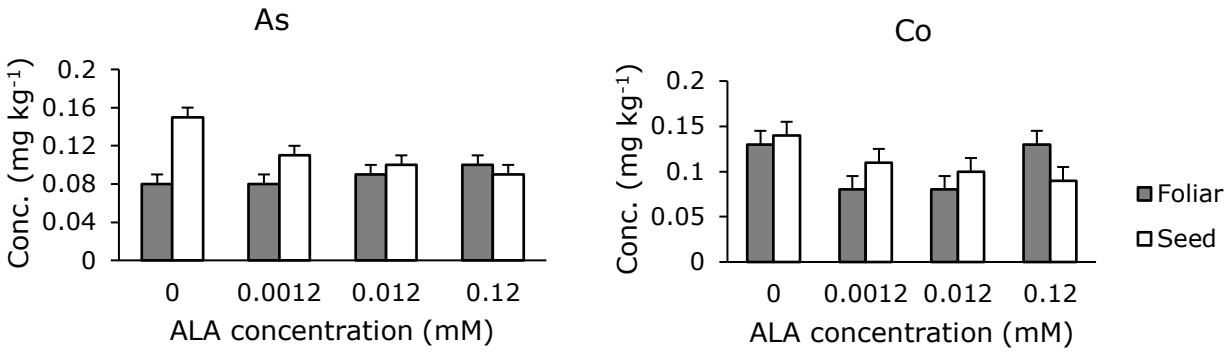

Figure 5.4. Mean concentrations (mg kg^{-1}) of foliar As (ANOVA: concentration × application method interaction, p=0.013; LSD = 0.032) and Co (concentration as a single factor, p=0.044; LSD = 0.034). The individual error bars are based on pooled variance estimate from the ANOVA with 16 degrees of freedom.

5.4 Discussion

5.4.1 Effects of ALA treatment on SPAD values and growth of kale

In this study, foliar spraying with, and pre-soaking seeds in, ALA solutions significantly increased the SPAD readings, which may be used as a proxy for chlorophyll content. These results are consistent with previous studies which demonstrated the same effect of ALA treatments on chlorophyll concentration. In accordance with these findings, (Akram and Ashraf 2013; Zhao *et al.*, 2014; Anwar *et al.*, 2018; Wu *et al.*, 2018) showed that ALA treatment improved the chlorophyll content in tomato, cucumber, melon and watermelon. Naeem *et al.* *(*2010) also demonstrated the role of ALA in regulating biosynthesis of chlorophyll and photosynthesis in *Brassica napus.* In addition, ALA application improved the accumulation of chlorophyll, soluble sugars and flavanoids in *Ginkgo biloba* (Xu *et al.*, 2011), which improved the leaf quality (Cheng *et al.*, 2011; Xu *et al.*, 2011).

Interestingly, in both this current experiment and the previous one (Chapter 5), a similar concentration of ALA (0.0012 mM) affected the SPAD values despite the difference in the time of measurement. In the previous experiment, measurements were undertaken at 18, 22 and 25 days after sowing, while the SPAD measurements for this experiment were on days 17, 24, 31 after sowing for the foliar treatment and on days 20, 27, 34 for the seed treatment.

Despite the ALA-enhancement of chlorophyll content, this was not translated into a demonstrable increase in yield or nutrient uptake. The present study showed that ALA treatment did not significantly affect root or shoot biomass.

5.4.2 Effects of ALA treatment on the foliar FTIR profiles

Fourier Transform InfraRed (FTIR) spectroscopy, a non-destructive analytical technique presents chemical information based on the IR spectrum (McCann *et al.*, 1992; Yu *et al.*, 2004). FTIR is necessary for structural interpretation, and for understanding binding capacity, affinity and metal ion sites (Magarelli et al., 2010). Spectroscopy is generally used for identifying functional groups in the material, which are illustrated by the intensity of peak absorbance (Munajad et al., 2018).

Yu and Irudayaraj (2005) proposed that FTIR analysis should be used to study biochemical transformations in the plant tissue. The biochemical changes in a plant which are marked by the change in spectral fingerprint could be determined by combining FTIR spectra with multivariate data analyses (Manrique and Lajolo, 2004; Dokken and Davis, 2007).

In this study, IR analysis illustrated typical bonds and stretching of functional groups present in the kale shoots treated with the foliar and seed amendments of ALA. Chen *et al*. (2003) discovered that the ALA spectrum shows strong absorbance peaks for the C=O, C-H(2) and O-H bonds. Foliar spraying and seed soaking with ALA showed a strong peak at 1577 cm^{-1} and 1622 cm^{-1} and was attributed to the bending vibration of the C=O double bond (COOH), followed by the peak observed at 1394 cm^{-1} and 1395 cm^{-1} which was assigned to O-H bending of the carboxylic and phenolic groups (Magarelli *et al*., 2010; Tarangini and Mishra, 2013). Based on this information, it could be presumed that this is evidence of the presence of ALA in the kale leaves. However, one might expect any exogenous ALA to have been metabolised by the time of harvest.

What is of particular interest are the different responses to the ALA solutions when applied as a seed dressing or as a foliar spray. High concentrations of ALA in previous experiments within this thesis were detrimental to plant growth, most likely due to oxidative stress. The different profiles for the spectra relating to phenolics appear to have shifted slightly (1394 versus 1395 cm^{-1}) depending on ALA application method. There is also a greater separation of the spectra derived from plants subjected to the foliar spray than those treated with the seed dressing. The protein components (1700-1800 cm^{-1}) are notably different depending on mode of ALA application. This could be a stress response since kale produces glucosinolates and there may be a correlation between glucosinolate production and proteins (Maodzeka *et al.*, 2019). Alternatively, the large absorbance peaks associated with the highest ALA concentration and observed in plants that were sprayed, may be associated with oxidative stress as an intermediate-term response to the high concentration of ALA. Either that, or in

this context, seedlings were more sensitive to lower concentrations of ALA than were the mature plants.

Since low concentrations of ALA promote growth and stimulate synthesis of soluble sugars, it is perhaps not surprising that the spectra for plants amended with the 0.0012 mM seed dressing, had higher peaks associated with sugars/carbohydrates (1200-1500 cm^{-1}) than plants which had seeds soaked in more concentrated ALA solutions. It is likely that the plants subjected to a low concentration of ALA in the seed dressing benefited from this at the seedling stage, and this metabolic advantage was maintained through to maturity. Data from previous chapters (e.g. Chapter 4) corroborate the benefit seedlings receive when grown with low concentrations of ALA, even though growth was not affected here. Ideally, spectra would be available at different life stages of the plant. However, the SPAD values suggest that the lower concentrations of ALA increased leaf chlorophyll content and therefore photosynthetic capability. The data demonstrate that metabolic changes took place in the experimental plants which were not observed in cruder biomass measurements at the time of harvest. This highlights the benefit of using FTIR to observe changes in the plant resulting from treatment effects.

The evaluation of plant components, such as lignin, cellulose, proteins and lipids is based on the output of FTIR measurement (Naumann *et al.*, 2007). The C-H bond that was shown in the sprayed and seed-soaked plants with the centred peaks at 2912 cm^{-1} and 2975 cm^{-1} respectively, demonstrated the lipid component in leaves of kale (Centeno and Shamir, 2008; Tarangini and Mishra, 2013). These results are in accordance with those of (Buensanteai *et al.*, 2012) who found the CH stretching and CH bending in cassava related to the lipid

structure of the cell membrane. The lipid spectra (2000-3000 cm^{-1}) in the leaves of sprayed versus seed-treated plants, suggest a similar ALA-related seedling sensitivity as before.

The characteristic region between 800-1200 cm^{-1} is attributed to the C-O bond of the carbohydrate fingerprint (Thumanu *et al.*, 2015). FTIR spectra show absorbance peaks at wavelengths of 900-1500 cm^{-1} which are identified as cellulose (Munajad et al., 2018). The foliar spraying and seed soaking application of ALA with peaks at 1092 cm^{-1} and 1049 cm^{-1} were associated with the cellulose component in the leaves. The higher cellulose component in plants treated by seed soaking suggested that ALA application by pre-soaking the seeds improved the plant carbohydrate content.

The FTIR spectra appear to be sensitive to metabolic differences in the plants that were not necessarily obvious with other analytical methods in this Chapter.

5.4.3 Effect of ALA treatment on macro- and micro-nutrient concentrations in kale leaves

Macro- and micro-nutrients are essential elements in plant development (Dimkpa and Bindraban, 2016) and efficiency of uptake and distribution of these nutrients is vital for plant growth (White and Brown, 2010). In this study, ALA application had little significant effect on foliar elemental content, with some notable exceptions (e.g. Cs and As). In contrast, Al-afifi and Al-shrouf (2010) found that ALA improved macro- and micro-nutrient content of tomato (*Solanum lycopersicum*) and *Brassica napus* (Ali *et al.*, 2013). ALA treatment significantly

improved the macro-nutrients (N, P, K, Ca and Mg) and micro-nutrients (Cu, Fe, Mn and Zn) in the leaves and roots of cucumber (Anwar *et al.*, 2018).

Some of the common heavy metals are also micro-nutrients which are essential for plant growth, such as Zn, Cu, Mn, Ni and Co, while other metals have unknown biological function, such as Cd, Cr, Pb, Co, Ag, Se, Hg (Gaur and Adholeya, 2004). The toxicity of heavy metals affects plant physiology, including inhibiting nutrient uptake (Stefanov *et al.*, 1995; Sharma and Dubey, 2017).

For many years, ALA has been proven to mitigate environmental stresses, such as chilling, dark, drought, heat and salinity in several plants, such as lettuce, spinach, lettuce, pepper and cucumber (Härtel *et al.*, 1996; Nishihara *et al.*, 2001; Korkmaz and Korkmaz, 2009; Zhang *et al.*, 2012; El Gawad and El-Shora, 2014; Aksakal *et al.*, 2017).

In this experiment, application of ALA significantly reduced the potentially toxic elements, particularly As and Co. These results are in line with El Gawad and El-Shora (2014) who demonstrated that exogenous application of ALA ameliorated arsenic phytotoxicity. ALA application also alleviated Cd stress, increased the plants' capacity to store essential nutrients and promoted plant growth in *Brassica napus* and brown rice (Ali *et al.*, 2013; Feijuan *et al.*, 2018; XU *et al.*, 2018). Foliar application of ALA alleviated Pb uptake and enhanced plant growth, chlorophyll content and photosynthetic parameters in *Brassica napus* (Tian *et al.*, 2014). ALA has a role in reducing Cr accumulation in cauliflower (Ahmad *et al.*, 2017).

In the current experiment, much of the elemental data were not significant, but consistent observations were made which, whilst statistically are not robust, they

might be biologically relevant. For instance, there were trends towards enhanced elemental concentrations when plants were treated with low to intermediate doses of ALA, although many were for potentially toxic elements, e.g. Pb, Ni and Cs.

Plant growth is associated with carbon and nitrogen fixation. Light intensity and the availability of nutrients are the stimulators for carbon acquisition and biomass distribution (Irving, 2015). Carbon is utilized for both maintenance and growth (Amthor, 1984). Nitrogen is an essential component which supports all fundamental processes in plants. The role of nitrogen in plants, includes promoting leaf and root growth, improving fruit quality, driving uptake of other nutrients and increasing crop production (Leghari *et al.*, 2016). Uptake of nitrogen by plants is essential for the formation of amino acids as the backbone for protein synthesis (Arnold et al., 2015). Leaves are the primary sites of nitrogen assimilation and the influence of light in stimulating this is indicative of a close relationship with photosynthesis (Foyer and Noctor, 2002). However, ALA amendment here did not significantly affect plant N, although the lowest ALA concentration (0.0012 mM) when applied as a seed dressing, increased foliar N concentration. In contrast,(Wei *et al.*, 2012) reported that exogenous ALA application enhanced absorption and assimilation of nitrogen.

Up to now, several researchers have reported the role of ALA in regulating chlorophyll biosynthesis, nutrient uptake, photosynthesis accumulation, carbon and nitrogen fixation, fruit quality and yield (Alkhateeb *et al.*, 2006; Zhang *et al.*, 2006; Maruyama-Nakashita *et al.*, 2010; Naeem *et al.*, 2010). However, in this current experiment, ALA treatment did not affect foliar carbon content despite increasing SPAD values and therefore potentially enhancing photosynthesis. This

contrasts with the findings of Awad (2008) who showed that ALA application by foliar spray at 0.18-0.6 mM, promoted photosynthetic carbon fixation and decreased dark respiration. In addition, Wu *et al.* (2018) also stated that exogenous ALA positively influenced carbon assimilation. The fact that ALA did not increase yield or nutrient uptake here, but did in the previous experiment, is interesting and may be related to some unmeasured environmental parameter.

5.5 Conclusion

The application of ALA significantly improved the chlorophyll content of kale (measured as SPAD values) and treatment-related metabolic changes were detected by FTIR. ALA also alleviated the concentration of some toxic elements. However, these observations were not expressed as increased in foliar yield or increased nutrient efficiency.

6 GENERAL DISCUSSION

The key findings from each experimental chapter are summarized in the table below.

Table 6.1. Summary of key findings from Chapters 2, 3, 4, 5 and 6

Chapter	Key Findings
Chapter 2: Identification of the isolated bacterium and quantification of ALA production by the bacterium.	• Using 16S DNA methodology, the bacterium isolated from peat soil was identified as *Bacillus paramycoides*. • *B. paramycoides* produced detectable concentrations of ALA in culture. • Bacterial ALA production in culture was increased by adding glutamate (as a precursor) or levulinic acid (as an inhibitor of ALA dehydratase).
Chapter 3: Effect of *B. paramycoides* on growth of cress Petri dishes) and kale (soil).	• *B. paramycoides* promoted cress growth in a dose-dependent manner. Low concentrations of bacterial metabolites in the growth medium stimulated growth, while high concentrations inhibited germination and growth. • Nutrient strength of the bacterial growing medium influenced germination index (GI), with a 1:100 dilution of half-strength LB broth being optimal. Bacteria grown with low concentrations of nutrients stimulated germination, but did not affect yield. • Bacterial metabolites did not affect growth of kale in soil (pots). • Whilst *B. paramycoides* produced ALA *in vitro*, neither the bacterium nor its metabolites benefited plant growth when inoculated into soil.

	• Parallel tests with ALA were conducted to verify its effects on cress. • Use of a low concentration of chemical ALA (0.0012 mM) as a seed dressing before sowing in soil increased % germination of kale seeds and SPAD readings. • A higher concentration of chemical ALA (0.12 mM) used as a seed dressing stunted kale root and shoot biomass.
Chapter 4: The effects of ALA on the growth of cress, radish and kale.	• The promotive effect of ALA depended on the concentration. Based on the growth response curve, the best ALA dose for increasing cress growth was 0.012 mM and for radish, 0.0012 mM when grown in Petri dishes. • When grown in peat-based soil, ALA applied as a foliar spray increased SPAD values for kale and cress but not for radish. • When grown in peat-based soil, ALA at low concentrations applied as a seed dressing increased SPAD values but high concentrations reduced SPAD readings. Effect of ALA concentration were plant species specific. • ALA as a seed dressing generally lowered root and shoot biomass relative to foliar application.
Chapter 5: Effects of ALA on kale yield, nutrient content and biochemical differences measured by FTIR.	• Plants were grown in loam-based soil. • Low concentrations of ALA (0.0012 mM) increased SPAD values. • ALA did not affect biomass. • ALA did not affect plant macronutrient content, but As, Cs and Pb concentrations depended on an ALA concentration × ALA application method interaction.

	• FTIR spectra presentation was different in plants treated with the foliar spray compared to the seed dressing. • FTIR spectra suggest that plants are more sensitive to the negative effects of ALA (stronger concentrations) when applied as a seed dressing compared to a foliar spray on a more mature plant.

6.1 ALA production by *Bacillus paramycoides* as a potential plant growth promotor

The rationale behind the work undertaken here was to determine if a soil bacterium previously isolated and with suspected ALA production capability, could be used as a plant growth promotor. The first stage was to quantify and optimise ALA production by the bacterium and then test it against a range of plant species.

The bacterium in question was originally isolated from a peat soil, and as part of this project it was identified as *Bacillus paramycoides* with 100% similarity using 16S DNA. Interestingly, Noha and Shixue (2018) recently reported that *Bacillus paramycoides* improved the growth of pea plants (*Pisum sativum*).

Previous pilot work by the thesis author indicated that the cultured isolate was able to enhance growth of rice and it was hypothesised that 5-aminolevulinic acid (ALA) was the growth promotor responsible. ALA is a key precursor of tetrapyrrole compounds and is categorized as a plant growth regulator because it is a precursor of chlorophyll (Wu *et al.*, 2018). ALA application has been shown to increase the following: Seed germination, root formation, leaf greenness,

seedling vigour, tillering, photosynthetic activity, chlorophyll content and yield of radish, kidney beans, potato, cabbage, tomato, spinach, lettuce (Hotta *et al.*, 1997[b]; Al-afifi and Al-shrouf, 2010; Smoleń *et al*., 2010; Xu *et al*., 2012; Funada and Iwai, 2013; Pokluda *et al*., 2018). Use of a plant growth promoting soil bacterium that can produce ALA would have potential benefits for sustainable agriculture. Therefore, key aims of this project were to establish whether *Bacillus paramycoides* is able to produce ALA and if so, does the bacterium have any potential as a plant growth promotor.

ALA is very expensive to commercially synthesise. Therefore, there has been and still is, much interest in utilising bacteria as an alternative and inexpensive means of producing ALA. Most work to date has focused on photosynthetic bacteria (e.g. *Rhodopseudomonas* spp. and *Rhodobacter* spp.) and on algae (e.g. *Chlorella* spp.). ALA production has been tested using various reaction precursors and even recombinant *Escherichia coli* (Lin *et al*., 2009).

In this current study, ALA production by *B. paramycoides* was detected in the growth medium and bacterial production was successfully optimised by adding glutamate and/or levulinic acid at critical points during the linear growth phase of the bacterium. The early exponential phase was the best time to add the precursor glutamate, in order to maximise ALA production. The result corroborates the study by Ahn (2007) who encouraged *Bacillus cereus* to produce 2.0 mM of ALA after using glutamic acid, glucose and acetic acid as precursors. The utilization of glutamate by *B. paramycoides* indicated that it synthesises ALA by the C5 pathway (Nishikawa and Murooka, 2001).

To perform an initial test on the effectiveness of the ALA (and/or other metabolites also present in the bacterial culture broth), samples were centrifuged

to remove cells and the supernatant tested on cress seeds to quantify germination and seedling growth in Petri dishes. The improvement in germination and biomass of the cress indicated a dose-dependent stimulation of plant growth by the bacterial metabolites. Low concentrations of the broth (i.e. high dilutions) encouraged germination whilst higher concentrations did not. Koh and Song (2007) reported that improvement of seed germination was supported by bacterial metabolism which translated into plant growth promotion.

One of the attempts to improve bacterial production of the active compound, was to employ a nutrient-stress-strategy by reducing the strength of the culture medium as suggested by Buitelaar and Tramper (1992). A 1:100 dilution of half strength LB broth (plus bacterial metabolites) stimulated the germination index but did not affect biomass. A possible explanation for this might be that low nutrient conditions enhanced metabolite production, perhaps as a facultative strategy of either cooperation or competition and these metabolites unexpectedly also supported seed germination. Whilst some growth or germination benefits were observed, they were not substantial and separating effects of the control (bacterium-free) broth from those of broth containing bacterial metabolites was not straightforward. An experiment to test the separated and ruptured cells rather than the broth was carried out (data not shown), but did not yield any better germination results, probably because it is known that ALA is an extracellular metabolite (Pinu *et al*., 2017). Whilst the bacteria may have been synthesizing and secreting ALA with dose-dependent effect, ALA was not measured in these cultures. Bacteria produce and release other metabolites, e.g. microbial volatile organic compounds (mVOCs) which have similar growth promotion and inhibition characteristics to ALA (Tahir *et al.*, 2017). The recent research related to VOCs by Wu *et al.* (2019) showed the VOC's role in promoting

plant growth depending on the dose. Some *Rhizobacteria* emit a blend of volatile components that increase growth of *Arabidopsis thaliana* (Ryu *et al.*, 2003), whereas others inhibit growth or tend to be phytotoxic (Lemfack *et al.*, 2014).

Nevertheless, the small degree of positive stimulation of seedling growth that was attributed to the bacterial metabolites indicated the usefulness of testing the bacteria and their metabolites in soil. However, no bacterial metabolites effects were observed when tested against plants growing in soil.

Therefore, whilst stimulation of ALA production by the bacterium was successfully conducted under laboratory conditions by employing the use of a precursor and a degradative inhibitor, the ALA produced gave variable results with regard to either enhancing or preventing seed germination and plant growth. This may be for any number of reasons, but one that had to be tested was whether commercially supplied ALA affects the experimental plant species.

6.2 The effect of ALA on the vegetative growth of kale, radish and cress

A range of ALA concentrations were tested on kale, cress and radish plants grown in a peat-based compost. ALA was applied as a foliar spray or as a seed dressing. Radish responded less to ALA than the other species, both of which generally exhibited increased SPAD values at low ALA concentrations and lower SPAD readings at high ALA concentrations. These findings are in line with published work and suggest a transition from beneficial to herbicidal behaviour depending on the ALA concentration (e.g. (Fu *et al.*, 2014; Wu *et al.*, 2018). Any pH effects as a result of increased concentration had been addressed in a previous

experiment. The between-species differences in response may be attributed to the effectiveness of ALA in stimulating growth between plant species (Yang *et al.*, 2014). Another key observation is that of reduced biomass in plants subjected to the seed-soaking application method, compared to the foliar spray. This could be explained because a seedling will be more sensitive to ALA than a more mature plant. This observation makes sense in light of the FTIR data (albeit those are from a different set of plants), which suggested that the kale was more sensitive to seed-based ALA treatments than to the foliar spray. However, this pattern is not so readily rationalised because all seeds were soaked in either an ALA solution or in deionised water. Therefore, the only difference between the two 0 mM treatments is that one set of plants received an additional foliar spray treatment. Therefore, spraying the leaves increased biomass irrespective of ALA treatment and this is consistent across all three species. It is possible that misting the plants removed surface dust, or alternatively they benefited because of a transient increase in humidity. Although the plants were watered as required, the pots were only 9 cm diameter and it is noteworthy that this pattern was not observed in the following pot experiment which was undertaken in larger pots. Kale responded to the ALA seed dressing treatment with a slight increase in shoot biomass at low concentrations and a decline at the highest.

In the following pot experiment where a loam-based compost was used in addition to larger pots, there were no significant treatment effects on either shoot or root biomass. However, SPAD values were affected by ALA treatment, with the lowest concentration (0.0012 mM) giving the highest readings, yet the potential for increased photosynthesis was not realised; the foliar carbon content was unaffected by treatment, although these data are for total C and partitioning was not measured. Foliar nitrogen was slightly higher in plants amended with the

0.0012 mM seed dressing which corresponds with the SPAD data. One can tentatively suggest that the SPAD data can imply foliar nitrogen content. Whilst no calibration was performed to enable calculation of either chlorophyll or N from the SPAD data, there is an accepted relationship between chlorophyll and nitrogen content because most of the leaf nitrogen is associated with proteins in the thylakoids and the Calvin cycle (Evans, 1989). Therefore, there is some evidence that the plants treated with the lowest ALA concentration (seed dressing) had a greater photosynthetic capacity than plants assigned to other treatments. The unanswered question is why was this not reflected in plant biomass? Previous work by Roy and Vivekanandan (1998) showed that pre-soaking seeds with 10 mg L^{-1} of ALA increased plant growth, dry matter accumulation and chlorophyll content of three *Vigna* species (*Vigna catjung*, *V. mungo* and *V. radiata*) and Wei *et al.* (2012) highlighted the promoting effect of ALA in nitrogen absorption and assimilation.

6.3 The effects of ALA on foliar elemental concentrations

Foliar elemental concentrations were little affected by ALA treatment or by mode of application. Nevertheless, there were some broad patterns in the data. Plants which had undergone seed soaking with one or other of the two lowest ALA treatments (0.0012 mM or 0.012 mM) had higher concentrations of Cs (0.058 mg kg^{-1}; grand mean = 0.017 mg kg^{-1}; $p<0.001$); P (0.089 mg kg^{-1}; grand mean = 0.0045 mg kg^{-1}; $p=0.052$); Al (13.1 mg kg^{-1}; grand mean = 4.9 mg kg^{-1}; $p=0.069$); Pb (0.135 mg kg^{-1}; grand mean = 0.045 mg kg^{-1}; $p=0.052$). In tandem with this, some elements, e.g. P and K were present in lower concentrations in plants which originated from seed treated with the higher ALA

concentrations. Whilst these trends were only weakly significant, this observation may nevertheless have some bearing on the metabolic status of the foliage – for instance, P is important for carbohydrate production. Wu *et al.* (2018) discovered that exogenous ALA enhanced photosynthetic carbon fixation and assimilation; the data here suggest that this is predicated on the optimum ALA concentration.

The FTIR spectra illustrate the relative biochemical changes in the plants treated with one or other of the two ALA application methods and the different responses to the treatments. These data suggest that ALA treatment by pre-soaking seeds increased the foliar carbohydrate content. The spectra for plants amended with the 0.0012 mM seed dressing, had higher peaks associated with carbohydrates than plants which had seeds soaked in more concentrated ALA solutions. It is likely that the kale subjected to a low concentration of ALA in the seed dressing gained a metabolic advantage. It is interesting that the low-concentration-ALA-seed-soaking treatment increased the SPAD values, thus indicating that a consequence of the enhanced relative chlorophyll levels was a greater presence of carbohydrates which were not detected when just considering total foliar C. From analysis of other peaks, it seems apparent that seedlings were more sensitive to ALA treatment (seed soaking) than the plants when sprayed since these were older. The FTIR spectra appear to be sensitive to metabolic differences in the plants that were not necessarily obvious with other analytical methods in this Chapter.

The effectiveness of ALA in increasing carbon fixation, nitrogen assimilation, plant growth and yield were highlighted by (Nakashita *et al.*, 2010).

6.4 General conclusions and recommendations

Based on the findings of this study, application of ALA consistently improved chlorophyll content and biochemical profiles based on FTIR, and inconsistently enhanced shoot and root biomass and number of leaves on kale, radish and cress plants.

A key finding was the importance of selecting the correct ALA dose in order to illicit a positive and not a negative response, but also that the mode of delivery and/or the growth stage of the plant when treated affects its biochemistry. Furthermore, since different results were obtained from the two pot experiments, further work on the impact of different soil types and environmental stresses on the actions of ALA would be worthwhile in addition to investigating co-treatment with a range of macro- and micro-nutrients may be beneficial.

The initial assays quantifying and then optimising ALA production by *Bacillus paramycoides* have potential for further development. Ideally, the bacterium might be utilised as a growth promotor for sustainable agriculture, but if not, the species has an advantage over bacteria currently being used for ALA production in bioreactors which is, that it is not photosynthetic. This means large-scale production would potentially require less energy. Prior to development as a growth promotor to use in the field, understanding the effects of microbial volatile organic compounds (mVOCs) is another important consideration since these have similar characteristics to ALA.

The seed soaking treatment resulted in plants that were generally more responsive to ALA than those treated with the foliar spray more effective than the foliar spray.

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