Application of biostimulants on waterlogged cabbage to improve uptake of nutrients

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MASTER'S THESIS

Nadya Alexandra Buga

Zagreb, July 2023

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Supervisor:

Assoc. Prof. Marko Petek, PhD

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STUDENT'S STATEMENT

ON ACADEMIC RECTITUDE

I, **Nadya Buga**, JMBAG 0178131012, born on the 27th September 1993 in the Reading, UK, declare that I have independently written the master's thesis under the title of:

APPLICATION OF BIOSTIMULANTS ON WATERLOGGED CABBAGE TO IMPROVE UPTAKE OF NUTRIENTS

With my signature I guarantee:

- that I am the sole author of this thesis;
- that all literature references, published or unpublished, are adequately cited or paraphrased, and listed at the end of this paper;
- that this thesis does not contain parts of other papers submitted at the Faculty of Agriculture or other higher education institutes, for the reason of completing studies;
- that the electronic version of this thesis is identical to the printed one approved by the mentor, Professor Marko Petek, PhD;
- that I am familiar with the regulations of the Ethical Code of Students of the University of Zagreb (Art. 19).

In Zagreb,

Date: ______________________ Student signature: ______________________

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REPORT

ON EVALUATION AND MASTER THESIS DEFENSE

Master thesis written by Nadya Alexandra Buga, JMBAG 0178131012, under the title of

APPLICATION OF BIOSTIMULANTS ON WATERLOGGED CABBAGE TO IMPROVE UPTAKE OF NUTRIENTS

is defended and evaluated with the grade ________________ , on ____________________ .

Committee for thesis evaluation and defense:

1. Assoc. Prof. Marko Petek, PhD (mentor) ___________________________
2. Assoc. Prof. Klaudija-Carović-Stanko, PhD ___________________________
3. Assoc. Prof. Boris Lazarević, PhD ___________________________

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Abstract

In Croatia, short-term spring flooding is expected to be an increasing issue for young crops due to climate change. Three biostimulants were tested on young, waterlogged cabbage (*Brassica oleracea* var. *capitata*) to study the effect on nutrient status. The biostimulants were ExelGrow®, Organico, and EcoGreen, based on algae extract, microorganisms and amino acids, and micronized calcite respectively. The findings suggest that young cabbage plants are not highly susceptible to short duration waterlogging. Furthermore, the biostimulants did not have a large impact on the nutrient status of treated plants and, in some cases, there was a downward trend in treated plants. Multiple studies claim the application of algae extracts, microorganisms and nano-particles to aid plants under abiotic stress. Further research is required to assess the effect of these ingredients in biostimulant formulations when applied in response to flooding and waterlogged conditions.

Keywords: Abiotic stress, *Aschophullum nodosum*, nano-$CaCO_3$, plant growth promoting bacteria (PGPB)

1. Introduction

Climate change is a global concern due to its effect on temperature and weather patterns around the world and the impact this will have on various sectors, one of which is agriculture. Alongside warming and changing precipitation patterns, climate change is marked by an increase in the intensity and frequency of extreme events such as heatwaves, droughts, and heavy precipitation (IPCC, 2019). Heavy rainfall events, and their increasing intensity and frequency due to climate change, constitute a serious threat to agriculture, as soil flooding negatively impacts plant growth and can lead to plant death (Mustroph, 2018). There is an increasing amount of observational evidence that confirms an increase in heavy rainfall events in Europe of approximately 45% in the years 1981-2013 compared to 1951-1989 (Fischer & Knutti, 2016).

For the Republic of Croatia, some predictions state that the agriculture sector is the sector that will suffer the most damage as a result of climate change effects, with yields expected to decrease by 3-8% by 2050 (Ministry of Environment and Energy, 2018). It is estimated that 15% of Croatian territory is at risk of being flooded (Beslik & Causevic, 2019). In a recent study of farmers across various regions in Croatia, 80% of participants reported having experienced flooding on their field plots in the period 2015-2020 (Senko *et al*., 2022). Short-term flooding events (maximum 7 days) caused the most issues, with flooding occurring most frequently in the early growth stage before the appearance of the 5th leaf. This information gives reason to investigate measures that could aid in the protection of young crops against heavy precipitation events in Croatia and the European region.

Brassica crops are among the 10 most economically important vegetable crops globally (Francisco *et al*., 2017), and in 2021 represented 16.2% of all vegetables grown in Croatia (Ministarstvo poljoprivrede, 2022). Furthermore, in 2020, cabbage (*Brassica oleracea* L. ssp. *capitata* (L.) Duchesne) production reached 95% self-sufficiency in Croatia, the highest of all vegetables (Mesić *et al.*, 2016). Young cabbage seedlings are very sensitive to excess soil moisture, and require well-drained soil for optimal growth (Ghosh & Madhavi, 1998; Hsu & Wu, 2018). The nutrient content in *Brassica* crops is strongly influenced by environmental factors, including water availability, during growth (Francisco *et al*., 2017). Huđ *et al*. (2023) found that cabbage plants subject to short-term flooding showed significant changes in macro- and micro-elements; those flooded at an early stage had decreased content of P, K, Zn and Cu, and increased content of Mn, while plants flooded at a later stage (10 days later) only showed a significant decrease in K and Zn.

There is a growing body of research that supports the use of biostimulants to enhance plant growth and development, nutrient efficiency, stress tolerance and crop quality traits (Du Jardin, 2015; García-Sánchez *et al.*, 2022; Godlewska *et al.*, 2019). Biostimulants differ from fertilizers in that they do not supply essential nutrients to plants, but rather they enhance both nutrient uptake by the root system and transport in the plant (Godlewska *et al.*, 2019). This function is especially important during flooding, when nutrient uptake might become inhibited and eventually result in reduced yields (Jackson & Drew, 1984). Additionally, biostimulants have the potential to offer a natural alternative to synthetic products, thereby reducing environmental pollution, and their use in agriculture is predicted to become standard practice in the coming years as this sector moves towards more ecological management (Godlewska *et al.*, 2019).

1.1. Objective of Research

This research aims to investigate the effect of foliar biostimulant application on the nutrient content of young, waterlogged cabbage plants, simulating flooding conditions that are commonly reported by farmers in Croatia. The hypothesis is that biostimulant application will increase the nutrient content of waterlogged cabbage as compared to no biostimulant application. The specific aims are as follows:

1) Measure the macro- and micronutrient content in water-logged cabbage plants under the control treatment (no biostimulant application) and three different biostimulant treatments (Exelgrow®, Organico and Eco Green).
2) Determine if there is a significant difference in nutrient content between the control and biostimulant treatments.
3) If there is a significant difference, to determine which biostimulant treatment is the most effective at increasing nutrient content for water-logged cabbage plants.
4) Investigate if and how nutrient content is affected by earlier or later flooding (7 days later) under the control and biostimulant treatments.

2. Literature Review

There is a growing body of research that investigates the effects of biostimulant application to various crops experiencing biotic or abiotic stress. When considering abiotic stress conditions, most studies tend to focus on drought, salinity, or heavy metals exposure, and there is less information available on the effect of biostimulants on flooded or waterlogged crops. However, a major concern with waterlogging is oxidative stress and the impairment of the plant's antioxidant system. There are several papers that investigate flooding, oxidative stress and biostimulants, and these will be further explored in this section.

2.1. *Brassica oleracea*, Flooding and Nutrients

Brassica crops are considered a vital component of a healthy diet due to their high nutrient content and phytochemicals. However, the nutrient content in *Brassica* crops has been found to vary considerably according to the environmental factors during growth (light, temperature, water availability, soil fertility, etc.) (Francisco *et al*., 2017). Cabbage grows optimally in well-drained soil under relatively cool, moist weather, and exhibits good vegetative growth in the temperature range 15-20°C (Ghosh & Madhavi, 1998). Overwet environments create stress for plants by significantly slowing gaseous diffusion and preventing aerobic mechanisms that are vital for plant health (Jackson & Drew, 1984).

The main impacts of climate change felt by the agriculture sector in Croatia are: changes in vegetative period of crops, frequent droughts, and more frequent flooding/stagnation of surface water (Landau *et al.,* 2008). 'Flooding' is a general term for excessively wet conditions, and care must be taken to use consistent terminology for the type of excessive water conditions studied in an experiment. Four types of flooding have been suggested for use in flooding and low O_2 stress research (Sasidharan *et al*., 2017): (1) 'waterlogging' in which only the root-zone is flooded; (2) 'partial waterlogging' in which the root-zone is partially flooded; (3) 'submergence' in which the entire plant (root and shoot) is under water; and (4) 'partial submergence' in which the entire root system and only part of the above-ground organs are under water.

Plants generally respond in the following ways to flooding: overall decrease in shoot growth, inhibition of leaf growth, inhibition of stem extension, and inhibition of photosynthesis (slower increase in dry weight) (Jackson & Drew, 1984). Generally, root growth and function are facilitated by aerobic respiration, in which O_2 is supplied from the rooting environment and acts as the terminal electron acceptor in the electron transport chain (ETC) (Jackson & Drew, 1984). Excessive rainfall (e.g. spring floods) can create conditions that lead to root hypoxia or anoxia, and cause plants to switch to anaerobic metabolism, which negatively impacts root growth and activity (Arduini *et al.*, 2019; Blokhina *et al.*, 2003; Mustroph, 2018). Anaerobic metabolism is unable to provide sufficient energy for ion pumps and causes nutrient uptake via roots and radial transport to become immediately inhibited in nonwetland species, which subsequently reduces the quality and yield of crops (Chang *et al.*, 2016; Jackson & Drew, 1984). The severity of flooding impact depends on the duration of the flood, the type of flooding, the growth stage of the plant, and the plant species (Jackson & Drew, 1984). When only the root system is flooded (i.e. waterlogging), flooding effects on the shoot result from changes in the internal flow of substances (water, photosynthate, inorganic nutrients, hormones or precursors, and toxins) between the root and shoot (Jackson & Drew, 1984).

2.2. *Brassica oleracea* and Oxidative Stress

Several -oxic terms have been suggested for distinguishing different oxygen conditions during research: (1) 'normoxia', the reference condition for normal O_2 availability in air; (2) 'hypoxia' in which O_2 concentrations are below normoxic and specific processes might be affected; (3) 'anoxia', which describes a complete absence of O_2 in a system; and (4) 'hyperoxia' in which O_2 concentrations are above normoxia (Sasidharan *et al*., 2017). Periods of heavy precipitation (e.g. spring flooding) can lead to anoxic stress for many intolerant species, such as cabbage. Waterlogging-induced hypoxic or anoxic conditions impair plant metabolism and can result in oxidative stress, which occurs through the overaccumulation of reactive oxygen species (ROS) free radicals (superoxide anion, hydroperoxyl radical, alkoxy radical, and hydroxyl radical) and nonradical molecules (hydrogen peroxide and singlet oxygen) (Blokhina *et al.*, 2003; Loreti *et al.*, 2016; Mehla *et al.*, 2017). ROS interact with phytohormones and play an important role in stress signalling pathways, however, the build-up of ROS causes damage to important cellular components such as carbohydrates, proteins, lipids and DNA (Hasanuzzaman *et al.*, 2020; Raja *et al.*, 2017).

Excessive ROS accumulation is considered one of the most crucial consequences of abiotic stress, resulting from disequilibrium between the generation of ROS and their detoxification by the antioxidant defence system (Hasanuzzaman *et al.*, 2020). The antioxidant defence system comprises enzymatic (superoxide dismutase [SOD], catalase [CAT], ascorbate peroxidase [APX], glutathione reductase [GR], monodehydroascorbate reductase [MDHAR], dehydroascorbate reductase [DHAR], glutathione peroxidase [GPX], guaiacol peroxidase [GOPX], glutathione S-transferase [GST], Ferritin, nicotinamide adenine dinucleotide phosphate [NADPH], oxidase-like alternative oxidase [AOX], peroxiredoxins [PRXs], thioredoxins [TRXs], glutaredoxin [GRX]) and nonenzymatic (ascorbic acid [AsA], glutathione [GSH], phenolic acids, alkaloids, flavonoids, carotenoids, α-tocopherol, and nonprotein amino acids) antioxidants (Hasanuzzaman *et al.,* 2020). These antioxidants work in a coordinated manner to directly or indirectly scavenge ROS and/or inhibit their overproduction in the ascorbate-glutathione (AsA-GSH) cycle.

Several crop species can survive waterlogging conditions for various durations by activating their antioxidant defence systems (Hasanuzzaman *et al.,* 2020). For example, in maize, higher activity of SOD, peroxidase (POX) and CAT was noted under waterlogging conditions (Li *et al.,* 2018). When oxygen is unavailable and unable to act as the terminal electron acceptor in the electron transport chain (ETC), intermediate electron carriers become reduced, which interferes with redox-active metabolic reactions (Blokhina *et al.*, 2003); the antioxidant system can become impaired, with limited recycling, *de novo* synthesis and transport of antioxidants. *De novo* synthesis of antioxidants relies on a sufficient energy supply, which cannot be sustained during anoxic conditions (Blokhina *et al.,* 2003). This is where the antioxidant potential of biostimulants becomes useful.

2.3. Biostimulants

Biostimulants are agrochemical products that are formulated from natural substances and/or microorganisms and improve plant health/development in some way (García-Sánchez *et al.*, 2022). Biostimulants differ from fertilizers as they are only applied in small quantities and do not contain sufficient quantities of nutrients by themselves, but instead enhance nutrient uptake by the root system and transport within the plant (Du Jardin, 2015; Godlewska *et al.,* 2019). For this reason, biostimulants are considered metabolic enhancers that can increase the effectiveness of mineral fertilizers (Jannin *et al*., 2013).

Biostimulant application to crops has been reported to stimulate vegetative growth, improve nutrient uptake efficiency and distribution, increase tolerance to biotic and abiotic stress, improve plant vigour, increase antioxidant capacity, and ultimately enhance crop yield and quality (García-Sánchez *et al.,* 2022). The 5th Biostimulant World Congress reported nine categories of substances that act as biostimulants: (1) humic substances, (2) complex organic materials, (3) beneficial chemical elements, (4) inorganic salts, (5) algae extracts, (6) derivatives of chitin and chitosan, (7) antiperspirants, (8) free amino acids and N-containing substances, and (9) plant growth promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF), and *Trichoderma* spp. (García-Sánchez *et al.*, 2022). This study used biostimulants based on algae (ExelGrow®), microorganisms and amino acids (Organico), and nano-$CaCO_3$ (Eco Green). For this reason, only these biostimulants are the focus of this literature review.

2.4. Algae Biostimulants

ExelGrow® is a biostimulant made from fermented seaweed extract from *Ascophyllum nodosum*; it also contains K_2O (min 3.5%), organic carbon (min 9.6%), glycine betaine and salicylic acid (ADAMA, 2022; ADAMA, n.d.). According to the manufacturer, it is reported as improving nitrogen uptake efficiency, stimulating growth of the root system and protecting the plant from damage caused by abiotic stress.

A. nodosum is a brown inter-tidal seaweed that is the most widely researched seaweed in terms of biostimulant use (Shukla *et al.*, 2019). *A. nodosum* extracts (ANEs) are a source of phytohormones (abscisic acid, cytokinins, auxins), organic osmolites, macro- and micronutrients, vitamins, amino acids, and several bioactive compounds: poly- and oligosaccharides (laminaran, fucan, alginate), antioxidants, peptides, betaines, and secondary metabolites (sterols) (De Saeger *et al.*, 2020; Lola-Luz *et al.*, 2013). The precise composition of ANEs depends on the extraction method used (e.g. water-based extraction, acid/alkaline hydrolysis, microwave-assisted extraction, etc.) (Shukla *et al.*, 2019). ANEs are able to regulate molecular, physiological and biochemical processes in plants, improve plant growth and defence, and mitigate some abiotic and biotic stresses (Shukla *et al.*, 2019).

Several modes of action are proposed for ANEs (Figure 1). For *Brassica oleracea*, the mode of action relates to isothiocyanate, phenolic compounds and flavonoid compounds (Shukla *et al.*, 2019). Lola-Luz *et al.* (2013) found that the application of *A. nodosum* extract increased the content of phenolic and flavonoid compounds in *B. oleracea*, however, this did not translate to a significant increase in total yield compared to the untreated control, despite this effect being reported in other studies. Generally, the effects of ANEs on plants have been attributed to their content of phytohormones, micronutrients, and alga-specific polysaccharides, betaines, polyamines and phenolic compounds (De Saeger *et al.*, 2020).

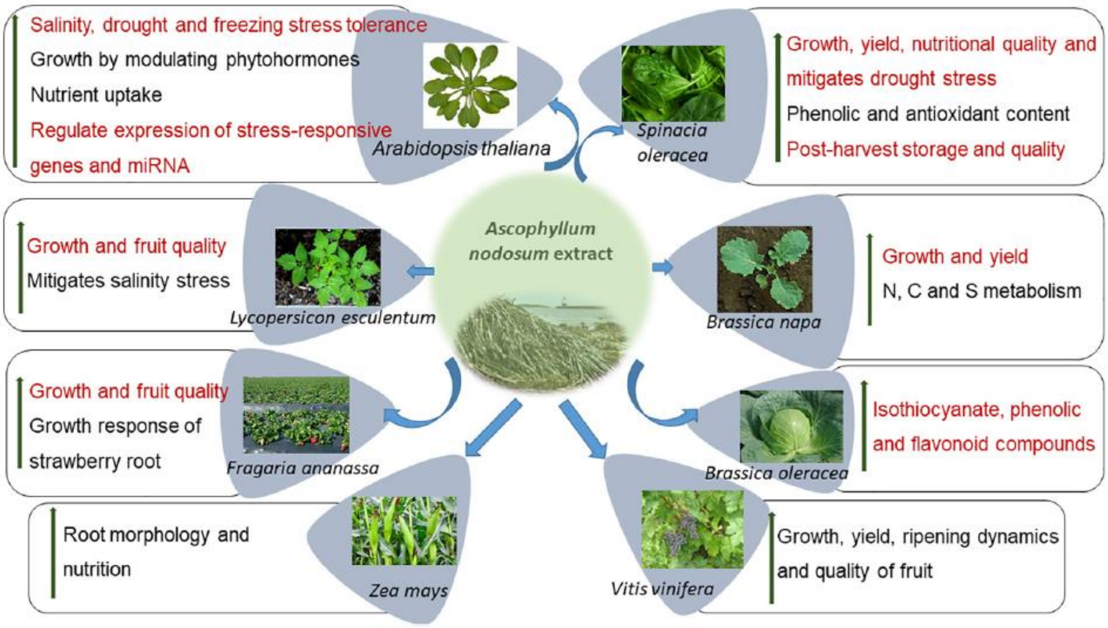

Figure 1: *Ascophyllum nodosum* extract (ANE) improves the growth of several crops by different modes of action (Shukla *et al.,* 2019).

2.4.1. Ascophyllum nodosum and Nutrient Acquisition

ANE applications have been found to increase nutrient availability and uptake, and nutrients from ANEs can be readily absorbed by leaves through stomata and cuticle hydrophilic pores (Hidangmayum & Sharma, 2017; Shukla *et al.*, 2019). Several studies report an increase in nutrients following ANE application, although the effect varies for different crops. In tomato, *A. nodosum* increased content of N, P, K, Ca, S, Mg, Zn, Mn and Fe (Di Stasio *et al.*, 2018). In olive, only K, Fe and Cu increased (Chouliaras *et al.*, 2009). In broccoli, there were increases in dry matter, yield, and content of P, K, Ca, and Mg (Gajc-Wolska *et al.*, 2012). Manusco *et al.* (2006) believe entry through the stomata to be the preferential route for K^+ and Ca^{2+} ion uptake, and additionally suggest that chelating compounds in seaweed (e.g. mannitol) are responsible for increasing nutrient availability and absorption. Other studies suggest that the increased nutrient availability and uptake is likely due to changes in the regulation of genes involved in nutrient acquisition (Shukla *et al.*, 2019). Through their microarray analysis, Jannin *et al.* (2013) identified approximately 1,000 genes whose expression was significantly affected by application of seaweed extract to *Brassica napus*. The most affected pathways involved carbon and photosynthesis, cell metabolism, and N and S and responses to stress. Specifically, they found the enhancement of genes that encode proteins involved in uptake and assimilation of N and S.

Several studies have noted that the beneficial effects of ANE application are dose dependent, with the best results being obtained on plants receiving a medium dosage. In their experiment, da Silva *et al.* (2012) found that the early development of *Brassica oleracea* L. improved the most with a 3.80 ml/L application of *A. nodosum* extract as compared to no application or a higher application of 4-6 ml/L. Similarly, Hidangmayum and Sharma (2017) found that onion growth parameters increased the most with a 0.55% treatment while higher concentrations showed a decreasing trend.

2.4.2. Protection From Abiotic Stress (Waterlogging)

One type of algae biostimulant protection from abiotic stresses stems from increased antioxidant activity (Godlewska *et al.,* 2019; Du Jardin, 2015). Plant tissues (e.g. seaweed) contain several enzymes and products that control the level of ROS and protect cells from stress. These include scavenging ROS enzymes, detoxifying lipid peroxidation (LP) products, and low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and tocopherols) (Blokhina *et al.,* 2003). ANE application has been found to increase glutathione reductase activity and enhance levels of ascorbate and glutathione in plants under stress (Ayad, 1998; Hasanuzzaman *et al.,* 2023). Glutathione has a reduced form (GSH) and an oxidised form glutathione disulfide (GSSG); they act together to maintain redox balance in cellular compartments (Blokhina *et al.,* 2003). Furthermore, through the AsA-GSH cycle, GSH can regenerate the water-soluble antioxidant ascorbic acid. Ascorbic acid is the main compound in the aqueous phase to detoxify ROS, as it is able to donate electrons in a variety of enzymatic and non-enzymatic reactions (Blokhina *et al.,* 2003).

Jannin *et al*. (2013) report on multiple studies of seaweed extract application to crops such as grapevine, strawberry, soybean, tomato, and maize. These studies found that seaweed extract application accelerated crop development cycles, increased total dry weight, increased proliferation of secondary root systems, enhanced leaf chlorophyll content, improved development, and increased crop growth (leading to increased yield, quality and size of harvested products). In the studies, authors suggest that phytohormones, such as auxins or cytokinins contained in the seaweed extract, are likely responsible for these effects. Jannin *et al.* (2013) propose that the beneficial effects may be the result of several components (phytohormones, betaines, polymers and nutrients) working together synergistically.

2.5. Microorganism and Amino Acid Biostimulants

Organico is an organic microbiological biostimulant that contains the beneficial microorganisms *Bacillus subtilis* 10^8, *B. megaterium* 10^7, and Saccharomyces sp. 10^6 in addition to: phytohormones (auxins, gibberellins, cytokinins), amino acids (proline), antibiotics, vitamins D, E, K and B complex (B1, B2, B3, B5, B6, B7, B9 and B12), chitinase, siderophores, glutathione (made from amino acids glycine, cysteine and glutamic acid), glucan, microencapsulants, and wetting agents (Biogeist, n.d.). According to the manufacturer, Organico can protect plants from extreme weather and disease, accelerate development, and increase the speed of reaction to stressful conditions and disease. In general, Organico has stimulating, bacterial and fungicidal effects and its application activates the plant's natural defence system (induced resistance and induced systemic resistance). It can be applied foliarly and protection peaks between days 6-8, lasting approximately 15 days (Biogeist, n.d.).

2.5.1. Bacillus spp. and Plant Growth Promoting Bacteria (PGPB)

Bacillus spp., such as those found in Organico, are popular bacteria used as biostimulants (Drobek *et al.,* 2019). Several *Bacillus* spp. have been found to suppress pathogens and promote plant growth, and for this reason they have been considered as plant growth-promoting bacteria (PGPB) (Sansinenea, 2019). PGPB are also known to change or release hormones in plants, produce plant growth-promoting volatile organic compounds, improve nutrient availability and uptake, and enhance abiotic stress tolerance (Ruzzi & Aroca, 2015). Some mechanisms of action are shown in Figure 2. With regards to water stress, attention is drawn to phytohormone level modulation, increased antioxidant activity, osmolyte production, and secretion of exopolysaccharides (EPS). While the majority of PGPB inhabit the rhizosphere and are isolated from the soil environment, some bacteria migrate aboveground and can be found on aerial parts of plants, although in decreasing bacterial density compared to rhizosphere populations (Compant *et al.*, 2010).

One method of using PGPB as a biostimulant in agricultural crops is through foliar application, in which a formula containing the PGPB is sprayed onto the leaves of the plant (Efthimiadou *et al.,* 2020). Foliar applications of PGPB (*B. subtilis*) have been found to stimulate plant growth and increase yield in apple varieties (Pirlak *et al.,* 2007), and increase the photosynthetic rate of kale by up to 89% (Kordatzaki *et al.*, 2022). Efthimiadou *et al.* (2020) also found foliar application of PGPB (*B. subtilis* and *B. megaterium*) to have a positive effect on the photosynthetic rate, yield, and dry weight of maize but noted that soil application gave better results than foliar application.

2.5.2. PGPB, Nutrients and Phytohormones

Many studies have investigated the effect of PGPB inoculations (*B. subtilis* and *B. megaterium*) on *Brassica* spp. and have found increases in nutrient status and phytohormone changes. PGPB inoculations lead to increased content of N, Ca, P, K, Mg, S, Mn, Fe, Cu, and Zn in Chinese cabbage and cauliflower (Ekinci *et al.*, 2014; Kang *et al*., 2019). In cauliflower, the highest recorded concentrations for N and P were from the *B. megaterium* inoculation and the highest concentrations of Ca, Na and Fe were from *B. subtilis* inoculation (Ekinci *et al.*, 2014). PGPB inoculations also improved the growth and development of host plants under stress conditions through the release of bioactive secondary metabolites including acetic acid, cytokinins, jasmonic acid, gibberellins, salicylic acid and abscisic acid (Kang *et al*., 2019; Pan *et al.*, 2020).

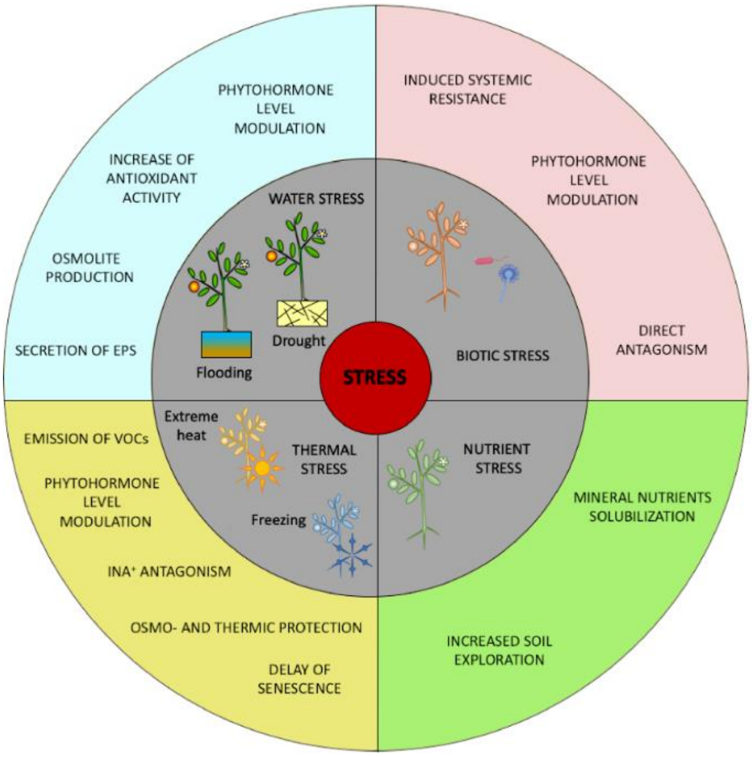

Figure 2. Schematic view of the protective mechanisms exerted by microbial biostimulants in relation to the stresses to which plants are subjected (Sangiorgio *et al.,* 2020).

The phytohormones mentioned above are plant growth regulators that are involved in various processes and stress responses, and their foliar application has been found to assist plant response to abiotic stress. Auxins (e.g. acetic acid) play an important role in the development of adventitious roots and promote ethylene (ET) biosynthesis as a response to waterlogging, while ethylene production can promote the transport of auxin (Pan *et al.*, 2020). Cytokinins play a role in delaying stress-induced leaf senescence, and induce the accumulation of proline (Bielach *et al.*, 2017). Jasmonic acid (JA) is also involved in the defence response to abiotic stress, and the application of JA can reduce damage to plants under waterlogging conditions (Kamal & Komatsu, 2016; Pan *et al.*, 2020; Xu *et al.*, 2016). Exogenous JA can also increase the content of ET, which aids in relieving waterlogging stress (Pan *et al.*, 2020). Kim *et al.* (2015) found that gibberellins (GAs) significantly increase under waterlogging in waterlogging-tolerant and waterlogging-resistant lines of soybean compared to waterlogging-sensitive lines. GAs are essential for regulating growth and development of plants, mainly by controlling the size and number of cells (Pan *et al.*, 2020). Under flooding conditions, the accumulation of abscisic acid (ABA) in above-ground parts of plants improves their resistance to environmental stress (Pan *et al.*, 2020).

With regards to phyllosphere bacteria and foliar application, nitrogenase activity and indol-3-acetic acid (IAA) production have been considered the most important plant growth promoting traits (Abadi *et al.*, 2020). Phyllobacteria also stimulate plant growth by nitrogen fixation, phosphorous solubilisation, and siderophore production (Abadi *et al.*, 2020). Due to their ability to meet plant nutrient requirements, PGPB in the phyllosphere are being investigated for use as biofertilizers to promote crop growth (Abadi *et al.*, 2020). In particular, the *Bacillus* genus was found to have a greater number of strains with plant growth promoting traits. However, when considering microbial biostimulants, they are applied in too small quantities to be considered as biofertilizers.

2.5.3. PGPB, Waterlogging and Oxidative Stress

One way plants respond to waterlogging is by the rapid accumulation of ethylene (ET) (Pan *et al.*, 2020). ET is produced by many plants as a generalised response to stress and its production is reliant on the action of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Del Carmen Orozco-Mosqueda *et al.*, 2020). Various stressors, including flooding, induce the transcription of ACC synthase genes. However, during anoxia ACC oxidase is unable to complete the conversion of ACC (the precursor of ethylene) into ethylene since it is oxygen-dependent (Sangiorgio *et al.,* 2020). Instead, ACC is translocated from waterlogged roots to aerial parts of the plant, where it is converted to ethylene and causes wilting, leaf chlorosis/necrosis, flower/fruit drop, growth inhibition, reduced yield or even death (Bradford & Yang, 1980; Glick *et al.,* 2014). PGPB act as a biological sink for ACC, since they possess the enzyme ACC deaminase that cleaves ACC into ammonia and α-ketobutyrate, thereby decreasing ACC in plants and ethylene in the leaves (Del Carmen Orozco-Mosqueda *et al.*, 2020; Honma & Shimomura, 1978, Glick *et al.,* 2014, Xu *et al.*, 2014).

With regards to other phytohormones, spraying exogenous salicylic acid (SA) was found to significantly increase the activities of ethanol dehydrogenase, POX, CAT, and proline in leaves and roots, which alleviated stress from waterlogging (Wang *et al.,* 2015). ABA application was also found to initiate the antioxidant defence system, resulting in reduced oxidative damage and improved waterlogging tolerance (Liu *et al.,* 2012).

The foliar application of *B. subtilis* has been found to increase the activity of the ROS scavenging enzymes polyphenol oxidase (PPO), SOD and POX, which play an important role in alleviating oxidative stress in plants (El-Gendi *et al.,* 2022). Another study found that the foliar application of *B. megaterium* under abiotic stress (drought) resulted in high expression of stress-related genes and also increased content of total sugars, proteins, proline, phenolics, K, Ca, ABA, and IAA (Devarajan *et al.,* 2021).

2.5.4. Fungi and Waterlogging Response

Saccharomyces cervisiae, found in Organico, is a plant growth promoting yeast (PGPY) that is used for soil fertilization or as a foliar application on plants (Ismail & Amin, 2014; Salim *et al.,* 2021). Yeasts (such as *S. cervisiae*) act as a natural stimulator and are rich in protein, carbohydrates, nucleic acid, lipids, minerals (Na, Fe, Mg, K, P, S, Zn, Mn, Cu, Si, Cr, Ni, Va, Li), thiamine, riboflavin, pyridoxine, hormones, biotin, B12 and folic acid (Nagodawithana, 1991). In general, PGPY benefit host plants through influencing phytohormone production, improving soil fertility and nutrient availability, and enhancing abiotic stress resistance (Nimsi *et al.,* 2023). Yeasts are also a direct source of enzymes and amino acids, and are Generally Recognized As Safe (GRAS) for field application (Nimsi *et al.,* 2023). This makes them a useful tool for sustainable agricultural production.

Various studies support yeast's positive effect on plant growth, total yield, quality, chemical constituents, formation of photosynthetic pigments, and increased antioxidant activity (Agamy *et al.,* 2013; Arokyaraj & Jagathan, 2018; Attia & El-Araby, 2016; Ismail & Amin, 2014).

Similarly to bacteria, they also contain and promote the regulation of plant growth hormones such as auxins, gibberellins, and cytokinins (Attia & El-Araby, 2016; Hegazi *et al.*, 2013; Ismail & Amin, 2014; Nimsi *et al.*, 2023; Salim *et al.*, 2021). As a general note, the cultivation of yeast with other PGPB enhances its ability to promote cell activation, root division and vegetative growth (Nimsi *et al.*, 2023; Salim *et al.*, 2021). This suggests that biostimulants containing fungi and bacteria together (i.e. Organico) would be more effective than biostimulants containing one of them on their own. Furthermore, it should be noted that positive effects of foliar application on plant growth are sometimes only seen at certain stages of development; for example, enhanced growth of shoots was seen in the first stage of wheat growth but not later on (Ismail & Amin, 2014). This should be taken into consideration when planning biostimulant application schedules.

2.5.5. Amino acids, Nutrients and Abiotic Stress

Amino acids are compounds that contain an amine functional group and a carboxylic acid functional group (Halpern *et al.*, 2015). They serve various functions in plants, including biotic and abiotic stress protection, signalling, N storage and chelation of metals (Vranova *et al.*, 2011). Commercially available amino acids tend to be short peptides known as protein hydrolysates, which have been isolated from plant, animal or microorganism material (Du Jardin, 2012). These can be applied foliarly and are absorbed by plants through the leaves via diffusion (Kolomazník *et al.*, 2012).

The application of amino acids as biostimulants has demonstrated positive effects on plant growth, yield and stress mitigation (Halpern *et al.*, 2015). Amino acids, such as cysteine, glycine and glutamic acid, are primary metabolites that can act as compatible solutes in cells experiencing abiotic stress (Ganie, 2021); accumulation of compatible solutes is one of the basic strategies employed by plants for their protection and survival during stressful conditions (Chen *et al.*, 2007). Haghighi *et al.* (2020) applied an amino acid mix containing L-cysteine, L-glycine and L-glutamic acid (among others) to drought stressed cabbage, with the effect of increased antioxidants and increased phenolic, protein and proline concentrations. In another study, the use of amino acid extracts enhanced soybean resistance to hypoxic stress (De Andrade Silva *et al.*, 2023).

Some studies have found that amino acid application can improve nutrient uptake (Sarojnee *et al.*, 2009). Exogenous amino acid application to plant leaves has been shown to increase uptake and nutrient-use efficiency of macro- and micronutrients, in particular N, Fe, Zn, Cu, and Mn

(Halpern *et al.*, 2015). Different crops vary in the way they respond to amino acid treatment; in some crops (apples and tomatoes) Ca uptake was improved with amino acid application, however there was no effect in other crops (kiwifruit) (Maini, 2006; Otero *et al.,* 2006). The mechanism for amino acid improvement of plant nutrition via foliar application involves direct changes to the plant physiology: improving nutrient mobility, changing root morphology and increasing activity of NO_3-assimilation enzymes (Halpern *et al.*, 2006). Amino acids can also chelate metals (Fe, Zn, Mn and Cu) and facilitate their absorption through the leaves (via specific amino acid transporters) and translocation within the plant (Ghasemi *et al.,* 2012; Halpern *et al.*, 2015; Jie *et al.*, 2008).

2.6. Mineral biostimulants

Eco Green is a micronised natural mineral powder (nano $CaCO_3$) obtained from calcite by tribomechanical activation (TMA) (Agroledina, 2014). The powder is dissolved in water and applied foliarly. According to the manufacturer, Eco Green increases plant immunity and resistance to pests, diseases and environmental stress. Furthermore, calcium carbonate penetrates into the leaf and is dissolved into calcium oxide (CaO) and then carbon dioxide (CO_2), enhancing photosynthesis in the leaf (Agroledina, 2014). Calcium oxide supports and intensifies primary and secondary metabolic processes and other physiological processes in the plant. Eco Green has been found to increase plant production by 10-30% compared to untreated plots, and treated leaves are larger and darker green (Agroledina, 2014).

Nanotechnology, a form of precision agriculture, is becoming progressively more prevalent in crop production due to its ability to enhance crop productivity (Elemike *et al.*, 2019). Plant products based on nanomaterials have several advantages over conventional products, including increased efficacy, reduced input requirements and lower environmental toxicity (Fu *et al.*, 2019). When applied as an ameliorative agent against plant stress, the success of nano-products is due to their small size, which improves their contact with leaf pores and facilitates translocation in plant vessels (Sajyan *et al.,* 2020). Additionally, foliar applications are beneficial to plants as they work rapidly and independently of soil conditions (Nassef & Younes, 2012).

Calcium (Ca^{2+}) is an important macronutrient for plant growth and development; it increases yield, nitrate absorption, chlorophyll content, transpiration and photosynthetic rates, and stomatal conductance (Seydmohammadi *et al.,* 2019; Tantawy *et al.,* 2014). Nano $CaCO_3$ has been applied foliarly to various ornamental plants and agricultural crops, resulting in increases in plant size, dry and fresh weight, yield, and improved nutrient absorption in shoots (Seydmohammadi *et al.*, 2019; Tantawy *et al.*, 2014). Currently, the body of research investigating nano $CaCO_3$ as a nano-fertilizer is greater than that investigating it as a biostimulant. In particular, the nano-fertilizer Lithovit has been extensively studied on various crops. Lithovit is a natural CO_2 nano-fertilizer made from micronized calcite that acts as a source of Ca and Mg (Farouk, 2015). Due to their similar composition, the results from the foliar application of Lithovit might be relevant when investigating the effects of foliar Eco Green application.

Similarly to Eco Green, foliarly applied Lithovit also dissolves into CaO and CO_2 and increases the intensity of photosynthesis (Bahloul *et al.*, 2021; Sajyan *et al.*, 2020). When applied foliarly to cucumber, broccoli, *Echinacea purpurea*, soybean, and potato, the beneficial effects were numerous: increased vegetative growth characteristics (plant height, number of leaves, leaf area and dry matter), increased chemical constituents (chlorophyll, carotenoids, total carbohydrates, nutrients), increased productivity and total yield, and increased total sugars and phenol compounds (Bahloul *et al.*, 2021; El-Aal & Eid, 2018; El-Baset, 2018; Farouk, 2015; Nassef & Younes, 2012).

Regarding nutrients, Lithovit has been found to increase absorption and content of certain elements. The nutrients influenced depend on the crop involved. In chilli peppers, Ca and Mg content was significantly higher in the foliage of treated plants (Sajyan *et al.*, 2020). This is to be expected, due to Lithovit containing Ca and Mg and acting as a foliar fertilizer. On salt stressed tomato plants, Lithovit contributed to better absorption of Ca, Mg, Si, Fe and Mn (Sajyan *et al.*, 2018). Once again, this is believed to be due to Lithovit containing Ca, Mg, and micronutrients such as Fe and Mn. In cucumber, Lithovit increased N, P, K, Ca, Zn and B in the foliage (Bahloul *et al.*, 2021). In *Echinacea purpurea*, Lithovit increased N, P and K content (El-Baset, 2018), while in soybean foliage, the content of N, P, K, Ca, Mg and Fe increased (El-Aal & Eid, 2018).

3. Materials and methods

3.1. Materials used for the experiment

Cabbage plants were produced by placing seeds of white cabbage (*B. oleracea* var. *capitata* cv. Varaždinski) into seedling propagation containers containing the substrate Klasmann Substrat 1 (Geeste, Germany). A pot experiment was then conducted under greenhouse conditions with small cabbage plants at the stage of four leaves. The experiment consisted of 3 different waterlogging regimes (according to the definition by Sasidharan *et al.*, 2017) and 4 experimental treatments.

Under waterlogging regime 1 (WR1, also referred to as "earlier flood"), soil was flooded (Day 1) and plants experienced waterlogging for seven days before the soil was drained. Plants received foliar treatment with biostimulants two and nine days after the end of the waterlogging (Day 9 and Day 16). Plants were sampled seven days after the final biostimulant application (Day 23). Under waterlogging regime 2 (WR2, also referred to as "later flood"), soil was flooded seven days later (Day 8) and these slightly older plants experienced the same waterlogging conditions for seven days before the soil was drained. The same biostimulant treatments were applied to the plants two and nine days after the end of the flood (Day 16 and Day 23). Plants were sampled seven days after the final biostimulant application (Day 30). Under the control waterlogging regime (WR0), the soil was not flooded and plants did not experience waterlogging. These plants were sampled on Day 23 and Day 30 to be mimic the age of plants in WR1 and WR2.

Regarding the experimental treatments: treatment 1 (T1) consisted of the foliar application of Exelgrow®, an algae extract biostimulant, at a dose of 0.5 mL/0.5 L water; treatment 2 (T2) consisted of the foliar application of Organico, a microorganism and amino acid biostimulant, at a dose of 2.5 mL/0.5 L water; treatment 3 (T3) consisted of the foliar application of Eco Green, a biostimulant with fine calcium carbonate nanoparticles, at a dose of 1.5 g/0.5 L water; the control (T0) consisted of plants grown the foliar application of water only. There were 3 pots per treatment, representing 3 replicates for each condition. There was one plant in each pot.

3.2. Mineral analysis

Leaf samples were dried at 105 °C and ground to a fine powder. Total nitrogen (% dry matter) was determined using the Modified Kjeldahl Method, AOAC 2015: digestion in H_2SO_4 + salycilic acid at 420°C (FOSS DT220 Digestor with SR 210 Scrubber), followed by distillation with 35% NaOH (FOSS Kjeltec 8100 Distillation unit) and then titration with 0.01 M HCl. For the other elements, the samples were digested in a microwave oven (Milestone, ETHOS UP Microwave) with HNO_3 and $HClO_4$. Total phosphorous (% dry matter) was determined using the Spectrophotometric Molybdovanadophosphate Method, AOAC 2015 (Thermo Scientific Evolution 60S UV-Visible Spectrophotometer). Total potassium (% dry matter) was determined using flame photometry (Jenway PFP-7 Flame photometer). Total macroelements calcium and magnesium (% dry matter), and microelements iron, zinc, manganese and copper (mg/kg dry matter) were determined using atomic absorption spectrometry (Thermo Scientific, SOLAAR M Series Atomic Absorption Spectrometer).

3.3. Statistical Analysis

The samples, collected in triplicate, were analysed individually and the results are displayed as average values. Statistical data analysis followed the analysis of variance (ANOVA) according to general linear model (GLM). Fixed factors were waterlogging treatment and biostimulants treatment. The SAS System for Win program was used. ver 9.1 (SAS Institute Inc.), and Tukey's Honestly Significant Difference (HSD) test (SAS, 2002-2003) was used to test the results.

4. Results and Discussion

4.1. Comparison of earlier (WR1 T0) and later (WR2 T0) flooding control with no flooding control

In general, 7-day flooding (earlier or later) did not have a large impact on the young cabbage plants for the parameters tested. In neither of the flooding conditions (WR1 or WR2) were there any significant differences compared to the control (no flood) for % dry weight, fresh weight, number of leaves, K, Ca, Mg, Fe and Mn (Tables 1-2). Regarding plant biomass, these results are consistent with Huđ *et al.* (2023), who reported no significant difference in mass for 3-day flooded small cabbage plants compared to the unflooded control. For the earlier flood (WR1), there was additionally no significant difference with the control for N and Cu. A statistically significant difference was noted for P and Zn, which were lower (5.4% and 19.7% respectively) under WR1, suggesting that waterlogging conditions might affect these nutrients. Interestingly, Huđ *et al.* (2023) also reported a significant decrease for P (34.3%) and Zn (30.5%), however, their experiment resulted in additional decreases in K (27.2%) and Cu (24.1%), which were not seen in this case.

Table 1

Mean values for nutrient content and ANOVA results comparing no flood (WR0 T0) with the earlier flood control (WR1 T0). Different letters represent significantly different values according to the Tukey HSD test, p≤0.05. The non-letter values are not significantly different.

Treatment	Dry mass (%)	N	P	K	Ca	Mg	Fe	Zn	Mn	Cu	Fresh mass (g)	Number of leaves
WR0 T0	10.03	2.25	0.37 a	3.64	1.71	0.29	31.68	28.31 a	21.94	9.26	3.04	10
WR1 T0	10.09	2.05	0.35 b	2.78	1.63	0.25	29.27	22.72 b	25.86	9.83	3.58	8
Fexp	0.01	1.76	49.00	10.32	0.29	10.56	1.07	37.48	0.63	0.14	5.38	3.57
Pr>Fexp	0.9344	0.3161	0.0198	0.0848	0.6432	0.0831	0.4101	0.0170	0.5094	0.7415	0.1462	0.1994

Table 2

Mean values for nutrient content and ANOVA results comparing no flood (WR0 T0) with the later flood control (WR2 T0). Different letters represent significantly different values according to the Tukey HSD test, p≤0.05. The non-letter values are not significantly different.

Treatment	Dry mass (%	N	P	K	Ca	Mg	Fe	Zn	Mn	Cu	Fresh mass (g)	Number of leaves
WR0 T0	8.88	2.06 b	0.43	3.65	1.32	0.20	39.17	29.18	47.13	7.36 a	2.79	8
WR2 T0	8.61	2.82 a	0.46	3.57	1.41	0.17	25.88	22.63	40.87	5.77 b	3.66	10
Fexp	0.29	26.55	3.00	0.07	1.94	1.58	1.24	8.93	1.31	41.93	2.32	4.00
Pr>Fexp	0.6417	0.0357	0.2254	0.8134	0.2981	0.3356	0.3812	0.7735	0.3716	0.0230	0.2669	0.1835

For the later flood (WR2), there were no significant differences with the control for P and Zn, indicating mixed results. The only statistically significant differences in WR2 were for N (27.0% higher than the control) and Cu (21.6% lower than the control). Generally, the results seem to indicate that young cabbage plants are not very sensitive to 7-day waterlogging conditions and agree with findings from Huđ *et al.* (2023), who suggest that cabbage plants are more sensitive to floods of longer duration. For example, Casierra-Posada & Cutler (2017) found cabbage to show little tolerance to 25-day flooding, which caused significant changes in growth parameters and chlorophyll content.

4.2. Biostimulant Treatment Results for the Earlier Flood (WR1)

Under the earlier flood (WR1), no significant difference was found for ExelGrow® (T1) and Organico (T2) compared to the control for % dry weight, fresh weight, number of leaves, N, P, K, Ca, Fe, and Zn (Table 3). The only statistically significant difference is lower Mg in both T1 (24.0% decrease) and T2 (24.0% decrease), and higher Mn (36.4% increase) and lower Cu (23.6% decrease) in T1 only. In other studies, following *A. nodosum* extract (ANE) application, Mg increased in some crops (tomato, broccoli), while in other crops there was no difference (olive)

Table 3

Mean values for nutrient content and ANOVA results comparing the earlier flood (WR1) biostimulant applications (T1-3) with the control (T0). Different letters represent significantly different values according to the Tukey HSD test, p≤0.05. The non-letter values are not significantly different.

Treatment	Dry mass (%)	N	P	K	Ca	Mg	Fe	Zn	Mn	Cu	Fresh mass (g)	Leaves (number)
WR1 T0 (control)	10.09	2.05	0.35	2.78	1.63	0.25 a	29.27	22.72	25.86 b	9.83 a	3.58	8
WR1 T1 (ExelGrow®)	11.10	2.20	0.32	2.58	1.52	0.19 b	57.86	23.78	40.64 a	7.51 b	4.13	8
WR1 T2 (Organico)	10.22	2.22	0.34	2.76	1.54	0.19 b	33.21	24.43	33.99 ab	9.08 ab	5.07	8
WR1 T3 (Eco Green)	10.65	2.18	0.34	2.63	1.75	0.23 a	34.68	23.64	31.44 ab	9.15 ab	4.52	9
Fexp	2.02	0.75	0.40	0.41	0.94	30.71	0.80	0.28	2.62	3.99	1.42	0.53
Pr>Fexp	0.2121	0.5604	0.7557	0.7535	0.4795	0.0005	0.5355	0.8349	0.1455	0.0704	0.3269	0.6761

(Chouliaras *et al.*, 2009; Di Stasio *et al.*, 2018; Gajc-Wolska *et al.*, 2012). Interestingly, there was no report of significant decreases in nutrients following ANE application. This confirms that, regarding nutrient uptake, different crops respond differently to ANE application, and this is expected to be seen with ExelGrow® applications. Higher Mn was seen in some studies (Di Stasio *et al.*, 2018), however, no studies considered here reported an effect of ANE application on Cu. In this case, the data suggest that ExelGrow® applications can lead to a decrease in Cu. With regards to Organico, despite studies reporting beneficial effects of microbial and amino acid applications on plant nutrient uptake, this cannot be confirmed here under the earlier flooding regime.

For Eco Green (T3) no significant differences were found in any of the parameters measured (% dry weight, fresh weight, number of leaves, macronutrients and micronutrients) compared to the control. Unlike T1 and T2, Mg was not lower than the control with Eco Green treatment; this is consistent with previous studies that have found micronized calcite to be a source of Mg as well as Ca (El-Aal & Eid, 2018; Sajyan *et al.*, 2018, 2020). However, there were no beneficial effects on the other parameters measured compared to the flooded control, suggesting that Eco Green treatment may be unnecessary for young cabbage plants experiencing this type of flooding regime.

4.3. Biostimulant Treatment Results for the Later Flood (WR2)

Under the later flooding regime (WR2), for all the biostimulant treatments (T1-T3) % dry matter was significantly higher than the control (T0), while there were no significant differences for Ca and Fe (Table 4). Flooded plants generally demonstrate a slower increase in dry weight than those growing in optimal soil (Jackson & Drew, 1984), so this increase indicates a positive influence of biostimulants on waterlogged plants. Furthermore, increase dry matter takes place when photosynthesis is greater than respiration, indicating sustainable plant growth and development (Bhattacharya, 2019). In their study, Zeng *et al.* (2020) found that insufficient absorption of nutrients by the roots led to reduced dry matter accumulation. It is interesting that dry matter was increased in T1-T3, despite many lower nutrients in some treatments (e.g. Organico). Dry matter accumulation is also important for determining crop yield formation (Cai *et al.*, 2023), although this has not been investigated in this study of cabbage.

Plants receiving ExelGrow® treatment (T1) had lower Mg (17.6% decrease) and Cu (42.8% decrease) than the control, and fewer leaves (10% decrease). The lower Mg and Cu results for WR2 match results from the earlier flood. Although Cu experienced a significant decrease in the WR2 control compared to the no-flood control, the decrease under T1 is much larger. This suggests that the age of the plant influences nutrient uptake to some extent (since Cu was unaffected in younger flooded plants), but also that the application of ExelGrow® did not counteract the decrease that occurs in slightly older plants. In fact, the application of ExelGrow® in this case had a negative effect on Cu content.

Plants treated with Organico (T2) had significant differences for N (26.2% decrease), P (19.6% decrease), K (13.4% decrease), Mg (11.8% decrease), Zn (23.7% increase), Cu (35.0% decrease), fresh weight (30.9% decrease) and number of leaves (20% decrease) compared to the control. This contrasts greatly with results from the earlier flood, in which the only significant differences were for Mg, Mn and Cu. Similarly to the other biostimulant treatments under WR2, Mg was also lower here compared to the control. The result for Cu is consistent with the later flood control, in which Cu was significantly lower in the flooded condition than the non-flooded condition. Zn was the only nutrient that increased under this treatment; as this nutrient was higher in the unflooded controls, this indicates a positive influence of the biostimulant. However, considering that most nutrients were unaffected by the later flood control, the results suggest that Organico application might generally have a negative effect on nutrient status of cabbage under waterlogged conditions.

Eco Green (T3) had significantly lower Cu (27.6% decrease) than the control, but significantly higher Mn (27.9% increase). The lower Cu result here is consistent with the other WR2 treatments; flooding at a later stage does result in lower Cu, and the application of Eco Green in this case

Table 4

Mean values for nutrient content and ANOVA results comparing the later flood (WR2) biostimulant applications (T1-3) with the control (T0). Mean values for nutrient content and ANOVA results comparing the earlier flood (WR1) biostimulant applications (T1-3) with the control (T0). Different letters represent significantly different values according to the Tukey HSD test, p≤0.05. The non-letter values are not significantly different.

Treatment	Dry mass (%)	N	P	K	Ca	Mg	Fe	Zn	Mn	Cu	Fresh mass (g)	Leaves (number)
WR2 T0 (control)	8.61 b	2.82 a	0.46 a	3.57 a	1.41	0.17 a	25.88	22.63 b	40.87 b	5.77 a	3.66 a	10 a
WR2 T1 (ExelGrow®)	9.62 a	2.31 ab	0.42 ab	3.18 ab	1.35	0.14 b	30.67	22.30 b	43.30 b	3.30 c	3.36 ab	9 b
WR2 T2 (Organico)	10.06 a	2.08 b	0.37 b	3.09 b	1.35	0.15 b	29.31	29.65 a	47.10 ab	3.75 bc	2.53 b	8 b
WR2 T3 (Eco Green)	9.68 a	2.32ab	0.43 a	3.30 ab	1.43	0.17 a	34.66	23.84 ab	56.71 a	4.18 b	3.14 ab	9 ab
Fexp	10.88	3.31	4.73	2.41	0.21	15.29	1.50	3.20	3.65	46.21	2.47	7.00
Pr>Fexp	0.0083	0.0991	0.0505	0.1694	0.8890	0.0032	0.3077	0.1048	0.0829	0.0002	0.1594	0.0219

has not benefitted Cu nutrient status. Overall, none of the biostimulant treatments (T1, T2, T3) were able to prevent the decrease in Cu caused by waterlogging conditions. With regards to Mn, the only other condition in which higher Mn was recorded was in the ExelGrow® treatment (T1) under the earlier flood (WR1). The reason for these results is unclear with the present data.

Eco Green (T3) had significantly lower Cu (27.6% decrease) than the control, but significantly higher Mn (27.9% increase). The lower Cu result here is consistent with the other WR2 treatments; flooding at a later stage does result in lower Cu, and the application of Eco Green in this case has not benefitted Cu nutrient status. Overall, none of the biostimulant treatments (T1, T2, T3) were able to prevent the decrease in Cu caused by waterlogging conditions. With regards to Mn, the only other condition in which higher Mn was recorded was in the ExelGrow® treatment (T1) under the earlier flood (WR1). The reason for these results is unclear with the present data.

4.4. Biostimulant effects on cabbage roots

In the present experiment, only the aboveground organs of the cabbage plants were analysed. Despite this, visual inspection of the root systems revealed differences under different treatments (Figure 3). The largest visual difference is seen in the unflooded control, which has a much more extensive root system compared to the flooded control and other treatments. This is to be expected, as cabbage plants are known to grow optimally in soils with good drainage that are not waterlogged. Out of the biostimulant treatments, T1 (ExelGrow®) appears to improve the root system most under flooding conditions compared to no treatment and the other biostimulants tested. Of course, laboratory analysis would be required to confirm this.

In future studies, it would be interesting to conduct full analysis on the root system in addition to aboveground plant organs, as this may contribute to a more thorough understanding of the effect of biostimulants on the overall growth of the plant. A more extensive root system would enable greater nutrient acquisition once waterlogged conditions recede. Therefore, these plants might be expected to show increased nutrient status after more time has elapsed under normal soil water conditions, compared to those with a less developed root system. It is possible that greater differences might have been detected if the plants had been sampled at a later date.

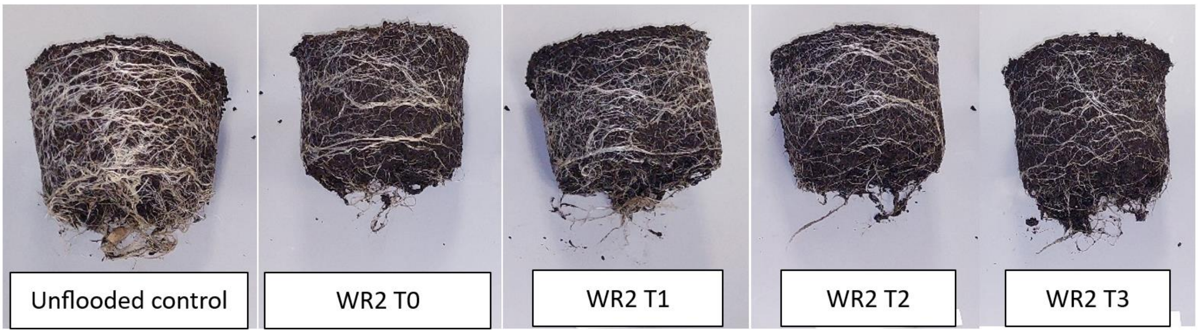

Figure 3. Root systems of *Brassica oleracea* var. *capitata* under different treatments: Unflooded control, later flooding control (WR2 T0), later flooding ExelGrow® treatment (WR2 T1), later flooding Organico treatment (WR2 T2), and later flooding Eco Green treatment (WR2 T3).

5. Conclusions

The effect of 7-day flooding (waterlogged conditions) on young cabbage plants (*B. oleracea* var. *capitata*) at the stage of 4 leaves was investigated. Three biostimulants were tested: ExelGrow®, Organico, and Eco Green, based on algae extract, microorganisms and amino acids, and micronized calcite respectively. In addition to this, the effect of earlier flooding and later flooding (7 days later) on cabbage plants were compared to unflooded controls. In agreement with previous studies of this kind, the results indicate that young cabbage plants are not very sensitive to short duration waterlogging.

Overall, there were not many significant differences in nutrients in treated plants compared to untreated controls, and the data suggests that biostimulant applications did not improve nutrient uptake in young cabbage plants following 7-day waterlogging stress. The application of ExelGrow® confirms that different crops respond differently to ANE applications, and different nutrients are affected in different ways. In the later flood, plants treated with Organico had many nutrients in lower quantities than untreated plants, indicating that Organico application might have a negative effect on nutrient status of cabbage under waterlogged conditions. Further studies are needed to investigate this finding. For plants treated with Eco Green, there were no beneficial effects on any of the parameters measured in the earlier flood compared to the control. In the later flood, Cu decreased and Mn increased in treated plants, but there were no significant differences in any of the other parameters measured.

Considering the visual differences in the root systems under different treatments, it is recommended to conduct full analysis on the root system in addition to aboveground plant organs in future studies. It is also recommended to test phytohormone content under different treatments, as it is possible that some growth characteristics (e.g. roots) might be affected due to phytohormones, despite little difference being found in nutrient status. It is also possible that greater differences might have been detected under longer flooding conditions, or if plants had been allowed to grow for a longer amount of time once flooding conditions had receded. It would be interesting to grow crops until harvest to determine the effect of biostimulants on yield and marketable characteristics. As farmers in Croatia report 7-day flooding as being the most problematic, it may be more worthwhile to investigate other crops that are more sensitive to this duration of flooding.

6. References

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Curriculum Vitae

Nadya Buga was born in Reading, UK, on September 27th 1993. She was home-educated for the first years of her life and then joined the formal education system at fourteen years old, where she successfully completed her GCSEs. At 16, she joined Reading College where for two years she studied Biology, Psychology, Law, and English, having an interest in many different subject areas. Several years of full-time work in the customer service industry, with intermittent periods of traveling, allowed Nadya to explore Australia, New Zealand, Asia, South America and several European countries, during which she developed a passion for and interest in the earth and environmental protection. Inspired to study abroad, in 2017 Nadya began an undergraduate degree in Earth Systems at the University of Malta, where she later graduated at the top of her class with first class honours. Throughout her undergraduate degree, she became interested in the vast areas of land dedicated to agricultural production, the land management practices employed, and the implications these have on environmental health. This led Nadya to the decision to turn her attention to agriculture with a focus on the environment. Currently, Nadya is completing her master's degree in Agriculture, Environment and Resources Management at the Faculty of Agriculture, University of Zagreb.