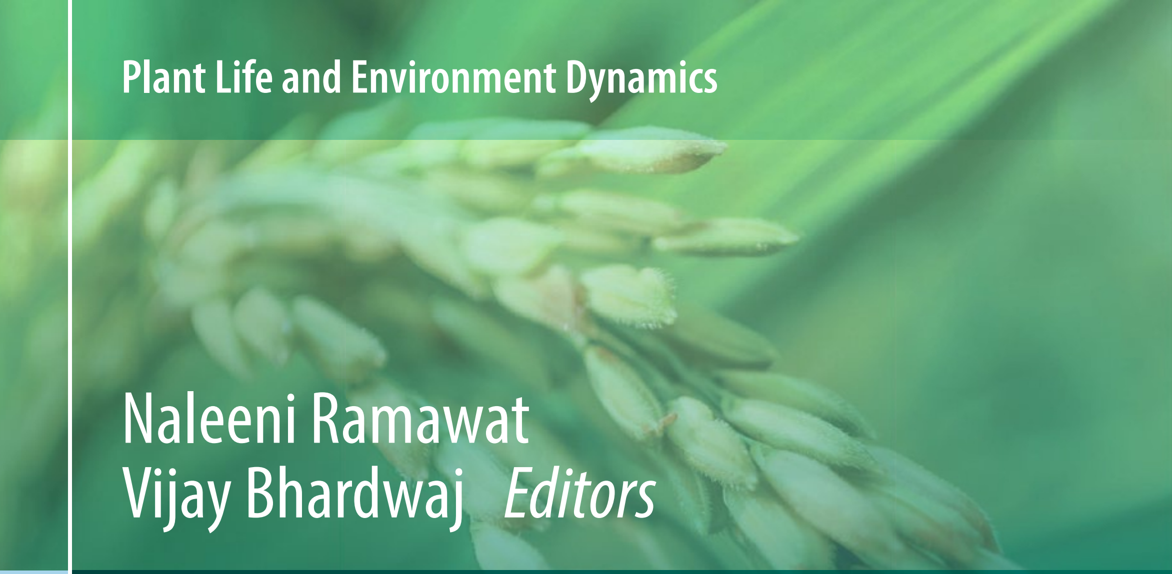

Plant Life and Environment Dynamics

Naleeni Ramawat
Vijay Bhardwaj *Editors*

Biostimulants: Exploring Sources and Applications

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Biostimulants: Exploring Sources and Applications

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ISSN 2730-6755 ISSN 2730-6763 (electronic)
Plant Life and Environment Dynamics
ISBN 978-981-16-7079-4 ISBN 978-981-16-7080-0 (eBook)
https://doi.org/10.1007/978-981-16-7080-0

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Preface

"Biostimulants" are materials of natural origin. An urge to search for new preparations based on natural substances that can replace or provide ecological alternatives to the application of chemicals in agriculture has attracted the interest of industry, academia and researchers to this category of products. Growing consumer awareness for safe and sustainable food vis-à-vis organic agriculture industry is driving demand for such organic products. Scientific evidence supporting the beneficial properties and their role in alleviating stress is also leading to popularity of biostimulants among both organic and conventional farming community.

Plant biostimulants may be defined as "materials containing substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality". Biostimulants are a new class of products for which legal definitions do not exist and regulatory pathways are unclear. Although for almost a decade, these biostimulants are existing in market yet a research-based industrial portfolio has recently been emerged. The role of biostimulants in sustainable agriculture is still being studied with interest by scientist, academicians and regulators. Amid its multifunctional roles, it is difficult to categorize biostimulants as pesticides or fertilizers; hence, the regulatory framework in many countries is still in its nascent stage.

Biostimulants is a potentially novel approach regulating physiological processes in plants to enhance their inherent stress-induced limitations and simultaneously stimulating growth and development and ultimately increasing yield. It is known that the biological functions of the plant can be positively moderated through application of these biological materials, or their mixtures. But an explicit mode of action has not been defined. Given the difficulty in determining a "mode of action" for these biological materials and recent developments in regulatory framework to attain legitimate status, biostimulant research and validation of its effects necessitates the need to determine broad mechanism and mode of action of various biostimulants. However, there is lack of availability of recently compiled work on biostimulants and its role in regulating plant growth and development, physiological processes and underlying mechanisms providing strength to plants under stress conditions.

Therefore, through this book an attempt has been made to address the diverse and complex ways in which biostimulants affect the mechanisms for plant survival and offers alternative to chemical farming. Information has been collected worldwide regarding advances made in research on soil micro-organisms and chitin, protein hydrolysates, biochemical materials, humic acids, organic acids as fulvic acid, extract from seaweed and other similar materials in order to make these outcomes easily accessible to researchers, academicians, industry and students at one place. *Biostimulants: Exploring Sources and Applications* summarizes and offers an insightful introduction to biostimulants, their categories, modes of actions, sources and their biostimulant application strategies as well as biostimulatory effects.

The book is composed of 11 chapters. Chapter 1 deals with introduction to the subject of plant biostimulants and an overview of categories and effects. Chapter 2 provides an insightful biostimulant potential of seaweed extracts derived from Laminaria and Ascophyllum nodosum. Chapter 3 deals with borage extracts as biostimulants of plant growth and development. Chapter 4 discusses the role of simple organic acids as plant biostimulants. Chapter 5 describes the protein chemistry of both the raw materials and the hydrolyzed protein, excursus on the main analytical methods used for its characterization vis-à-vis animal-derived hydrolyzed protein and its biostimulatory effects on plants have been discussed. Chapter 6 explains protein hydrolysates as biostimulants for plant growth and Development highlighting the technologies used in PH-based biostimulant productions their enzymatic processing, and recent advances in biostimulant research and development, as well as the incorporation of new phenomics and transcriptomic technologies to elucidate the mode of action of these biostimulants. Chapter 7 describes various bioactive compounds present in the agri and food waste industry and their utilization to produce high value product as biostimulants. Chapter 8 deals with the Foliar application of microbial and plant-based biostimulants on plant nutrition. Chapter 9 elaborates the role of biostimulants in plant growth, developments and abiotic stress management covering recent insights in this field. In continuation, Chapter 10 describes the role of biostimulants in agriculture including enhanced plant metabolism, improved yield and crop quality, increased tolerance against abiotic stresses among others. Chapter 11 elaborates further on emerging trend and opportunities in biostimulants discussing microbial and non-microbial biostimulants and the development of tools to address the need to create harmonized regulatory processes encouraging global market for plant biostimulants.

The global market for biostimulants was valued at $2.19 billion in 2018 and is projected to reach a compound annual growth rate of 12.5% from 2019 to 2024. The book also presents the market potential of biostimulants, available products as well as legislation.

The primary objective of this book is to explore sources of biostimulant production, its influence plant growth and development and regulatory status of plant biostimulants for better understanding and opening new vistas for future research.

The book will give insight into various aspects of biostimulants and would be very useful for academicians, researchers, farmers, students and all the stakeholders of the biostimulant industries.

Jodhpur, Rajasthan, India — Naleeni Ramawat
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About the Editors

Dr Naleeni Ramawat, PhD is working as Associate Professor (Agronomy), College of Agriculture, Agriculture University, Jodhpur, Rajasthan. Earlier she has worked as a Director at Amity Institute of Organic Agriculture, Amity University Uttar Pradesh, Noida, India. She has to her credit meritorious service of over 17 years in teaching, research, and administration in the discipline of agricultural crop production and management. She has a doctorate in agronomy with specialization in crop production and crop modeling. Her primary area of research is crop modeling, plant nutrition, organic agriculture, and abiotic stress management. She is extensively involved in academics, research and extension, and institution building activities, such as being the director of Amity Institute of Organic Agriculture for the past one decade. She has been recipient of BIRAC—Grand Challenges India Award as Co-PI for Agri-Nutrition and also Young Scientist Award in 2016. Her excellence is reflected in her more than 60 peer-reviewed national/international publications. Also, she has delivered a plethora of oral and poster talks. She has also experience in handling research projects sponsored by MoA, GOI, DST, BIRAC, and Nanomission on zinc biofortification, agri-voltaics, and organic agriculture.

Dr Vijay Bhardwaj, PhD, MBA is director of Bioatlantis India who received his PhD degree in Agriculture Entomology from the Himachal Pradesh Agriculture University. He was a recipient of an Indo-Israel Research fellowship in the year 2006. He has been associated with the area of biostimulants since the last 10 years, having worked across various geographies to grow biostimulant acceptance among growers and businesses. He has been a member of the Board of Studies of Amity Institute of Organic Agriculture and has been an industry expert and a mentor to postgraduate students in food and agriculture.

Plant Biostimulants: Overview of Categories and Effects

1

Radjassegarin Arumugam and Gabriel Amalan Rabert

Abstract

Modern agricultural practices primarily rely on synthetic chemical fertilizers and pesticides to increase growth and yield. The indiscriminate use of chemical fertilizer/pesticides leads to devastating consequences of environmental quality, which has guided to the exclusion of several such chemicals. Therefore, any advancement in agricultural systems that results in increased productivity, reduces the harmful environmental effects on agriculture, and enhances the feasibility of the system is motivated. One such attitude is the recent shift toward the use of natural biostimulants in agriculture, and horticulture is becoming popular globally. In this chapter, we discussed the effects of various biostimulants such as humic substance, smoke water, protein hyrdrolysates and amino acids, polysaccharides, inorganic compounds, microorganisms, and seaweeds in terms of their response to plant growth, productivity and yield, primary and secondary biochemical content, plant resistance to microbial diseases, ameliorating biotic and abiotic stress, enzyme metabolism and activity, antioxidant activity, regulation of gene expression, fruit quality, storage time, and self-life. These biostimulants are renewable, easy to use, nonhazardous, environmentally friendly, biodegradable, conserve natural resources, protect the environment, and enhance the safety concern and health of the users. Moreover, it is economically feasible and profitable.

Keywords

Biostimulants · Plant growth and productivity · Disease resistance · Biotic and abiotic stress · Enzyme metabolism and activity

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_1

1.1 Introduction

Biostimulants are natural or synthetic constituents that would be useful to plants and soil. They change vital and organizational processes in order to impact plant growth through amended lenience to abiotic stresses and increase quality and quantity of yields. Kauffman et al. (2007) first introduced the term biostimulant. Biostimulants improve the overall growth and progress of plants and guard them against infectious diseases. In the fields of agriculture and horticulture, the biostimulants would be used very effectively for positive modifications (Drobek et al. 2019). Biostimulants never supply nutrients directly, so many of them will not be considered as fertilizers. They sustain the metabolic activities; also, they may expedite the acquisition of nutrients (Tavarini et al. 2018). The biostimulants are available in the form of small particles, powders for soil application, and liquids for foliar spray (Kocira et al. 2018). The biostimulants interact with the environmental stressors, and plant genotypes have developed physiological modifications (Du Jardin 2015). They reduce fertilizers necessity and escalate the growth of the plant parameters and also abiotic stress resistance. They are competent, favoring the upright recital of the plant's vital practices and letting high yields and well-intentioned products (De Vasconcelos and Chaves 2019).

The biostimulants contain microorganisms, and organic and nonorganic ingredients. The biostimulants enhance the efficacy of nutrient uptake, improve crop quality and ameliorating abiotic stresses (Rouphael et al. 2018). In agriculture and horticulture fields, the biostimulants would be effectively utilized for better growth. They progress overall health, growth parameters, and vitality and protection from various infections (Drobek et al. 2019).

1.2 Sources of Biostimulants

Seaweed extracts, fungi, bacteria, proteins, humic substances, smoke water, protein hydrolases, polysaccharides, and inorganic compounds are used as biostimulants.

According to Du Jardin (2015), there are different categories of biostimulants as listed below:

1. Humic substances—Organic acids naturally present in the soil by the process of decomposition of various biological residues.
2. Smoke water—Enhances germination and seedling growth and vitality.
3. Protein hydrolysates (PHs) and amino acids – They are a significant group of plant biostimulants, comprising peptide mixtures and amino acids having multi-functional activity.
4. Polysaccharides—Promote seed germination, seedling growth, crop yield, and also prevent plants from various stresses.
5. Inorganic compounds—Silica and selenium amplify plant growth and tolerance to different stress.

6. Microorganisms—Fungi: Arbuscule-forming mycorrhiza (AMF) promote nutrition efficacy and increases yield and tolerance to different stress factors.

   Bacteria: Interaction with plants in biogeochemical cycles, improve nutrient efficiency, initiation of resistance for different diseases, and improvement of stress amelioration.
7. Seaweeds—Fresh seaweed products and their organic matter have protective compounds and are used as biostimulants.

Uncomplimentary climatic variations have been leading to an enlarged threat of several biotic and abiotic stresses over the past few years; it causes massive damage to agricultural crops (Dixit et al. 2020). Teshome et al. (2020) reveal that the stress results in major economic fatalities in agriculture and forestry year after year. Agronomic eminence such as pod size, yield parameters, color, shape, firmness, fungal and bacterial resistance, vitamins, and nutrient content is decreased by the influence of various stresses (Di Vittori et al. 2020). According to the European Union's consent, chemical plant protection mediators are deliberate to be gradually replaced by natural provisions (Al-Juthery et al. 2020). The chemical plant protection agents are the reason for this adverse influence as well as on the plant crops health benefits. The pro-ecological agriculture is naturally maintained by the provisions of natural origin biostimulants. More usage of biostimulants would control inorganic fertilizers, thus reducing pollution; this is highly significant to limit global warming (Drobek et al. 2019).

The multifaceted biostimulants have beneficial effects on crops (Tarantino et al. 2018). Biostimulants can improve crop condition without causing any adverse side effects. The characterization of biostimulants is eclectic, and there a are few features that differentiate them compared to the others. They are plant protection agents and improve the plant growth (Du Jardin 2015). They cause adverse effects to the environmental threats and reduce chemical fertilizers. Moreover, they can be considered as eco-friendly alternatives (Vasseur-Coronado et al. 2021).

1.3 Humic Substance

Humic substances are naturally occurring heterogeneous mixture of many organic substances that have high molecular weight and refractory. It is the most important form of organic carbon, produced by chemical and biological conversion of plant and animal materials and from microbial metabolism on the surface of earth. Chemically they are the product of a saponification reaction by alkaline extraction from soils and sediments. Humic substances include humic acid and fulvic acid. In soil science, humic acid is a combination of numerous organic substances and a mixture of weak aliphatic and aromatic organic acids that are only soluble in aqueous alkaline solutions but are insoluble in aqueous acid solutions, while fulvic acid is the fraction of humic substances that are soluble in aqueous solutions at any pH conditions (Aiken et al. 1985). The composition of humic substances revealed the presence of soil organic matter, especially humic and fulvic acids (65–70%) and N and S in significant amount (Stott and Martin 1990; Stevenson 1994).

Humic substances have direct or indirect impacts on plant growth and development as of the multiple functions of humic and fulvic acid (Chen et al. 2004; Pal and Biswas 2005). The soil application of humic acid improves soil structure and aggregation, fertility, water permeability and holding capacity, air conditioning, promotes microbial activity, and cation exchange capacity (Chen and Aviad 1990; Marinari et al. 2000; Sharif et al. 2002; Mohamed 2012). When it is used as foliar spray, it is easily absorbed by the leaves, which in turn improve the metabolic rate by stimulating the enzymes in many biological processes, enhance photosynthetic rate by increased production of photosynthetic pigments, increasing resistance of plants to biotic and abiotic stress, prolong the fruit storage period, and thus improve the plant growth characteristics and productivity (Arancon et al. 2003; Abd El-Razek et al. 2012; Hagagg et al. 2013; Yakhin et al. 2017).

Govindasmy and Chandrasekaran (1992) isolated humic acid from lignite and applied it to the soil surface at 6 g/m^2, significantly promoting the cane yield, sugar content, and nutrients content in the leaves. Application of 1 g of solid humus kg^{-1} in the soil and 0.1% as foliar spray respectively improved the uptake of N and P, K, Mg, Na, Cu, and Zn of *Triticum durum* under saline stress (Asik et al. 2009). Foliar application of humic acid showed intermediate responses in terms of plant dry mass, yield, spike fertility, protein content, and Rubisco activity of durum wheat compared to control and split soil N application (Delfine et al. 2005). Humic substances (humic acid and fulvic acid) increase nutrient uptake and dry matter yield of rice (Eshwar et al. 2017).

Yildirim (2007) has reported that soil and foliar applications of humic acid at 20 mL/L concentration significantly increased total soluble solid, growth, and yield of tomato. IN contrast, Foliar application showed highest ascorbic acid (AA) content. In tomato, significant increases in plant growth, fruit number, medium–large-sized fruit yield, P and Ca content in fruits were observed when the plants were given foliar application of 0.8 or 1.1 g·L^{-1} of fulvic acid. Whereas treatment with higher concentration at 1.6 g·L^{-1}, significantly reduced the incidence of fruit cracking and blossom-end rot in tomato (Young et al. 2014). Application of humic acid at 14.4 kg·ha^{-1} to two tomato hybrids under hot continental climate results in increased vegetative growth, flowering parameters, and yield characters (Abdellatif et al. 2017).

Hernandez et al. (2015) studied the effect of humates isolated from vermicompost on lettuce. They noticed that 15 mg C L^{-1} humate application as foliar spray significantly reduced the production cycle without altering the quality and also increased the number of leaves/plants. The leaves of lettuce also showed enhanced enzyme activity (nitrate reductase and phenylalanine ammonia lyase), protein, nitrate uptake, and decreased total carbohydrate content, suggesting the use of soluble humic substance for enhanced production and quality of crops in urban agriculture. El-Hoseiny et al. (2020) reported the combined application of humic acid (0.30%) and boric acid (600 mg·L^{-1}) enhanced tree growth, flowering, yield, and fruit quality of mango than the individual applications. It also enhanced the photosynthetic pigments, nutrients, carbon assimilation, and phytohormones (auxins, gibberellins, and cytokinins) and protected tree against destructive oxidative reaction by reducing the content of abscisic acid that decreases floral malformation percentage and improves productivity. Further humic acid and boric acid applications also diminish the prevalence of alternate bearing from one season to another. Humic acid at 1000 mg/L promotes the root growth and significantly enhanced the N, P, K, Ca, Mg, Fe, and Zn contents of leaves and scapes of *Gerbera*. Whereas 500 mg/L showed better postharvest responses such as increased flower number and lengthened the vase life of cultivated flowers (Nikbakht et al. 2008). Yazdani et al. (2014) have reported humic acid and fulvic acid (particularly 50 mg L^{-1} fulvic acid) improved the quality (root architecture, nutrient uptake, and hormone-like activities) and quantity (increased flowers number up to 72% and extended vase life of about 8 days) of *Gerbera*. Humic acid (2 mL L^{-1} water) isolated from rock material Leonardite along with NPK (17:17:17) as foliar application resulted in increased growth and yield, and improved flower quality, longer vase life, and corm characteristics of gladiolus (Ahmad et al. 2013). *Impatiens walleriana* treated with 40 mg kg^{-1} of fulvic acid and humic acid respectively enhanced the plant growth and flower number significantly (Esringü et al. 2015).

Humic acid application on soil under continuous cropping of peanut significantly enhanced the yield (increased by 78.29%) and quality for continuously three years. The improved effect is attributed due to the improvement in soil parameters such as physical–chemical properties (increased soil nutrient contents, total soil N, P, K, available N, P, K, and organic matter contents), enzymatic activities (urease, sucrase, and phosphatase in soil), and microbial diversity of soil (increased Firmicutes in bacteria, Basidiomycota, and Mortierellomycota in fungi) (Li et al. 2019). Addition of mineral-derived and plant-derived liquid fulvic acid to albic black soil enhanced the nutrient availability, soil properties (physical, chemical, and biological), and hastened the crop (maize) growth parameters (plant height, biomass, stem diameter, and thousand-grain weights). It also increased the available N, soil organic carbon, and light fraction C contents (Sootahar et al. 2019). Sootahar et al. (2020) reported that application of fulvic acids (plant-derived solid, mineral-derived liquid, and plant-derived liquid) enhanced the physicochemical and biological conditions of soils with distinct texture (Aridisols, Vertisols and Mollisols). The result showed a significant increase in heavy fraction C (10–60%), light fraction C (30–60%), and available N (30–70%) and K (20–45%) in all soil types, whereas increased P content (80–90%) was noticed in Aridisols and Vertisols. Furthermore, plant-derived solid, mineral-derived liquid hasten the wheat growth parameters (plant height, biomass, thousand-grain weight, and spike grain weight) and nutrient uptake in Mollisols and Aridisols, respectively.

Humic acid also has direct hormone-like stimulatory effects such as cytokinin (Zhang and Ervin 2004) and auxin or gibberellin-like (Pizzeghello et al. 2001), along with indirect impact on plant metabolism (Piccolo et al. 1991). The auxin-like activity in the initiation of lateral roots by humic substance was proved in *Arabidopsis thaliana* through genetic and molecular approaches. Furthermore, this finding was supported by expression of the early auxin-responsive IAA19 gene and DR5::GUS synthetic element (Trevisan et al. 2010).

1.4 Smoke Water

Smoke generated by burning vegetation is documented to promote germination for seeds of many plant species from fire-prone and non-fire-prone environments (De Lange and Boucher 1990; Brown 1993; Baldwin and Morse 1994; Van Staden et al. 2000; Van Staden et al. 2006a, b). Smoke can be prepared from a wide range of plant parts such as straw, wood, or a mixture of fresh and dried plant materials (Chiwocha et al. 2009). De Lange and Boucher (1990) for the first time reported smoke compounds found in plant that promoting seed germination of some species by breaking seed dormancy. Smoke water derived from bubbling smoke in a container of water retains water-soluble compounds present in smoke by burning plant materials and is also recognized to be extremely useful in breaking seed dormancy and stimulating seed germination in wild plant species from many ecosystems (Dixon et al. 1995 ; Brown and van Staden 1997; Landis 2000; Pennacchio et al. 2007; Jain et al. 2008; Lindon and Menges 2008), increasing the number of secondary roots (Kulkarni et al. 2006), fresh weight of seedling (Sparg et al. 2006; Van Staden et al. 2006a, b), and also enhancing seedling growth and yield of many horticultural and agricultural crops (Brown et al. 2003; Light and Van Staden 2004; Kandari et al. 2012).

Many studies documented the seed germination and growth-promoting effects of smoke water on food crops such as rice (Doherty and Cohn 2000; Kulkarni et al. 2006), maize (Modi 2004), bean (Van Staden et al. 2006a, b), okra and tomato (Kulkarni et al. 2007), and onion (Kulkarni et al. 2010). Smoke water prepared from dry rice straw at 0.1% or 0.2%, v/v shortened the germination time and promotes seed germination of papaya. Anatomical study of seed revealed that smoke water treatment overcomes water impermeability barriers by rupturing the seed coat and allowing the radical to emerge faster and significantly enhances growth attributes of seedlings by improving the uptake of mineral nutrients (Chumpookam et al. 2012).

Seed germination and seedling growth of three Chinese medicinal plants such as *Astragalus membranaceus, Magnolia officinalis,* and *Panax notoginseng* were optimized under different temperature, light, and nutrient contents using smoke water (Zhou et al. 2012). Zhou et al. (2013) reported increased photosynthetic rate, transpiration rate, stomatal conductance, and improved rate of the photochemical organization of PS-II in leaves of *Isatis indigotica* treated with smoke water. Smoke water derived from burning germination paper and the wood of *Cecropia palmata* Willd. significantly increased seed germination of Amazonian tree species such as *Cordia goeldiana* Hub., *Ochroma pyramidale* (Cav. ex Lam.) Urb. and *Jacaranda copaia* (Aubl.), *Bertholletia excelsa* Humb. & Bonpl., and *Bellucia grossularioides* (L.) Triana. Moreover, *B. excels* treated with smoke water derived from germination paper showed most prominent effect, in which the germination time was reduced to

76 days compared to mean germination time of 108 days (control) (Ferraz et al. 2013).

Smoke water treatment (1:750 v/v) improved seedling fresh weight, and karrikinolide (10^{-8} M) significantly enhanced the acid phosphatase (1856 nkat mg-1) and alpha-amylase (3.225 mg min-1) two important hydrolytic enzymes and amino acid content of *Phaseolus vulgaris* during germination. This is due to the gibberellic acid-like activity of the biostimulants (Singh et al. 2014). Butenolide, 3,4,5-trimethylfuran2(5H)-one(2), obtained from plant-derived smoke when applied simultaneously with karrikinolide, hinders seed germination and significantly diminished the impact of arrikinolide. These antagonistic-opposing actions on seed germination of plant-derived smoke constituents have significant ecological importance in a post-fire environment and are valuable in understanding the physiology of breaking seed dormancy (Light et al. 2010).

Kumari et al. (2015) evaluated the effect of smoke water and Karrikinolide on in vitro pollen germination and pollen tube growth of many plant species. They noticed that smoke water (1:1000 and 1:2000 v:v) significantly promotes pollen germination and pollen tube elongation in *Aloe maculata* All., *Kniphofia uvaria* Oken, *Lachenalia aloides* (L.f.) Engl. var. *aloides,* and *Tulbaghia simmleri* P. Beauv. Whereas Karrikinolide (10^{-6} and 10^{-7}M) treatment also significantly promoted pollen germination and pollen elongation in *A. maculata, K. uvaria*, *L. aloides,* and *Nematanthus crassifolius* (Schott). Çatav et al. (2018) have reported that germination and seedling growth of 21 species out of 37 species from the Mediterranean Basin are enhanced by any one of the fire-derived compounds and/or chemicals (smoke water, karrikinolide, mandelonitrile, potassium nitrate, and gibberellic acid) tested. They also observed positive correlations in terms of seed germination with most of the fire-derived compounds tested. Smoke water has improved seed germination of many North American forage and prairie plants (Abu et al. 2016; Yao et al. 2017). Recently, Mackenzie and Naeth (2019) reported that applications of smoke water (1:20) and smoke water + KNO_3 increased seed germination of cold-stratified native plant species of boreal forest. Aqueous smoke solution prepared from grasses diluted to 1:2500 (v/v) and sub-diluted 25:75 (v/v) showed increased seed germination of lettuce to 91% compared to the control that showed 7–10% germination. For the first time, the amounts of karrikinolide 1 (KAR1), karrikinolide 2 (KAR2) (stimulatory), and trimethylbutenolide (TMB) (inhibitory) compounds in smoke water were reported using ultra-high-performance liquid chromatography–electrospray-positive ionization tandem mass spectrometry (Gupta et al. 2020).

The promising activity of smoke water to stimulate germination and seedling growth seems to be the presence of ethylene, gibberellic acid, and butenolide at low concentration in the smoke water (Chumpookam et al. 2012). The first major germination active stimulant identified in smoke water was butenolide (3-methyl-2H-furo[2,3 c]pyran-2-one) by Flematti et al. (2004) from combustion of filter paper and Van Staden et al. (2004) from aqueous smoke solution obtained from burned plants (*Passerina vulgaris* Today and *Themeda triandra* L.). Later this compound was referred to as karrikinolides or karrikins (Commander et al. 2008; Nelson et al.

2009). These researches pave the way for the identifications of other smoke-derived stimulatory compounds such as catechol, which promotes root growth and root hair elongation through ROS-mediated redox signaling (Wang et al. 2017) and hydroquinone-promoting seed germination of lettuce (Kamran et al. 2017). The applications of smoke water at low concentrations promote seed germination and seedling growth. However, higher concentrations of smoke water has inhibitory effect (Light et al. 2002). The search of inhibitory compounds in smoke water revealed small butenolide, 3,4,5-trimethylfuran-2(5H)-one (trimethylbutenolide) (Light et al. 2010) and furanones (Burger et al. 2018).

1.5 Protein Hydrolysates and Amino Acids

Protein hydrolysates are a mixture of amino acids, polypeptides, oligopeptides, and denatured proteins that can be manufactured from protein sources such as microbes, plants, or animals by chemical, enzymatic, and thermal hydrolysis (Schaafsma 2009; Nardi et al. 2016; Colla et al. 2017a). They have been recognized to play multiple roles in the performance of crops, such as enhance nutrient uptake, use efficacy, and N and C-metabolism (Cerdán et al. 2009; Ertani et al. 2009; Colla et al. 2015a, b; Halpern et al. 2015; Battacharyya et al. 2015a, b), minimize the impact of abiotic (heavy metal, thermal, nutrient, salinity, and drought) stress on crops (Botta 2013; Cerdán et al. 2013; Colla et al. 2013, 2014; Ertani et al. 2013; Lucini et al. 2015; Rouphael et al. 2017a), interfere hormonal activities, increase microbial and enzymatic activities in soil (García-Martínez et al. 2010; Du Jardin 2015; Colla et al. 2017b), and thus increase plant biomass and productivity (Paradikovic et al. 2011; Colla et al. 2014; Ertani et al. 2014).

In general, applications of amino acids and peptides as a nutrient solution to plants promote the growth and enhance the concentration of K, Ca, Mg, Fe, Cu, and Zn (Garcia et al. 2011). Total protein, phenol and flavonoids, and antioxidant activity of banana was significantly increased when treated with feather degradation products having amino acids and peptides (Gurav and Jadhav 2013). Protein hydrolysates of natural origin have been extensively used as biostimulants for horticultural crops because of their positive outcome on crop yield and nutritional quality (Colla et al. 2014). Quartieri et al. (2002) documented low molecular weight fractions (1–3 kDa) of animal-derived protein hydrolysates stimulated shoot and root growth of kiwifruit plants.

Ertani et al. (2013) tested the effect of protein hydrolysates derived from alfalfa plants (25 and 50 mL/L) and red grapes (50 and 100 mL/L) on *Capsicum chinensis* L and reported protein hydrolysates-treated plants showed increased concentration of chlorogenic acid and antioxidant activity. Meanwhile, the fruits had a high concentration of capsaicin. Ertani et al. (2014) also reported that plant-derived protein hydrolysate improved the contents of carbohydrates, phenols, ascorbic acid, and lycopene and also increased the antioxidant activity of *Capsicum chinense* L. grown in greenhouse. The effect of commercial formulations of protein hydrolysate on the phytochemical profile of *Melissa officinalis* L. was tested by Mehrafarin et al.

(2015). They noticed the contents of citronellal, neral, deltacadinene, germacrene, and geranial were increased significantly than that of untreated control plants. Applications of legume-derived protein hydrolysate enhanced the yield of tomato (7.0%) and improved the quality of fruit by increasing the contents of lycopene, total soluble solids, and K and Mg (Colla et al. 2017a; Rouphael et al. 2017b), thus increasing the quality of the tomato fruits. Exogenous foliar applications of hydrolysates (polyamines) derived from enzymatic hydrolysis of *Arthrospira platensis* promoted the growth of lettuce seedlings and increased the spermine content by 64% in the leaves (Mógor et al. 2018). Lucini et al. (2018) studied the morphological, physiological, and metabolomic changes in greenhouse grown melon treated with vegetal-based biopolymer. The treated plant showed a significant increase in weight of leaves, total biomass, root dry biomass, and root length. The UHPLC/QTOF-MS analysis revealed that the presence of brassinosteroids and their synergism with other hormones plays a major function in growth-promoting activity.

The biostimulating potential of foliar application of chicken feather hydrolysates increased the yield, nutrition, and grain parameters of maize (Tejada et al. 2018) and promoted the growth of wheat (Genç and Atici 2019) and lettuce (Sobucki et al. 2019). Protein hydrolysates application to maize seedlings in hydroponics enhanced root and shoot growth, mitigated abiotic stresses, and regulated the transcription of genes responsible for nitrate transport and ROS metabolism (Trevisan et al. 2019). Caruso et al. (2019) conducted an experiment to investigate the effect of legume-derived protein hydrolysate, Trainer®, and Tropical plant extract, Auxym®, on *Diplotaxis tenuifolia* (Wall Rocket) grown under greenhouse at different seasons. They observed that both hydrolysates enhanced plant growth and productivity, increased the biomass, oxalic acid and citric acids, P and Ca contents, phenols and ascorbic acid concentrations, and antioxidant activity. The winter–spring cycle extracted higher productivity, antioxidant content, and activity than the winter crops. Schmidt et al. (2020) compared the effect of protein hydrolysates derived from chicken feathers (FH) and a reference protein hydrolysate (RH) on barley and wheat in a pot culture experiment. They observed that shoot height and biomass of barley were increased by FH in pot culture. In a subsequent field experiment, RH significantly increased the yield and grain size of barley, but there is no significant impact on wheat by either hydrolysate. Furthermore, both hydrolysates encouraged the brutality of net blotch (*Pyrenophora teresmaculata*) on barley in the pot culture experiment, but reduced it in the field. FH treatment along with low-P supply promoted root colonization of AMF.

Protein hydrolysates improve plant resistance against pathogens (Lachhab et al. 2014; Figueiredo et al. 2015; Thatcher et al. 2016; Cappelletti et al. 2017) and control defense signaling pathways (Apone et al. 2010; Lachhab et al. 2014). Moreover, it also changes the prevailing environment for phytopathogens by altering the composition of the phyllosphere flora (Cappelletti et al. 2016) and enriching the amount of antifungal compounds (Luziatelli et al. 2019). The biostimulant activity of protein hydrolysates is due to the presence of signaling molecules such as peptides, amino acids, carbohydrates, and lignosulphonates (Du Jardin 2015). Moreover, many studies raveled the presence of bioactive peptides in protein hydrolysates showed hormone-like activities (Ito et al. 2006; Kondo et al. 2006). Further, several studies have confirmed plant-derived protein hydrolysates elicited auxin- and gibberellin-like activities (Schiavon et al. 2008; Matsumiya and Kubo 2011; Colla et al. 2014) and thus promoted crop performances. Leucine, glycine, alanine, arginine, glutamate, glutamine, proline, and valine are the major amino acids present in protein hydrolysates (Parrado et al. 2008; Ertani et al. 2009; García-Martínez et al. 2010). Alfalfa hydrolysate contains triacontanol and IAA (Ertani et al. 2013), free amino acid, and macro- and microelements (Schiavon et al. 2008).

1.6 Polysaccharides

In recent years, alginate-derived oligosaccharides received more interest owing to their immense prospective in the field agriculture and horticulture (Liu et al. 2013; Zhang et al. 2014; Aftab et al. 2014; Li et al. 2018). They are known to promote seed germination, seedling growth, crop yield (Hien et al. 2000; González et al. 2013), and also prevent plants from abiotic stress including heavy metals (Ma et al. 2010a, b), salinity (Tang et al. 2011), and water (Liu et al. 2013). In *Arabidopsis,* prolongation of the elongation zone and enlargement of meristem were noticed in the primary roots (Kučerová et al. 2016) due to oligosaccharide treatment. Exogenous application of Xyloglucan oligosaccharides extracted from xyloglucans of *Tamarindus indica* L. cell walls to *A. thaliana* significantly improved catalase gene expression and enzymatic activity, chlorophyll a/b ratio, and thus neutralized the harmful effects that arises due to oxidative stress under saline conditions (González-Pérez et al. 2018).

Foliar application of chitosan oligosaccharides enhanced the photosynthetic rate in maize by increased stomatal conductance and transpiration rate (Khan et al. 2002) and increased flowering in *Passiflora edulis* (Utsunomiya and Kinai 1994). Goñi et al. (2016) have reported chitosan oligosaccharides derived from commercial chitosans by enzymatic process significantly enhanced plant biomass and increased disease resistance against *Fusarium oxysporum* and induction of ISR markers of tomato cultivar. For the first time, Salachna et al. (2019) documented the effects of gellan oligosaccharides derived from Gellan gum and salt (100 mM NaCl) on the yield and quality of red perilla (*Perilla frutescens* var. crispa f. purpurea) leaves. Application of gellan oligosaccharides under non-saline conditions enhanced seedling growth and aboveground biomass, improved the accumulation of N, K, Mg, and total polyphenols, and increased antioxidant activity. Under saline conditions, gellan oligosaccharides-treated plants showed alleviated response to stress by minimizing the loss of biomass, macronutrients, and phenolic content. Furthermore, leaf extract from both treatments exhibited growth inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*. Yang et al. (2021) investigated the impacts of alginate-derived oligosaccharides on growth, development, and biophysical characteristics of *Hordeum vulgare* L. by soaking the seeds in alginate-derived oligosaccharide solutions with diverse molecular weights and mannuronate/guluronate ratio. Seeds received low molecular weight (500–3000 Da) alginate-derived oligosaccharides and higher mannuronate/guluronate ratio (>1) showed enhanced growth characteristic on the plant. They suggested the growth-enhancing effect of alginate-derived oligosaccharides was due to increased photosynthesis and roots growth and stimulated the expression of development-related genes including HvARF3, ARF17 (auxin response factor), and MAPK (mitogen-activated protein kinase), thereby improving plant growth.

1.7 Inorganic Compounds

1.7.1 Silicon (Si)

The second-most abundantly available element on earth on the basis of atoms, number, and weight is silicon (Si). The earth's crust contains Si dioxide of about 60%, which has very solid attraction with O_2. Si naturally presents as the combination of different metals to form silicates (Ma 2003). Si present in oxide form SiO_2 in plants is usually called silica (Lanning et al. 1958). The extensive usage of silicon in the form of fertilizers and soil amendments is essential for resolving such difficulties as eco-friendly farming activities and sustainable agriculture (Bocharnikova et al. 2010). Silicates are formed when silicon combines with other elements (Gunes et al. 2008).

Si has been found to mitigate different stresses and leads to amalgamation of silicates into numerous fertilizers (Currie and Perry 2007). This was first confirmed in cucumbers. Plants were treated with Si, verified higher activity of peroxidases, polyphenol oxidases, chitinases, and flavonoid phytoalexins, all of which may defend against fungal pathogens (Fawe et al. 1998); also these alleviate and have defense mechanisms as suggested by Rodrigues et al. (2003). The Si application can be alleviated metal toxicity, saline stress, drought stress, and temperature stresses by Liang et al. (2007). Si application brought decreased transpiration under drought stress (Epstein 1999).

Silicon has been acknowledged in the world since the middle of the eighteenth century. After 2000, their manufacturing moved further in many countries and has enlarged to 20–30% every year (Matichenkov 2004). It has been acknowledged that silicon is the second element to be found in abundance after oxygen on the globe. Soil contains around 5% of its active forms. In agriculture, the plants take up amorphous silicon dioxide from 30 to 700 kg/ha per year (Matychenkov et al. 2002). Water is vital for all living organisms and plays a very important role in plant metabolism. Water availability and quality can be restraining factors in plant growth (Lellis et al. 2019). Plants are exposed to various stresses that unfavorably affect plant metabolism (Kheybari et al. 2013). The environmental stresses such as salinity, drought, temperature, pesticides, and pH are major aspects limiting crop production because they distress almost all plant functions (Yue et al. 2011). For most of the crops, silicon is not considered as an important element, but it has been stated that application of silicon is favorable to many crops; accordingly, it encourages defense mechanism against pest attack (Epstein 1999). The application of Si mitigates drought stress and preventing membrane damage in sunflower cultivars. Si altered morphological parameters, membrane permeability, lipid peroxidation, nonenzymatic, and enzymatic antioxidant activities (Gunes et al. 2008).

Under water-stressed condition, the application of silicon to the wheat crops could ameliorate positively. Silicon increased the enzymatic activities. Si altered contents of photosynthetic pigments, soluble proteins, and showed significant changes of oxidative stress of proteins and acid phospholipase (AP). Silicon can be mitigated in the metabolic activities of water-stressed higher plants (Gong et al. 2005). Si improved saline stress, drought stress, or cadmium (Cd) stress tolerance in wheat. Under different stress conditions, Si conferred positively altered growth, tissue water, gas exchange, and membranes constancies. Si was highly valuable in extraordinarily affecting physiological occurrences and enhancing wheat growth parameters under abiotic stress (Alzahrani et al. 2018).

Si improves growth of the crop and its production in arid or semi-arid areas, but this method would not fully be a substitute for sufficient water supply (Kaya et al. 2006). In salt tolerance of wheat (*Triticum aestivum*) at germination and vegetative growth stages, silicon causes significant recovery from abiotic stresses. Shoot dry weight increased considerably after silicon application at 0.6% salinity. Chlorophyll content continued unaffected by addition of silicon at salinities. Silicon considerably reduced the Na+ content in flag leaves and roots under saline stress. Roots are increased with accumulative salinity and silicon levels (Ahmad et al. 1992). Silicon stimulates beneficial properties in plants; it further alleviates osmotic and oxidative stresses on *Glycyrrhiza uralensis* mediated by modifiable osmolytes to progress water status and antioxidants to sustain membrane stability (Zhang et al. 2018). Si treatment improved mango plants' efficient system for scavenging reactive oxygen under drought stress, and it guards the plants against negative oxidative reactions, thus improving the capability of the mango trees to endure environmental stress in arid regions (Helaly et al. 2017).

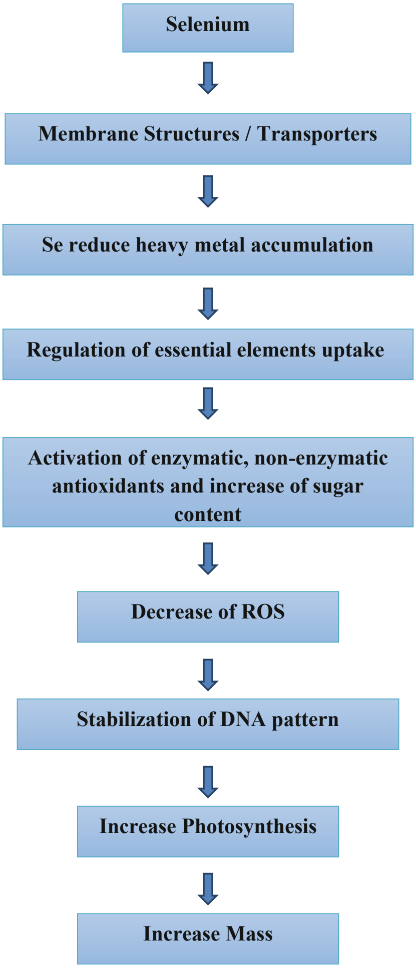
In sorghum plant, the application of silicon amplified the dry mass and relative water content. The drought stress tolerance in sorghum plant accomplished by adding silicon can be related to the enhancement of water uptake capability (Hattori et al. 2007; Gong et al. 2003). Treating the plants with Si during stressed conditions results in deposition in the various parts of the plant body (Richmond and Suaaman 2003). Hossain et al. (2007) have described that Si is responsible for the increase in the cell membrane extensibility. Ultraviolet-B radiation destructively disturbs plant physiological activities (Beckmann et al. 2012). Si escalates plant tolerance under UV-B radiation stress (Fang et al. 2011). Various research studies have revealed that though silicon is not a vital element it impacts the growth and yield of the crop positively (Ahmed and Khurshid 2011; Balakhnina et al. 2012). The research reports revealed that Si altered various growth, biochemical, and physiological functions in plants. Si plays an important role in different stress conditions. The resistance of plants to numerous stress conditions can be improved by increasing Si in plants. Silicon upsurges resistance to saline stress, photosynthetic activity, drought stress tolerance, and encourages active progress of roots and foliage. The upcoming research studies on Si will increase quality and adoptability of farming practice for a better yield (Balakhnina and Borkowska 2013).

1.7.2 Selenium (Se)

Selenium (Se) has been gaining attention in recent years; it is a vital nutrient for living organism, particularly the alleviating effects in plants under various stress conditions (Sieprawska et al. 2015). Se is available in the form of selenium dioxide. It is present in the air, engendered in the etiquettes of coal ignition (Babula et al. 2009). In the periodic system, selenium is in the VI group, existing in the environment. It is chemically bonded with metals and forms selenides. In minerals, Se is present as an anion like kullerudite (NiSe2), antimonselite (Sb2Se3), clausthalite (PbSe), and tiemannite (HgSe). Because of the chemical similarity Se moderately substitutes sulfur in some minerals. Some selenides are formed by having chemical bonding of selenium to metals (Fordyce 2013). In the environment, volcanic emissions are the added source of Se, and it was found during the years 1976–2007 (Calabrese 2009). On the global scale, the selenium concentration was in soil at 4 mg/kg. Based on the geological structure, the content of selenium varies within the nation. It is measured to be predominantly poor in this element (Fordyce 2013). The selenium content regulates gathering of elements. Se availability is contingent and is influenced by the potential of redox, pH, ions, activity of microbes, and organic matter of soil (Hawrylak-Nowak et al. 2015). The plants could enthrall Se from the soil in its organic forms (Keskine et al. 2010). Through the selenate supplementation, translocation of Se was stimulated (Mehdi et al. 2013), and selenite exists in soils (Li et al. 2008). Se is decreased by adenosine 5′-phosphosulfate reductase into selenite, triggered by ATP-sulfurylase-adenosine 5′-phosphoselenate (APSE) and glutathione lowered to selenide (Mehdi et al. 2013). Wrona et al. (2007) indicated that the defense mechanisms of biomembranes are called plasmalemma regarding the defense mechanism of Se.

Se activities can reduce the accumulation of toxic metals (Bernat et al. 2014). Se occurrence may impact the spreading of elements important for plant growth and improvement (Tobiasz et al. 2014). Owusu-Sekyere et al. (2013) and Turakainen et al. (2004) confirmed the Se-stimulated rise of carbohydrate concentrations. Se ameliorates oxidative stresses initiated by heavy metals (Łabanowska et al. 2012). Antioxidative enzymes such as catalase and peroxidase activities were altered by Se-supplemented plants growing under water stress conditions (Habibi 2013). Also, Ibrahim (2014) recorded the increased SOD activities in stressed environments. Se improves the plant tolerance against water stress and induced stimulation of the plant's antioxidant. Selenium ions could regulate the water intake and is involved in protective action in stressed crops (Proietti et al. 2013). Se encouraged increase in organic and nonorganic ions, which may cause improved water holding in wheat (Emam et al. 2014). Selenium (Se) is a significant microelement for humans and a valuable component for plants. Se application increased biomass accumulation, photosynthetic rate, and tolerance. It improved antioxidant capacity and enhanced

Fig. 1.1 Representation of selenium action on plant cells of some biochemical and physiological properties under stress factors



essential element content and amino acids through the root application (Yin et al. 2019). It also increased stem diameter, shoot length, and dry weight observed in Se application. The wild peach seedlings revealed different Se improvement ability, obviously amplified the dropping power, unimportantly increased the reducing sugars, and reduced the content of total sugars. Se achieved the growth parameter and physiological variations of wild peach seedlings. It is helpful for the production of Se-enriched fruits (Sun et al. 2020). Foliar application in Stevia (*Stevia rebaudiana* Bert.) selenium improves the adverse effects of NaCl (Shahverdi et al. 2018). Selenium (Se) is essential due to its antioxidant properties. Drought stress in paddy can be protected by selenium (Andrade et al. 2018). Comprehensive mechanism of Se metabolism is vital for operative Se biofortification. The physiological role of Se under abiotic stresses is highly significant (Hasanuzzaman et al. 2020) (Fig. 1.1).

1.8 Microorganisms

1.8.1 Fungi

Recent agriculture wants to expand its agricultural practices by incorporating opportunities coming from different sectors, including the bio-based industry (Rouphael and Colla (2020). The fungal-based products have been applied in agriculture to promote tolerance to stress and improve efficiency of nutrition, growth, and yield. The fungal endophytes can transfer the nutrients to their host and colonize roots. Mutualistic symbioses in the organisms contact each other and have beneficial relationships mutually (Behie and Bidochka 2014). There are growing attentions allowing for extensively recognized assistances of the symbioses to sustenance effectiveness, water stability, and biotic and abiotic stress defense of plants (Gianinazzi et al. 2010). The fungi and higher plants are coevolved with the concept of mutualism, and parasitism-extended relationships have evolved over the changing times (Johnson and Graham 2013). The heterogeneous group of mycorrhizal fungi would establish over 90% of plant species. The AMF are universal endomycorrhiza connected with horticultural crops where fungal hyphae of species of *Glomeromycota* enter root cells (Bonfante and Genre 2010). The plant inoculants increase in fungal endosymbionts. Largely *Trichoderma* spp. have been widely castoff for mycoparasitic and biocontrol, which can increase resistance capacity of diseases and used in various industries (Nicolás et al. 2014). The significant ecological and agricultural inferences in the fungal conduits permit interplant signaling (Johnson and Gilbert 2015). To obtain the assistances of the relations of mycorrhiza, plant cultivation would be improved to the communication with microbes (Sheng et al. 2011). The microbial relations in the rhizosphere are stimulated by metagenomics. The soils complement while using the inoculation of plant propagules (Candido et al. 2015; Colla et al. 2015a, b), convincing indication in plant reactions such as increase in nutrient use competence is also encouraged under various stress influences (Colla et al. 2015a, b). Based on the above research findings, the fungal organisms would be considered as suitable biostimulants for unpolluted agricultural activities.

1.8.2 Bacteria

The positive plant–microbe interactions are concomitant to plant growth including PGP *rhizobacteria*, non-pathogenic capable of enlightening nutrient accessibility in soil, plant nutrient application and assimilation, as well as associate nitrogen cycling (Lugtenberg 2015). In wheat, the *Azospirillum brasilense* discharges auxins and triggers auxin-signaling pathways intricate in root morphogenesis (*Triticum aestivum*). They improved lateral root density and root hair surface (Dobbelaere et al. 1999). They have specific strain, which will be used in agriculture. The plant growth-promoting rhizobacteria (PGPR) are guarded by this intricacy. By the changeable reactions, the acceptance of atmospheres, the invention of the inoculant's stretch rises to uneven results (Brahmaprakash and Sahu 2012).

In many ways, the bacteria could interact with higher organism. Niches of bacteria outspread from outer side to the inner side of plant cells, and certain bacteria are precipitously transferred through the seeds. Roles persuade contribution to biogeochemical cycles, upsurge in augmentation initiation of disease resistance, source of nutrients, and modulation of morphogenesis (Ahmad et al. 2008). The plant growth-promoting *rhizobacteria* (PGPR) have gained attention among the farmers for their welfare of crop. Multidisciplinary approaches improve the efficiency and mechanisms involved in developing the plant growth and production (Rathore 2014). Biostimulants significantly decrease the chemical contributions in agriculture. PGPR is a group of bacteria that improves plant vigor and yields parameters on various promoting constituents such as biofertilizers. The usages of helpful soil microbes for sustainable and safe agriculture have been amplified worldwide during the last couple of decades. They are accepted as effective soil microbes for the enhancement of agriculture and its yields (Mohammadi and Sohrabi 2012). *Rhizobium* improved the growth and yield of maize over control (Qureshi et al. 2013). *Rhizobium* improves the plant growth promotion, nodulation, and seed yield in urdbean (Singh et al. 2010).

Pseudomonas inoculation is induced significantly in root length, shoot length, and dry weight of tomato plants (Saravanan et al. 2013). *Phosphobacteria, Rhizobium*, and *Azospirillum* considerably increased plant growth and yield of cowpea and green gram (Senthilkumar and Sivagurunathan 2012). Using *Azospirillum brasilense*, *Azotobacter chroococcum*, and *Paenibacillus polymyxa* positively altered the growth and yield components of canola (El-Howeity and Asfour 2012). In the yields of maize (Montañez et al. 2009), wheat (Sala et al. 2007), and rice (Pedraza et al. 2009), notable positive modifications are intricated by *Azospirillum* over mechanism of BNF.

1.9 Seaweeds

Seaweeds are important marine renewable resources that have been primarily associated with human beings either directly or indirectly as a source of food, fodder, and manure from time immemorial (600 BC), particularly in densely populated nations. The utilization of seaweeds in agriculture and horticulture has a long history. Over the past few decades, seaweed has been well recognized for its growth-stimulatory effects on a number of agricultural and horticultural crops. They represent one of the important sources of organic matter and fertilizer available in different forms including seaweed liquid fertilizers, seaweed extract, and powder manure that have led to their use as soil conditioners and plant growth promoters (Blunden and Gordon 1986) and have been considered as an alternative source for conventional chemical fertilizers. Many investigations have revealed a board array of favorable effects of seaweed as fertilizer, such as increased seed germination, seedling growth, yield, and productivity, enhanced photosynthetic activity, improved resistance to diseases and stress (biotic and abiotic), and better postharvest shelf-life of perishable products (Beckett and van Staden 1989; Blunden 1991; Norrie and Keathley 2006; Khan et al. 2009; Gajc-Wolska et al. 2013; Sharma et al. 2014; Nabti et al. 2016; Mattner et al. 2018).

The positive impact of seaweed extracts on crops is a result of many components that may function interdependently at varied concentrations and are biodegradable, nontoxic, nonpolluting, and nonhazardous. A number of plant growth regulators such as cytokinins and auxin (Stirk et al. 2003; Stirk et al. 2004), gibberellins (Wildgoose et al. 1978), betaines (Wu et al. 1997), macronutrients (Ca, K, P) and micronutrients (Fe,Cu, Zn, B, Mn, Co, and Mo), and lower molecular weight compounds such as polyamines and brassinosteroids (Stirk et al. 2004; Stirk et al. 2014) have been reported from seaweed extracts. Blunden and Woods (1969) and Battacharyya et al. (2015a, b) reported that complex polysaccharides such as algin, mannitol, fucoidan, and laminarin of the large brown algae are important when those seaweeds were utilized as fertilizers that alter the physical nature of the soil and improve plant food value. Recent advances in seaweed research revealed that larger molecules such as polysaccharides and polyphenols present in seaweed extracts can also act as biostimulants, allelochemicals, and play an important role in alleviating different stresses (Klarzynski et al. 2000; González et al. 2013; Rengasamy et al. 2015; Aremu et al. 2015).

Rayirath et al. (2009) reported *Ascophyllum nodosum* extracts and its lipophilic fraction treatments to *A. thaliana* showed a significant improvement of tolerance to low temperature in in vitro and in vivo assays. Applications of *A. nodosum* extract to the roots of in vitro-grown *Arabidopsis* plants regulate the biosynthesis and accumulation of phytohormone cytokinins, particularly trans-zeatin-type cytokinins and cis-zeatin-type cytokinins, 24 and 96 h and 144 h after seaweed extract application. It also increased the abscisic acid (ABA) and ABA catabolite levels (Wally et al. 2013). Rengasamy et al. (2015) investigated the effect of phloroglucinol and eckol (phlorotannins) isolated from *Ecklonia maxima* and compared with commercial seaweed extract Kelpak® and commercial phloroglucinol and auxin (IBA). The isolated eckol promoted root and shoot growth and number of seminal roots in maize and also enhanced the enzyme activity (α-amylase) compared to commercial seaweed extract, phloroglucinol, and IBA. Moreover, eckol exhibited auxin-like activity in the mung bean root bioassay at 10^{-5} M. Aremu et al. (2015) have reported eckol and phloroglucinol isolated from *E. maxima* at 10^{-6} M concentration given as soil drench significantly increased the bulb size, fresh weight, root production, and bulb numbers, respectively, in *Eucomis autumnalis* (Mill.) Chitt. These compounds respectively at 10^{-5} M and PG 10^{-6} M concentration also significantly increased contents of p-hydroxybenzoic and ferulic acids of the test plant. Foliar application of *Kappaphycus alvarezii* extract to maize at grain-filling stage under drought conditions showed ameliorating effects on growth, yield, and biochemicals associated to pigments, photosynthesis, photoinhibition, lipid peroxidation, antioxidant defense system, and ROS (Trivedi et al. 2018). The transcriptome responses of maize roots to *K. alvarezii* extract as soil drench under normally irrigated and drought conditions were studied by Kumar et al. (2019). Application of

Kappaphycus extract showed advantageous changes in expression pattern of many genes, which are linked with diverse molecular tasks across the cellular components and enable the plants to survive under drought stress by altering the biological processes as a result of transcript changes occurring in roots. Łangowski et al. (2019) studied the effect of Sealicit, a biostimulant extracted from *A. nodosum,* on fruit development and seed dispersal trait in *Arabidopsis* and oilseed rape at both physiological and genetic aspects. They observed that Sealicitis reduce pod shattering and yield loss in oilseed rape by modulating the expression of the most important regulator of pod shattering, indehiscent, as well as disrupting the auxin minimum. Carmody et al. (2020) studied the comparative effects of two carbohydrate-rich formulations extracted from *A. nodosum* on heat-stressed tomato. Both extracts demonstrate significant improvement on flowering, pollen viability, and fruit yield with definite biochemical and transcriptional changes associated with enhanced thermo tolerance. Application of *Sargassum horneri* extract to tomato plant significantly increased the yield (4.6−6.9%) by improving the photosynthetic capacity of tomato leaves and hardness of tomato (10.2–19.8%), reducing the ripening time of tomato and thus achieving a high net returns (Yao et al. 2020).

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2 Biostimulant Potential of Seaweed Extracts Derived from *Laminaria* and *Ascophyllum nodosum*

Joël Fleurence

Abstract

Brown seaweeds belonging to the genus *Laminaria* or *Ascophyllum* are used in traditional and modern agriculture. They are an alternative way to the application of chemical pesticides or synthetic fertilizers frequently required to improve the agricultural production. Macroalgae extracts are used in many countries (India, China, France, Ireland) to improve the crop yield. Seaweed extracts from *Ascophyllum nodosum* and *Laminaria* have often been described to produce positive effect on the germination of seeds and morphological development of plants with an agronomic interest. The main effects are observed especially on the plant growth and root elongation. The presence of growth hormones such as auxins or auxin-like compounds in the seaweed extracts mainly explains these stimulation mechanisms. The positive effect of brown seaweed extract application on fruit quality is also well documented. In regard to these agronomic properties, seaweed extracts are also recommended to limit the biotic and abiotic stresses. The scientific literature gives some information on the bioactive compounds involved in these mechanisms of plant tolerance to these two types of stresses. Some algal polysaccharides, such as laminarin, are well known to induce the defense mechanisms of plants. These phytoelicitors are often used by foliar spraying. The goal of this chapter is to highlight the recent advances in the use of seaweed extracts as agronomic biostimulants. It also describes the main compounds involved in the stimulating activity.

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_2

Keywords

Laminaria sp. · *Ascophyllum nodosum* · Extracts · Abiotic and biotic stresses · Plant growth

2.1 Introduction

Marine algae have a long tradition of usage in agriculture, especially in North Europe. In the British, Irish, and French coasts (North Brittany), seaweeds washed up after the storm were harvested to provide nutrients to poor soils (Delaunay et al. 2016) or improve the soil structure. The most remarkable example is that of the island of Aran (Ireland), where the soil humus consists of sand and washed-up algae mixture. The main species concerned by these types of application belong to the genus *Laminaria*, *Ascophyllum,* or *Fucus*. Generally, the soil improvement was carried out from stranded algae called "goémon" in French. The goémon was mainly composed of a mixture of *Ascophyllum nodosum* (Black Wrack) and *Fucus vesiculosus* (Sauvageau 1920). Nowadays, seaweeds are used as fertilizers from solid or liquid products alone or in mixture. There are a lot of commercial products available for this type of application. The action mechanism of the seaweed extracts on the plant nutrition and development is now well documented by the scientific literature at least for some species with a great agronomic interest (e.g., maize) (Jeannin et al. 1991). In horticulture, the extracts of brown algae are often applied for their effects on growth promoting and improvement of the crop tolerance to abiotic stresses such as salinity, drought, or nutrient deficiency (Battacharyya et al. 2015). In regard to these aspects, seaweed extracts also contain various macromolecules such as polysaccharides with a behavior of plant mechanism defense inducer.

This chapter describes the main uses of the *Laminaria* and *Ascophyllum* extracts as biostimulants for the growth, the health of the cultures, and the crop quality. A description of the molecules involved in these agronomic properties is also proposed. The perspective to use brown seaweeds but also other species in agrobiology is discussed at the end of the chapter.

2.2 Historical and Traditional Uses as Fertilizers

In the past, the stranded brown seaweeds were used to improve the nutritional richness of the soil. The use of seaweed manure for the cabbage cultivation was recommended by the Roman Columella (Craigie 2011). In the fourth-century AD, another Roman, known under the name of Palladius, also recommended in March the application of marine algae manure to the roots of pomegranate and citrus trees (Craigie 2011). After this ancient period, seaweeds were often used as manure by the coastal populations. The additional practice into the soil differed according to region. The algae were mixed with the sand or the soil. However, the composting of seaweeds with straw or peat was also sometimes practiced by some farmers.

Table 2.1 Comparative effects of spreading algae manure and spraying seaweed extracts on plant development

Seaweed manure spreading	Seaweed extracts spraying
Turning soil to limit the production of sulfhydryl compounds	Rapid and positive responses of the plant (even at low concentration of the extract)
Possible inhibition of seed germination at least up to 15 weeks	Maintenance of Cu, Co, Mn, and Fe in soluble forms due to the presence of degraded or related fucoidan
Decrease of ionic nitrogen availability and increase in total N by the soil microorganism action	Stimulating soil microorganism and plant growth

After Craigie (2011)

In Ireland, the soil received a large amount of goémon (*Ascophyllum* sp., *Fucus* sp.) before the plantation of potatoes (Sauvageau 1920). In Brittany (France), farmers living near the coast collected the wrecked algae to spread on their fields of artichokes, potatoes, cauliflower, or in orchards of fruit trees (Perez 1997). In Denmark, the stranded seaweed was very popular as a fertilizer. The Japanese people also used brown algae for the fertilization of their rice fields (Sauvageau 1920). Other brown seaweeds different from *Ascophyllum* and *Fucus* have been also used as fertilizers, also known under the name of goémon. In the case of *Laminaria cloustonii* or *Laminaria hyperborea,* the stipe is perennial and the old blades break off in the spring when the young blades grow. The stranded blades are then called "April goémon" or "red goémon" in Brittany (France). *L. cloustonii* or *L. hyperborea* were also known under the names of "mantelets" in Normandy (France) and "may-weed" in the United Kingdom (Sauvageau 1920). The application of seaweed manure or meal improves the nutrient richness of the soil, but this practice has some disadvantages (Table 2.1). It needs to return the soil to reduce the production of toxic sulfhydryl compounds that can have a negative effect on the seed germination. This agricultural process, called "wheatered practice," is often a necessary step to optimize the effects to the addition of solid seaweed fertilizers. On the other hand, seaweed liquid preparation is also used as fertilizers. Generally, they are applied as soil or foliar spray and exhibit growth-stimulating activity. For this type of product acting on the plant development, the term biostimulant is more adapted than those of fertilizers. However, the stimulating activity does not concern only the plant growth. Other activities than those of the tolerance to various stresses or the quality improvement of the crop are also concerned by the application of seaweed extracts.

2.3 Biostimulant Uses

For most authors, biostimulant is defined as the "product, different to fertilizers that induce the plant growth when used in small quantities." It is the case of seaweed extracts that induce some positive effects on the plant growth by way of hormonal and nutritional responses. There are natural stimulants qualified as "biostimulants" in the commercial and scientific literature, and their use strongly contributes to the improvement of crop yields.

2.3.1 Plant Growth

The effects on the plant growth of seaweed manures or extracts are well documented and very different (Chatzissavvidis and Therios 2014) (Table 2.1). The seaweed extracts can be applied in several ways (Table 2.2) (Khan et al. 2011). According to the application method, the effects mainly concern the nutrition or the growth plant (Table 2.3). Generally, a soil application improves the soil structure with a stimulation of rhizobacteria [plant growth-promoting rhizobacteria (PGPR)]. This mechanism promotes the assimilation of nutrients available in the soil to increase; thus, the plant growth. On the other hand, a foliar spray application induces an improvement of shoot and root growth (Table 2.3).

In general, seaweed extracts and more particularly those of brown algae promote seed germination and plant growth. Most of these extracts are produced from Laminariales (*Laminaria* spp.) or Fucales (*Ascophyllum nodosum*). These liquid products are rich in degraded polysaccharides such as algin, fucoidan, or other sulfate esters (Fig. 2.1) that induce an immediate response of the plant. This response is obtained with an extract diluted (ca. 1/500), suggesting a strong biostimulating activity. The presence of these degraded molecules maintains the solubility of Cu, Co, Mn, and Fe that are important oligo-elements for the development of the plant. By this way, they improve the nutritional bioavailability of these elements for the plant. According to some authors (Craigie 2011), the introduction of degraded fucoidan or algin with strong polar activities should improve the crumb structure and aeration of the soil with a stimulation of the microorganism flora (PGPR) and root system.

Table 2.2 Different modes and methods of applying algal extracts

Application mode	Process
Aerial application	Seed soaking Seeding dip Foliar spray
Soil application	Soil drenching Addition of seaweed extracts to hydroponics

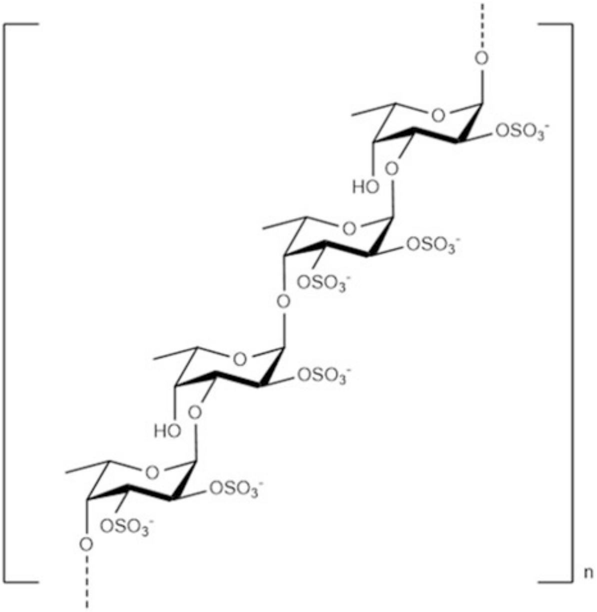
After Khan et al. (2011)

Table 2.3 Comparative effects of seaweed extracts on the plant growth according to the application method (aerial or soil application)

Foliar spray application	Soil application
Improvement of shoot and root growth Increase in yield crop	Promoting soil microorganism (PRGR stimulation) Increase in soil nutrients' bioavailability Improvement of soil aeration

After Chatzissavvidis and Therios (2014)

Fig. 2.1 Fucodain structure from *Ascophyllum nodosum*. (After Fleurence 2018)



The brown seaweed *Ascophyllum nodosum* is the dominant intertidal seaweed in the North Atlantic coastline. It represents a resource that is easy to harvest and available in great quantity. For this reason, this species is often included in the preparation of seaweed extract for agriculture usage. In regard to this economic consideration, a lot of studies also associate some biological properties to *A. nodosum* extracts.

The effects of *Ascophyllum nodosum* extracts on the seed germination or plant growth are often reported. The soaking bean seed in a solution containing *A. nodosum* has a positive effect on germination (Carvalho et al. 2013). The solution used contains an extract of seaweed at the concentration of 0.8 mL.L^{-1} with time soaking of between 5 and 20 min. The seeds soaked in seaweed solution show a germination rate higher (28.5%) than the control (e.g., soaking in water) regardless of immersion time. Foliar application of *A. nodosum* is also reported to increase some growth parameters such as plant height and dry mass (Ali et al. 2019). The use of extract to the concentration of 0.5% of seaweed induces an increase to 40% of plant height, 50% of leaf number, and 52% of dry mass. These results have been obtained from tomato and sweet pepper crops in tropical environment (Ali et al. 2019). In addition, treated plants showed an accentuated flowering and an increased number of fruits. The stimulating activity of *A. nodosum* on the plant growth has also been of other plants with an agronomic interest. It is the case of mung bean (*Vigna mungo* L.) and pak choi (*Brassica rapa chinensis* L.) (Sharma et al. 2012). The effects of the addition of seaweed extract to pak choi hydroponics were studied. The foliar application of brown seaweed extracts obtained from various species induces different results in dry matter yield. The best efficiency is reported with the application of *Ascophyllum nodosum* and *Sargassum muticum* extracts, respectively

Table 2.4 Effect of the alkaline extracts of different brown seaweed species on the dry matter content (%) of the mung bean plants

Ascophyllum nodosum	*Fucus vesiculosus*	*Laminaria hyperborea*	*Fucus serratus*	*Sargassum muticum*	Indole-3-butyric acid (control)
10.46	10.41	10.35	10.09	10.07	8.99

After Sharma et al. (2012)

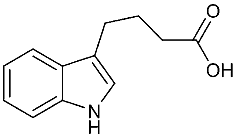


Fig. 2.2 Structure of indole-3-butyric acid phytohormone

Table 2.5 Effect of the acidic extracts of different brown seaweed species on the root number

Ascophyllum nodosum	*Fucus vesiculosus*	*Laminaria hyperborea*	*Fucus serratus*	*Sargassum muticum*	Indole-3-butyric acid (control)
11.09	14.04	10.40	11.21	9.95	31.20

After Sharma et al. (2012)

(Sharma et al. 2012). The dry matter yield is 5.6% after foliar spraying with *A. nodosum* extract at the concentration of 1/100 (weight/volume). It decreases to 4.54% when the extract applied is more concentrated (1/30). A similar result is observed with the *S. muticum* extract. The application of extract diluted to 1/30 promotes a decrease of 4.25% of the dry matter yield in regard to that obtained with 1/100 dilution. Concerning the mung bean plants, *A. nodosum* extract significantly increases the plant growth (+16.35% compared to control). This result is especially obtained with alkaline extracts. The application of acidic or neutral extracts of *A. nodosum* seems to be less efficient for growth promoting (+6.56% and 12.6%, respectively, compared to control). According to the brown seaweed species tested, the effect of alkaline extracts on the mung bean plant growth strongly differs (Table 2.4). Except *A. nodosum* extract, the best efficiency is reported for alkaline extracts of *Fucus vesiculosus* (+15.79% compared to control) and *Laminaria hyperborea* (+14.79% compared to control). In contrast, the acidic algal extracts significantly increase the root development of mung bean plants. The *A. nodosum* acidic extract is more efficient than the *L .hyperborea* and *S. muticum* extracts. However, its activity on the root development is weaker than those of the *F. vesiculosus* extract and vegetal hormone as Indole-3-butyric acid (Fig. 2.2) (Table 2.5). The effects of the *Ascophyllum nodosum* extract on the growth plant and root development therefore differ according to the chemical nature (neutral, acidic, alkaline) of the extract.

The application of commercial *Ascophyllum nodosum* extract (Goëmar GA 14) has been also successfully tested on the development of spinach plants cultivated in growth chambers (Cassan et al. 1992). After foliar spraying at the algal concentration

Table 2.6 Effect of foliar application of *Ascophyllum nodosum* extract on lettuce growth cultivated under normal (375 mg.L^{-1}) and deficiency (125 mg.L^{-1}) concentrations in potassium

Growth parameters	K (375 mg. L^{-1})	K (125 mg. L^{-1})	K 125 mg.L^{-1} + *A. nodosum* extract
Leaf number	42.7	41.2	46.7
Biomass (fresh weight g/plant)	365.4	295.4	371.4
Root (fresh weight g/plant)	35.5	28.1	39.8
Root length (cm)	24.2	18.0	22.7

of 2.5 g.L^{-1}, this extract improves by 12–15% the total fresh matter of leaves. This result is obtained after 8 weeks of culture of two varieties of *Spinacia oleracea* L. The same type of *A. nodosum* extract has been used by spraying on the maize (*Zea mays* L.) cultivated in growth chamber to reduce the environmental variations (Jeannin et al. 1991). The extracts have been applied at two concentrations of seaweed (3.3 g.L^{-1} and 6.6 g.L^{-1}). The foliar application of extract with the concentration of 3.3 g.L^{-1} gives the best performance in the matter of growth (+15.34% of fresh matter compared to control).

The positive effect of *A. nodosum* extract on the growth plant has been also reported with plants growing under potassium deficiency on hydroponic conditions (Chrysargyris et al. 2018). It is the case of lettuce plants when they are cultivated at 125 mg.L^{-1} of potassium. In these conditions, the production of biomass is strongly reduced compared to those obtained with a culture in the presence of potassium concentration of 375 mg.L^{-1}. The addition of *A. nodosum* extract then compensates the potassium deficiency in terms of biomass production, number of leaves, and length of roots (Table 2.6).

The *Ascophyllum nodosum* extracts are often used as biostimulants to improve the plant development. However, the action mechanisms are still partially known. The hormonal content, the oligo-element concentration with a nutritional interest, the presence of algal-specific polysaccharides, betaines, and/or various amine and phenolic compounds are often considered as being involved in the plant response (De Saeger et al. 2020). The phytohormones cytokinins and auxins play an important role in the plant development. The presence of cytokinins in a commercial extract of *Durvillaea potatorum*, a brown seaweed belonging to the order of Fucales as *Ascophyllum nodosum,* has been well described in the mid-1980s (Tay et al. 1985). The presence of numerous plant growth hormones has been reported for several brown seaweeds, especially *Ascophyllum* and *Laminaria* sp. (Table 2.7). In contrast, gibberellins are not detected in the extract of the two genera (Chatzissavvidis and Therios 2014).

Some studies report the presence of phytohormones in the commercial extracts obtained from *A. nodosum*. It is the case for Maxicrop product, which is an *A. nodosum* soluble powder. In this product, the presence of the auxin indole-3-acetic acid (IAA) (Fig. 2.3) has been detected at the level of 6.63 μg per gram of powder (Sanderson et al. 1987). The simultaneous presence of abscisic acid

Table 2.7 Presence of classic phytohormones in the extracts of *Ascophyllum nodosum* and *Laminaria* sp.

Phytohormone	Presence
Cytokinins	+
Auxins	+
Abscisic acid	+
Gibberellins	–

After Chatzissavvidis and Therios (2014)

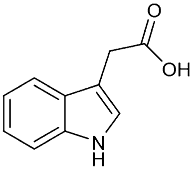


Fig. 2.3 Structure of indole-3-acetic acid phytohormone present in *Ascophyllum nodosum* extract. (After Sanderson et al. 1987)

Fig. 2.4 Structure of abscisic acid

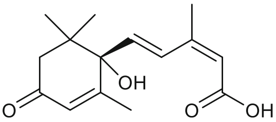


Table 2.8 Examples of plant growth hormone levels in different *Ascophyllum nodosum* extracts (ANE) (ng.g^{-1} dry matter)

Commercial algal extract	Indole acetic acid (auxin)	Abscisic acid	Zeatin-o-glucoside (cytokinin)
Canadian Atlantic ANE	35	1	5
French ANE	6	–	–
American Atlantic ANE	50	–	–
Irish ANE	–	–	–
Norwegian ANE	615	–	–

After Wally et al. (2013)

(Fig. 2.4), auxins, and cytokinins is well documented for some commercial extracts obtained from *Ascophyllum nodosum* (Table 2.8) (Wally et al. 2013).

On the other hand, there are also some examples to the presence of auxin and cytokinin-like activities in *A. nodosum* commercial extract. A cytokinin-like activity has been characterized on different products sold under the names of Marinure, Maxicrop, Seamac, and SM3 (Stirk and Staden 1997). Except SM3, which includes some Laminariaceae, other extracts only contain *Ascophyllum nodosum* as bioactive matter. These products improve the cellular division of the soybean callus, which is a characteristic action of the cytokinin hormone. The best result is obtained with Seamac. An improvement on rooting system of mung bean plants is also described,

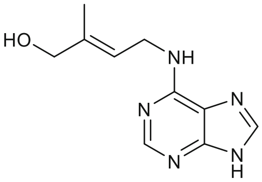


Fig. 2.5 Structure of trans-zeatin (gibberellin hormone)

Table 2.9 Examples of effects of *Ascophyllum nodosum* extracts on some vegetables crop

Vegetable	Seaweed extract	Effects
Bean	*Ascophyllum nodosum*	Increase in germination
Broccoli	*Ascophyllum nodosum*	Increase in biomass, leaf size, stem diameter
Cauliflower	*Ascophyllum nodosum*	Increase in crop yield
Cucumber	*Ascophyllum nodosum*	Increase in vegetative growth, crop yield
Eggplant	*Ascophyllum nodosum*	Increase in vegetative growth
Pepper	*Ascophyllum nodosum*	Increase in crop yield
Onion	*Ascophyllum nodosum*	Increase in crop yield
Watermelon	*Ascophyllum nodosum*	Increase in crop yield

After Battacharyya et al. (2015)

suggesting an auxin-like activity for these extracts. The best result is obtained for the Seamac extract at the *A. nodosum* concentration to 20% (w/v).

The induction of cytokinin-like activity in the plant treated by *Ascophyllum* extract has been also revealed. This phenomenon has well been described on the model plant *Arabidopsis thaliana* after treatment by a foliar application of commercial seaweed liquid extract (Simplex©) (Khan et al. 2011). The plant submitted to the application of the Simplex product, which contains an activity of 3 mL.L^{-1}, showed a cytokinin-like activity to 5 mL.L^{-1}. According to some authors (Wally et al. 2013), the application of *A. nodosum* extracts should activate the metabolic pathways of phytohormone synthesis. This hypothesis has in particular been verified on the model plant *Arabidopsis thaliana* (Wally et al. 2013). An increase in total cytokinin concentration (+50%) is reported in leaves of *A. thaliana* after treatment by seaweed extract. The increase is particularly significant for the trans-zeatin-type CKs (Fig. 2.5) 24 and 96 h after application of the *A. nodosum* extracts. A similar effect is observed for the cis-zeatin-type CKs 114 h after the seaweed extract spraying. In contrast, a reduction in auxin levels and an increase in abscisic acid are observed. These works suggest that the phytohormone content in the algal extracts is not sufficient (Table 2.8) to induce the development of the plant, but its compounds or others could activate the endogenous synthetic pathways of plant hormones. In this case, the term biostimulant is fully adapted to describe the action of *A. nodosum* extract on the plant growth. Nowadays, extracts including *A. nodosum* are often used for their effect on the growth of some vegetables (Table 2.9).

Regardless of the effect on plant growth, the brown seaweed extracts are also useful to reduce the effect of abiotic and biotic stresses.

2.3.2 Tolerance to Abiotic Stresses

Plants are submitted to various environmental stresses such as thermic shock, drought, and hypersaline concentrations. There are a lot of studies that describe the effects of seaweed extract on the improvement of plant tolerance against this type of stress. The winter hardiness and frost tolerance of barley have been well reported after application of *A. nodosum* extracts (Sharma et al. 2014). However, the mechanism of *A. nodosum* extracts involved to thermal variation resistance has been elucidated in some plants. For instance, in tomato plants (*Lycopersicon esculentum*), the thermotolerance is increased after the use of biostimulants of different carbohydrate fractions of *A. nodosum* (Carmody et al. 2020). Two types of preparations (C129, PSI 194) provided by an Irish company (Brandon Sciences) have been tested by foliar spray. C129 extract was obtained by enzymatic treatment of *A. nodosum* carbohydrate fraction by polysaccharides at low temperature (<30 °C). PSI-494 is produced by extraction in high temperature and under alkaline conditions from the same carbohydrate fraction. During the application of moderate thermal stress (24–31 °C) for 14 days, the tomato plants treated with application of PSI-494 extract show best tolerance to this abiotic stress. PSI-494-treated plants surpassed the heat tolerance effect of C129, significantly increasing pollen viability and fruiting (+86%) compared to untreated plants. The effect of PSI-494 on the transcription of three genes (HSP101.1, HSP70.9, HSP17.7 C-Cl) seems to be the mechanism involved in this heat tolerance (Carmody et al. 2020). Effectively, the use of PSI-494 increases the expression of HSP101.1 and HSP70.9 by 2- and 1.7-fold in comparison to untreated plants.

Drought is one of the most common abiotic stresses affecting plant growth and crop yield. Seaweeds contain some molecules that protect against some abiotic stresses such as osmotic imbalance or drought. The seaweed extracts are known contain betaines. It is the case of *A. nodosum* extract that possesses aminobutyric acid betaine, o-aminovaleric acid betaine, and glycine betaine (Fig. 2.6) (Sharma et al. 2014). Betaines have a behavior of cytoplasmic protector for the cells against the osmotic shock and drought.

The effects of *A. nodosum* extract on the plant physiology under drought conditions have been particularly studied in the model plant *Arabidopsis thaliana* (Santaniello et al. 2017). After 4 days of dehydration, the survival rate of experimental culture is near to 10% for untreated plants with seaweed extract (Fig. 2.7). In the same conditions, the survival rate of *A. thaliana* treated with *A. nodosum* extract is 75% for 4-day dehydration. The tolerance of the dehydration after seaweed extract treatment is mainly due to physiological and metabolic mechanisms. Indeed, the application of *A. nodosum* extract induces the partial closure of stoma plants

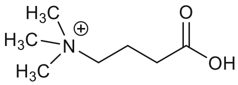


Fig. 2.6 Structure of 4-aminobutyric acid betaine

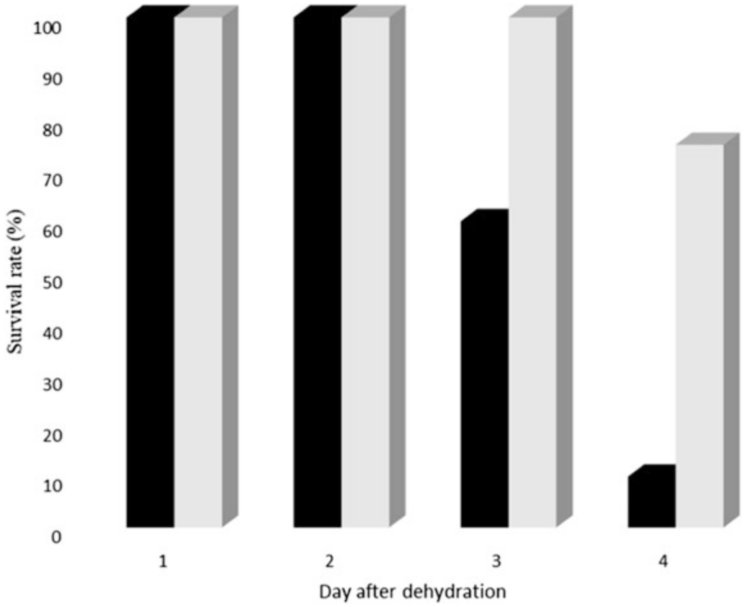


Fig. 2.7 Effect of dehydration on survival rate of *Arabidopsis thaliana* (%) untreated (■) and treated (□) with *Ascophyllum nodosum.* (After Santaniello et al. 2017)

reducing the water losses by sweat. An activation of level expression of genes involved in abscisic acid response and antioxidant pathways is also reported. These hormonal and metabolic activations allow treated plants to maintain a better photosynthetic activity compared to untreated plants. According to Santaniello et al. (2017), the plants treated with *A. nodosum* extracts can maintain a strong stomatal control and water use efficiency during the last phase of dehydration.

The impact on the application of *Ascophyllum nodosum* extract for the tolerance of plants to salt stress is well documented. Salinity is a factor that strongly limits the yield of some crops. It is the case of the cultivation of avocado (*Persea americana* Mill.) (Bonomelli et al. 2018). In saline conditions of irrigation (9 mM NaCl water), the fresh weight of all tissues is reduced to 50% compared to an irrigation without salts. The treatment with *A. nodosum* extract limits the effect of saline stress on the plant height up to 30 days after the application of the saline stress. However, the use of seaweed extract has no effect on the other plant growth criteria.

The effect of *A. nodosum* extract on the protection against saline stress has been also studied on the pepper plants (*Capsicum annuum* L.) (Yildiztekin et al. 2018). Under saline conditions (NaCl 100 mM), the induced stress strongly affects the growth parameters (leaf and dry weight). The application of algal extract improves the vegetative growth in all plants submitted to the saline stress. The best result is obtained with an extract at the concentration of 2 g.L^{-1} (+47%). On the other hand, an increase in antioxidant enzyme activities (e.g., superoxide dismutase, peroxidase, and catalase) is also reported for the plant under salt stress and treated by the algal extract. It suggests that seaweed extract protects against the oxidative stress produced by exposition to saline conditions. The molecular effect of *Ascophyllum nodosum* extract in the improvement of plant submitted to a saline stress is partially known. It has been elucidated on model plant *Arabidopsis thaliana* (Jitesh et al. 2012). In this species, the treatment with algal extract seems to induce a downregulation of genes that are negative regulators of salt tolerance.

2.3.3 Resistance to Biotic Stresses

The plants are able to set up natural defense mechanisms against pathogens (e.g., bacteria, fungi, virus) or various parasites (e.g., nematodes). Some commercial seaweed extracts have been described in the literature as potential inducer of these mechanisms (Table 2.10).

Table 2.10 Examples of commercial seaweed extracts including *Ascophyllum nodosum* and *Laminaria digitata* used for the plant protection

Commercial extract	Seaweed source	Application mode	Crop	Pathogen target
Algamare	*Ascophyllum nodosum*	Foliar spray	*Prunus salicina*	*Monilinia fructicola*
Stimplex	*Ascophyllum nodosum*	Foliar spray	*Solanum lycopersicum* *Capsicum annuum*	*Xanthomonas campestris pv. Vesicatoria* *Alternaria solani*
Ascophyllum nodosum extract	*Ascophyllum nodosum*	Foliar spray	*Fragaria* x *ananassa*	*Podosphaera aphanis*
Maxicrop	*Ascophyllum nodosum*	Foliar spray	*Brassica rapa* *Fragaria* sp.	*Botrytis cinerea*
Iodus or Vacciplant	*Laminaria digitata*	Foliar spray	*Vitis vinifera* *Triticum* sp. *Malus domestica*	*Botrytis cinerea* *Erwinia amylovora*
Ascophyllum nodosum extract (Acadian Seaplants)	*Ascophyllum nodosum*	Foliar spray	*Daucus carota*	*Alternaria radicina* *Botrytis cinerea*
Liquid seaweed extract (LSE)	*Ascophyllum nodosum*	Foliar spray	*Triticum aestivum*	*Fusarium graminearum*
Marmarine	*Ascophyllum nodosum*	Foliar spray Root drench	*Cucumis sativus*	*Phytophthora capsici*
Seasol	*Durvillaea potatorum* and *Ascophyllum nodosum*	Root drench	*Brassica oleracea*	*Plasmodiophora brassicae*

After Craigie 2011, Stadnik and Freitas (2014), Shukla et al. (2021)

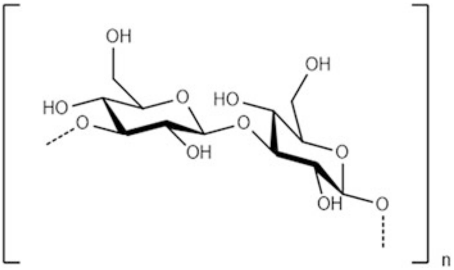


Fig. 2.8 Structure of laminarin from *Ascophyllum nodosum*. (After Fleurence 2018)

The application of Maxicrop, an alkaline extract of *A. nodosum*, blocks the spread of the gray mold (*Botrytis cinerea*) on strawberry fruit (Craigie 2011). This product has been also shown to be effective in limiting progression of powdery mildew on turnips. However, other tests performed with Goëmar BM 86©, which is obtained with the process of cell burst, show a lower efficiency against *B. cinerea* on the strawberry cultivar Elkat (Craigie 2011). It suggests that the processing method to produce the extract can influence the efficiency against a pathogen especially as a fungus.

The seaweed extracts such as *Ascophyllum nodosum* or *Laminaria digitata* contain systemic inducers of metabolic pathways involved in protecting plants (Moon and Anderson 2003). These inducers are mainly glucans. For instance, the liquid fertilizer processed from *A. nodosum* (GYFA 17 Goëmar) contains laminarin (Fig. 2.8), which is known to activate the phenylalanine ammonium lyase (PAL), key enzyme in the phenylpropanoid pathway. This metabolic way produces cinnamic acids (e.g., caffeic, hydroxycinnamic, ferulic acids) and amides conjugates (e.g., feruloyltyramine), which are produced in large quantities when the plant becomes resistant to a pathogen (Fleurence and Negrel 1989). Most of these molecules contribute to activate a novel formation of lignin, which is a mechanism for blocking the penetration of pathogen inside the cell (Fleurence 1988). This defense mechanism is well known under the name of hypersensitive reaction. The synthesis of other compounds with antimicrobial activities such as pathogenesis-related proteins (PR proteins) and phytoalexins is also increased by the action of laminarins.

The laminarin, also known under the name of laminaran, is present in *Laminaria digitata*. These molecules stimulate defense reactions in cell suspension of tobacco, grapevine, and rice (Stadnik and Freitas 2014). The addition of laminarin at the concentration of 200 μg mL^{-1} to the culture of tobacco cells promotes a strong stimulation of the PAL, caffeic acid O-methyl transferase, lipoxygenase activities, and the accumulation of salicylic acid (Klarzynski et al. 2000). The infiltration of laminarin (200 μg mL^{-1}) into tobacco leaf protects the plant against the infection by the pathogen bacterium *Erwinia carotovora* (Klarzynski et al. 2000). After spraying on tobacco or grapevine plants, laminarin induces the accumulation of phytoalexins and PR proteins without activation of hypersensitive response. An increase in the production of phytoalexins such as capsidiol is also reported after foliar spray with *A. nodosum* on pepper plant. This treatment significantly improves the resistance of the plant to *Phytophthora capsici* (Chatzissavvidis and Therios 2014). The process of laminarin supply therefore seems to play a role in the induced defense mechanisms. According to some authors, laminarin and other oligosaccharides mimic (Potin et al. 1999; Stadnik and Freitas 2014) the pathogen attack (bacteria, fungi, virus), and this activates the natural defense mechanism of plants (Potin et al. 1999; Stadnik and Freitas 2014).

Many commercial products containing laminarin, such as Vacciplant, are available in different countries to protect crops against fungi or bacterial diseases. Their application mode (foliar spay, soil, or root drench) differs according to the product formulation and the crop. Losses due to the soil nematodes are very important for the agriculture (US $100 billion per year) (Radwan et al. 2012; Chatzissavvidis and Therios 2014). In some cases, seaweed extracts could be an alternative to chemical pesticides for the crop protection against the damages due to nematode. The commercial product Algaefol© processed from *A. nodosum* has been tested against the nematode *Meloidogyne incognita,* which attacks tomato root system (Radwan et al. 2012). The product is applied by soil drenching at the concentration of 25 mL.kg^{-1} of soil.

A. nodosum extract significantly reduces the number of root galling (86.96%). It is more efficient than another bioproduct Plant Gard©, which is elaborated from the microscopic fungus *Trichoderma harzianum* (69.79%). At the concentration of 25 ml.kg^{-1}, Algaefol© significantly increases the root length (+47%) of the plants infected by *M. incognita*. However, at the concentration of 50 mL.kg^{-1}, the application of *A. nodosum* extract induces the tomato seedling death. The betains contained in *A. nodosum* extract seem to have a similar activity than that of chemical nematicide carbofuran (Chatzissavvidis and Therios 2014). However, the *A. nodosum* extract also could be active by another way than a biocide activity. Indeed, the betains of *A. nodosum* extracts seems to play a role in the suppression of the fecundity of *Meloidogyne javanica.* The treatment of the model plant *Arabidopsis thaliana* with an alkaline extract of *A. nodosum* (Maxicrop original) induces a significant decrease in the number of females that are present in the roots (−42%) and in the egg recovery (−41%) (Wu et al. 1998). A comparable activity is observed when the plants are treated with a betain mixture γ aminobutyric acid betain, aminovaleric acid betain, glycine betain. In this condition, a decrease of 34% in the number of females and 44% in the egg recovery is reported. Despite this, some results suggest that seaweed extract imparts a nematode resistance by modification of the auxin cytokinin ratio (Chatzissavvidis and Therios 2014).

2.3.4 Fruit Quality and Productivity

Some studies are focused on the impact of algal extract treatment on the fruit quality when they are still attached to the plant and after harvesting. Several results suggest that seaweed biostimulants can improve the fruit quality and productivity (Rodrigues et al. 2020). However, the impact seems to differ according to extract composition, way to apply, dose provided, and the plant concerned. For instance, in orange

Table 2.11 Impact of the Biozyme Crop Plus© treatment on the pomegranate fruit quality

Treatment	Dose (mL. L^{-1})	Fruit cracking (%)	Fruit length (mm)	Fruit diameter (mm)	Fruit weight (g)	Fruit volume (cc)
Control	Water spray	20	79.3	79.8	296.5	297.0
Biozyme	1	16.7	81.9	84.1	303.7	284.2
Biozyme	2	14.2	83.0	84.1	304.3	285.0
Biozyme	3	12.7	84.7	85.4	306	285.5

After Abubakar et al. (2013)

Table 2.12 Impact of the Cytozyme Spic© treatment on the pomegranate fruit quality

Treatment	Dose (mL. L^{-1})	Fruit cracking (%)	Fruit length (mm)	Fruit diameter (mm)	Fruit weight (g)	Fruit volume (cc)
Control	Water spray	20	79.3	79.8	296.5	297.0
Cytozyme	1	15.7	80.8	82.9	301.1	282.3
Cytozyme	2	14.0	82.8	83.1	302.8	283.8
Cytozyme	4	11.3	91.2	88.7	316.3	293.6

After Abubakar et al. (2013)

orchards the fruit productivity is increased. This treatment also increases the total soluble mass of the fruit and reduces its acidity (Rodrigues et al. 2020).

In the same way, *A. nodosum* extract significantly increases the yield of mandarin orange (*Citrus sinensis* cv Valence) by 11%. After three continuous years of foliar spraying, the yield fruit reaches about 25%. Although the yield is increased in this species, no increase in the size and weight of the fruit is reported. The treatment of pomegranate (*Punica granatum*) trees with the commercial product Biozyme Crop Plus© or Cytozyme Spic© that include *A. nodosum* in their composition has a positive effect on the pomegranate fruit quality. Biozyme Crop Plus© is a commercial preparation formulated from a seaweed extract (*A. nodosum*), enzymes, and hydrolyzed proteins. In comparison, Cytozyme Spic© contains *A. nodosum*, phytohormones (e.g., gibberellic acid, auxins, cytokinins), hydrolyzed proteins and trace elements. The fruit cracking, which is a quality disadvantage for the commercialization, is significantly reduced by the Biozyme Crop Plus© treatment (Table 2.11). Other quality parameters such as fruit length, diameter, and volume are improved (Table 2.11) (Abubakar et al. 2013). The best results are recorded with the Cytozyme Spic© at the concentration of 4 ml.L^{-1} (Table 2.12).

Evaluations of *A. nodosum* treatment on the ripening and fruit quality have also been performed. The effects of a sprayable *A. nodosum* extract on different cultivars of grapevines (cv Sangiovese cv Pinot Noir, cv Cabernet Franc) have been studied in particular (Frioni et al. 2018). The seaweed product is sprayed at the dose of 1.5 kg. ha^{-1} five times 2 weeks before the veraison. The treatment with algal extract improves the accumulation of anthocyanins in the berries of all cultivars studied.

Table 2.13 Effect of the treatment on the fruit quality parameters

Treatment	Titratable acidity (g.L^{-1})	Color index	Anthocyanins content (mg.100 g^{-1} dry weight)	Vitamin C
Control (water)	6.2	12.6	58.1	19.6
A. nodosum extract	6.9	31.5	186.1	16.2

After Soppelsa et al. (2018)

An increase in phenolic content is also especially observed in Sangiovese cultivar. Phenolic compounds are major criteria in the definition of wine quality, and anthocyanins are key substances for the color appearance of berries and therefore the quality of grapes. The impact of seaweed extract treatment on yield and fruit quality at harvest and during storage has been studied on apples (*Malus domestica*) (Soppelsa et al. 2018). The providing of *A. nodosum* extract mainly stimulates the apple tree growth (+20%) and increases the leaf photosynthetic rate. Other parameters such as fruit weight, soluble solids content, acidity, or flesh firmness are not significantly changed by the application of seaweed extract. The climatic conditions are the main criteria playing an important role during this experiment performed over 2 years (2016, 2017). On the other hand, the seaweed extract induces the accumulation of anthocyanins in the apple skin and has a positive effect on the fruit coloration (Table 2.13). Seaweed extract also reduces the incidence of "Jonathan spot," which is the main post-harvested disorder for this culture.

The action of *A. nodosum* extract on quality of numerous fruits has been often reported. The application of Goëmar BM86 on two tomato cultivars ("Esmeralda F1" and "Dual Plus F1") promotes the increase in lycopene and the reduction in other quality criteria such as carotenes or ascorbic acid (Rodrigues et al. 2020). For strawberry, the application of *A. nodosum* on the cultivar Clery increases the fruit coloration at the beginning of the season. However, on other cultivar (cv Elsanta), the effects reported are different.

On two cherry (*Prunus avium*) cultivars (Sweetheart and Skeena), the application of algal extracts induces positive effects on the fruit quality. A reduction in cracking index and an increase in the width, weight, and diameter of the fruits are reported (Rodrigues et al. 2020). However, no changes in the nutritional characteristics or fruit yield are observed after the application of seaweed extracts.

2.4 Conclusion

The seaweed extracts are frequently used as natural biostimulants in agriculture. According to the countries and the natural presence of some species on the coastline, the products differ in the use of species employed. The easy accessibility of algal resource is determinant to its use in the composition of the biostimulant product. *Ascophyllum nodosum,* which is a common intertidal species more accessible than *Laminaria digitata,* is the favorite species for processing. In other countries, other

Table 2.14 Examples of commercial products used as biostimulants in the world including brown seaweeds

Country	Commercial product	Algal species
Australia	Seasol	*Durvillaea potatorum*
Australia	Seaweed	*Durvillaea antarctica*
Australia	Liquid kelp	*Ascophyllum nodosum/Posidonius australus*
Canada	Stimplex	*Ascophyllum nodosum*
China	Gofar	*Ascophyllum nodosum* *Laminaria* sp. *Sargassum* sp.
France	Goëmar	*Ascophyllum nodosum*
Germany	Algifol	*Ascophyllum nodosum*
India	Biovita	*Ascophyllum nodosum*
Ireland	Ecolicitor	*Ascophyllum nodosum*
Italy	Rygex	*Ascophyllum nodosum* *Laminaria digitata*
Mexico	Agrokelp	*Macrocystis pyrifera*
New Zealand	AgriSea	*Ecklonia radiate*
South Africa	Afrikelp	*Ecklonia maxima*
USA	Maxicrop	*Ascophyllum nodosum*

After Sharma et al. (2014)

brown seaweeds are included in the formulation of biostimulants (Table 2.14), but to the world *A. nodosum* remains the main species exploited for this application. The properties of biostimulants are numerous and various. They mainly concern the plant growth and resistance to important agronomic disorders such as drought or microbial diseases. They are a suitable alternative to the use of chemical pesticides that have a negative impact on consumers' health. However, the efficiency of these products strongly depends to the composition of algal extract and also of the process applied. The effects can also vary according to the crop targeted. The main advantage to applying seaweed extracts in agriculture remains the plant development and the tolerance to abiotic and biotic stresses. It is due to the presence of natural phytohormones such as cytokinins and auxins that induce the activation of plant endogen hormones. The presence of specific oligosaccharides such as laminarin in the brown seaweed is also an advantage to use the algal extract as plant vaccine.

Concerning the fruit quality, the results are interesting on some species affected by the cracking such as pomegranate. The impact of algal extract application is also interesting to improve the color appearance of some fruits such as grapes, apples, or strawberries. In the future, a coupling between a sustainable valorization of seaweeds and the agricultural production seems to be a promising way for the consumers and the populations.

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Borage Extracts as Biostimulants of Plant Growth and Development

3

Giulia Franzoni, Roberta Bulgari, and Antonio Ferrante

Abstract

Plants can be an important source of bioactive compounds that can be used for several purposes. In agriculture, there is an increasing interest in biostimulant production as innovative agronomic tools to enhance crop productivity and tolerance against abiotic stresses. Biostimulants are usually obtained from raw materials rich in bioactive molecules, and botanicals can represent a source for the industrial production of biostimulants. Among the different plant species, there is a particular interest in borage (*Borago officinalis* L.) since is rich in bioactive compounds and has been largely used in popular medicine and in the foodstuff. Recently, borage has been used for biostimulant production. The bioactive compounds have been extracted through maceration in water. The extract has been active in the increase yield and stimulating the plant secondary metabolism. The application of borage extract increased the yield and photosynthesis of lettuce, and at biochemical level the activation of phenylpropanoids with an increase in antioxidant compounds with positive effect of produce quality was done.

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_3

Keywords

Antioxidants · Biostimulants · Borage Extract · Bioactive components · Photosynthesis

3.1 Plant Extracts

Plants have been used since ancient times not only as food but also as a source of biologically active agents, typically for the treatment of diseases in traditional medicine of several cultures. They were applied on an empirical basis, without any knowledge of their activity or components. Botanical active substances are defined as one or more components found in plants, obtained by subjecting plants or parts of plants to different processes of extraction. Even if a decline in interest of their application occurred in the past years, plants offer a unique resource of biological active substances due to their structural and botanical diversity. Secondary metabolites' plant composition might vary in the qualitative and quantitative traits because it is influenced by the environmental conditions. Nowadays, plant extracts are gaining much importance due to their potential and the change in consumers' behaviour. Indeed, besides their medical or pharmaceutical use, the request of plant extracts is increasing as food, beverage additive, and cosmetics. Moreover, their market also includes their application in the food preservation industry and agricultural sector as raw materials for agrochemicals or biostimulant products.

One of the most investigated groups of natural products is represented by secondary metabolites. They are defined as natural products that are not essential for vegetative growth, but they have an adaptive role in plants. For example, they could enable plants to resist pathogens and deter insect or other animal attack due to their toxic nature. They could be involved in signalling processes as defence mechanism regulator molecules or in different types of communication to attract pollinators or in interplant. Generally, they exert a vital function in ecological interaction between plants and the surrounding environment. Secondary metabolites can be divided into three distinct groups: terpenes, phenolics, and N- and S-containing compounds (Starmans and Nijhuis 1996; Mazid et al. 2011; Naboulsi et al. 2018). Terpenes are generally insoluble in water and are responsible of the odours of plants. They are classified as monoterpenes, sesquiterpenes, diterpene, triterpenes, and polyterpenes on the basis of their chemical structure. Phenolic compounds are one of the most studied classes of secondary metabolites due to their wide range of biological functions. They are responsible for the colour of plants and are involved in several physiological mechanisms during plant growth and reproduction. Based on their chemical formula, they can be divided into phenols, coumarins, lignin, lignans, tannins, phenolic acids, and flavonoids. Nitrogen and sulphur-containing secondary metabolites are mostly involved in plant defence mechanisms. The first class is represented by alkaloids; the second includes GSH, GSL, phytoalexins, thionins, defensins, and allinin.

3.1.1 Extraction Methods

A wide range of extraction methods and technologies are available for the separation of these compounds from the inactive or inert components (Handa et al. 2008; Azmir et al. 2013; Azwanida 2015). Most of these techniques exploit the different power of extraction of various solvents and the application of heat, pressure, and/or mixing. Infusion, maceration, digestion, decoction, percolation, and Soxhlet extraction are some of the conventional extraction techniques. These methods use organic solvents (such as hexane, acetone, methanol, ethanol) or water and are generally carried out under atmospheric pressure. Alternative approaches include accelerated solvent extraction (ASE) (Mustafa and Turner 2011), supercritical fluid extraction (SFE) (Lang 2001), microwave-assisted extraction (MAE) (Chan et al. 2011), ultrasound-assisted extraction (UAE), subcritical water extraction (SWE), pulsed-electric field extraction (PEF), enzyme-assisted extraction (EAE) (Puri et al. 2012), and rapid solid–liquid dynamic extraction (RSLDE) (Naviglio 2003). These methods have emerged in order to mitigate the limitations of the traditional ones, reducing the extraction time and the number of organic solvents used. These techniques also improve the yield and bioactivities of the extracts. The choice of the method, the solvent, and the temperature depends on different factors such as the nature of the targeted compounds (polarity or thermosensitivity), the organ of plants used (leaves, flowers, roots, or seeds, used individually or in combination), and the economic feasibility of the process to the particular situation. Moreover, also the time of the extraction affects both the yield and composition of the extract. Thus, standardized extraction methods that take into consideration all these parameters are required in order to obtain a stable and high-quality extract.

However, even if the newest extraction techniques promote the efficiency of the extraction of specific components, sometimes the high costs make them unaffordable. For example, maceration and decoction extract methods resulted in more applicable, convenient, and economical for small and medium enterprises or in developing countries compared with the modern technique (Sithisarn et al. 2006; Vongsak et al. 2013).

3.1.2 Plant Extract in Agriculture

To protect from insects or pathogen attacks, plants have developed the ability to synthesize specific molecules with a toxic or repulsive effect on their enemies. These properties have been exploited over centuries in the preparation of many pesticides products, both in small-scale application and as raw material for commercial formulation (Benner 1993; Siegwart et al. 2015). Their use declined since the 1940s, where they have been almost entirely replaced by synthetic pesticides. Nevertheless, nowadays the interest in natural-based products is increasing since modern agriculture is moving toward a more sustainable use of the resources, minimizing the input and harmful effects of several practices. Moreover, the limitations in the application of synthetic products increased due to the high costs and their potential negative impacts on the environment and human health. These reasons motivated researches to look for different solutions, and plant-based products are become an interesting alternative to replace, or at least reduce, the application of conventional chemical products also because plant extracts are generally cheap, environmentally friendly, and readily available. Thus, based on their traditional application and by studying the diverse properties of many different plant species, several trials have been done in order to investigate the effectiveness of plant extracts. Some of these products are still applied in small-scale agriculture or in organic farming due to the facility in their preparation or the prohibition to use synthetic alternatives (Gurjar et al. 2012; Naboulsi et al. 2018).

Besides their use in plant protection, the use of natural supplementations as green approach for promoting plant performance is highly requested. Several preparations based on plants showed interesting effects stimulating plant growth, improving quality traits, or enhancing tolerance to abiotic stresses (Bulgari et al. 2019a). Besides the application as crude extracts, plants might be considered as the raw material for the extraction of several bioactive compounds that can be used in the formulation of more complex products. For example, different biostimulant products contain protein hydrolysate, amino acids, or mineral compounds obtained from plant material. The interest in plant extracts with biostimulants properties is increasing as they have been shown to improve plant growth and quality (Culver et al. 2012; Abdalla 2013; Pardo-García et al. 2014; Szparaga and Piskier 2017). Moreover, the application of plant extracts as a potential alternative or additional products to the currently used agrochemicals fits the need for a more sustainable agriculture (Chojnacka 2015; Xu and Geelen 2018; Toscano et al. 2019). Thus, several companies and research groups are focused on the study and development of innovative products based on plants. Plants are a rich source of diverse bioactive compounds; however, the high variability in their composition, the difficulties in the choice of the best extraction method, and the stability of the final product are just some of the problems in the formulation of new plant-based products (Lapornik et al. 2005; Azmir et al. 2013; Naboulsi et al. 2018). Hence, several experiments need to be carried out to assess their effects.

3.2 Borage as Biostimulant Source

Borage (*Borago officinalis* L.) is an annual crop that is cultivated for several purposes; its beneficial properties are widely acknowledged, and it has been used in pharmaceutical and culinary fields since ancient times (Asadi-Samani et al. 2014). Nowadays, interest in this herb has been renewed due to the high level and quality of gamma-linolenic acid (GLA) in seeds, which is used in medicine, cosmetic (Flider 2005; Hebard et al. 2008), or as food supplement (Fan and Chapkin 1998). Moreover, the antioxidant activity of borage extracts has been exploited in food preservation (de Ciriano et al. 2009; Aliakbarlu and Tajik 2012) and packaging industry (Gómez-Estaca et al. 2009; Bellés et al. 2017, 2018; Zając et al. 2020). Besides the applications in human or animal fields, recent studies showed a biostimulant effect of borage extracts on lettuce and rocket plants (Bulgari et al. 2017, 2020; Franzoni et al. 2020).

3.2.1 Taxonomical Classification

Kingdom*: Plantae*

Subkingdom*: Tracheobionta*
  Superdivision*: Spermatophyta*
  Division*: Magnoliophyta*
  Class*: Magnoliopsida*
Subclass*: Asteridae*
Order*: Lamiales*
Family*: Boraginaceae*
Genus*: Borago L.*
Species*: Borago officinalis L.*

3.2.2 Botanical Characteristics

B. officinalis is an annual plant belonging to the family of Boraginaceae, with an indeterminate vegetative growth habit. The Borago genus only has five species around the world, among which *B. officinalis* is the most important and interesting species. Normally, one primary stem grows from basal rosette of leaves, although sporadically multiple stems appear equally dominant. From the main stem, primary, secondary, tertiary, and sometimes quaternary axillary stems develop (Janick et al. 1990). Stems are hollow, succulent, cylindrical, and occasionally susceptible to lodging. Borage is generally an erect plant whose height ranges from 70 to 100 cm, but low plant densities affect its habit, leading to a spreading growth.

Leaves are simple, alternate with an obtuse apex and an entire crenate margin. The shape may be obovate, ovate, or oblong and range from 5 to 15 cm long and 3 to 8 cm wide. Basal leaves are stalked, arranged in a rosette, and the petiole is decurrent while upper leaves are sessile or have a short petiole. Leaf colour is green, but the shade is darker on the upper surface and lighter on the lower page. White, tough, and unicellular trichomes cover both leaves, stems, and calyces. Borage flowers are normally bright blue or blue violet, but sometimes pink or white-coloured flowers may appear. Five ovate–lanceolate petals are united to form a star-shaped corolla, which is approximately 2 cm in diameter. Five short white scales with pink–violet tips are present at the base of the corolla where petals fuse together. Each petal is about 1 cm long and 0.5 cm wide. The single, short, gynobasic style with a capitate stigma is surrounded by five black stamens attached near the base of the corolla and the introse anthers form a kind of conical structure. Each flower is supported by a long pedicel ending with a deeply clefy calyx composed of five green and conical sepals. The ovary is situated above the flower parts (superior) and changes to a fruit with 3–4 green nutlets when immature, turning brownish-black at maturity. Since flowers are produced continuously and mature over an extended period, seeds of different developmental stage are present on the plant at the same time (Beaubaire and Simon 1987; Simpson 1993a). The rooting system is a taproot where a network of small and long roots emerges from a single dominant large structure. For this reason, borage does not tolerate transplants well.

3.2.3 Origin and Cultivation

Most of the studies report that borage is native to Mediterranean areas, probably to North Africa, and then were transferred into Spain and other regions of Europe and Asia Minor. Indeed, unlike other species from *Borago* genus restricted to northwest Africa or limited in some Italian regions, *Borago officinalis* is a widespread species distributed in several countries of and beyond the boundaries of the Mediterranean basin. Here it is present as wild weed, cultivated as garden plant or as crop vegetable (Selvi et al. 2006; Mansion et al. 2009). Otherwise, some authors say that the plant is native to India and Iran where the leaves have been used to prepare tea for years (Kalhor and Ashrafian 2017). Nowadays it is cultivated all around the world, such as in Canada, Australia, the United States, or New Zealand, for the production of its seed oil rich in GLA. However, information about cultivation and best management practices is still limited and not well defined. Borage is a very adaptable plant; it can grow in different types of soil and in a pH scale range from 4.5 to 8.2. It requires low to moderate moisture; it can survive in drought conditions, but a wet and well-drained medium with a pH around 6.6 is preferred. Borage plants are sensitive to salt stress, affecting their growth in terms of leaf area, dry weight, stem length, and diameter (Jaffel et al. 2011; Jaffel-Hamza et al. 2013; Chakovari et al. 2016). Also a decrease in plant fecundity, resulting from abnormalities during pollen developmental process, has been observed in plants grown in saline environment (Torabi et al. 2013). Nevertheless, due to its ability to withstand saline condition and to uptake sodium and chlorine, the possibility to use it in bioremediation has been taken into consideration (Badi and Sorooshzadeh 2011).

Borage plants grow better under moderate exposure to sun because intense radiations change their status to rosette leaves (Asadi-Samani et al. 2014). They have high resistance to cold, and the suitable period of cultivation is spring or autumn. Temperature is a critical but essential point in plant cultivation, and it influences seeds germination, the duration of the growing cycle, and the quality of the production. For these reasons, several trials have been performed in order to find the cardinal temperatures for borage cultivation and define the better sowing and harvesting dates. Ghaderi et al. 2008 reported that the minimum, optimum, and maximum germination temperatures for Iranian accessions of borage are 5, 30, and 40 °C, respectively. A further experiment conducted with a thermogradient table determined minimum, optimum, and maximum temperatures for borage germination of 9, 23, and 30 °C, respectively (Gilbertson et al. 2014). A recent study reported that borage growth was stimulated at 24 °C compared to 21 °C, but impaired at 27 °C caused an impaired growth and a decrease in flower buds number (Descamps et al. 2018). Indeed, even if borage plants are able to grow below 6 °C they flower only when the temperature is higher, affecting also the production of the seeds (Berti et al. 2002). The minimum amount of GLA that most of the processing companies require is 22%, which is not easy to obtain at latitudes lower than 38° (Berti et al. 2010). Therefore, borage is usually grown at higher latitudes. The accumulation of GLA occurs in later stages of development, and the higher level of GLA is not reached until physiological maturity. Its accumulation in borage seeds is affected by temperature, and, in agreement with other oilseed crops, GLA increases as temperature decreases during seed filling (El Hafid et al. 2002). It means that a variable mixture of seed at several maturity stages and different fatty acids content is harvested. Moreover, once seeds reach the physiological maturity, they are often lost due to shattering. All these aspects strongly affect seed yield and composition and make harvest management decisions very difficult. Several studies aim to define the best harvest time (El Hafid et al. 2002; Simpson 1993b; Mhamdi et al. 2010a, b) and method (Galambosi et al. 2014) and, also, to identify nonshattering borage mutants (De Haro-Bailón and Del Rio 1998; De Haro et al. 2004). On the contrary, flowering twigs are collected before the start of seed formation when borage is cultivated for medical purpose.

Plant density is another important point in borage cultivation; to avoid a spreading growth habit, a value of 100,000 shrubs per hectare, sowing about 5–7 kg is recommended. Researchers report also 30 cm distance between rows and 10 cm distance between plants on row as the best attendance (Asadi-Samani et al. 2014). The maximum yield was obtained with a planting density between 172,222 and 205,000 plant per hectare, at 60 and 40 cm between raw, respectively, and with a seeding rate of 7 kg per hectare (Berti et al. 2002). Other experiments indicated that a sowing rate of 16 kg per hectare may be positive if seed germinability is poor, but generally 8 kg is enough (Laurence 2004). Fertilization is not always adopted in borage cultivation because the amount of nutrient in soil is usually enough. However, several trials have been performed to evaluate the effects of different fertilization strategies on borage plants. The majority of them reported a positive effect of nitrogen and potassium fertilization on plant growth, number of branches, dry matter and grain yield, number of flower, mucilage percentage, and essential oil yield (Ebrahimi et al. 2010; Hendawy and El-Gengaihi 2010; Berti et al. 2010; Mahmoodi et al. 2018); another study showed an opposite effect on GLA concentration in borage seeds, according to the source of nitrogen (urea or ammonium nitrate) added (El-Gengaihi et al. 2000). In some cases, the N P K fertilization did not significantly affect plant growth and biomass, probably due to the high fertility of the soil (El Hafid et al. 2002; Berti et al. 2010). Furthermore, a positive response to sulphur application was observed in seed yield (Berti et al. 2010).

Irrigation as well as fertilization is not generally applied in borage cultivation even if it has been observed that water stress negatively affects flower development, nectar sugar content, pollen viability, and grain yield (Ghassemi-golezani et al. 2013; Tafazolli et al. 2016; Descamps et al. 2018).

3.2.4 Phytochemical Constituents

Over the years, many studies have been conducted to study the chemical composition of *B. officinalis*. The phytochemical analyses were carried out to identify different kinds of compounds from stems, leaves, flowers, and seeds, and several extraction techniques have been used (Ramezani et al. 2019). Since its importance in pharmacological, medical, and nutritional application, most of the studies focused on the isolation of the most active compounds, in particular GLA from seeds, and little work has been reported about minor and trace elemental composition of leaves and flowers.

Several studies described the fatty acid composition of borage, and even with some differences in the obtained results, almost all of these confirmed that borage leaves are rich in polyunsaturated fatty acids (PUFAs) especially α-linolenic, stearidonic, linoleic, and γ-linolenic acids. Besides PUFAs, some monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) were identified. MUFAs were represented by oleic, hexadecenoic, and palmitoleic acids; SFAs by palmitic, arachidic, and stearic acids (Griffiths et al. 1996; Mhamdi et al. 2007b; Borowy et al. 2017; Wannes et al. 2017). In addition, others fatty acids such as meristic, lauric, eicosenoic, behenic, lignoceric, and nevronic acids have been detected in green parts of borage plants by del Rio-Celestino et al. (2008). Furthermore, others lipid classes like phospholipid, glycolipid, and neutral lipids were investigated (Stähler et al. 2011; Wannes et al. 2017). Fatty acid composition of borage flowers and leaves was similar; few differences in the percentage of the components were observed, and flowers were characterized by high content of linoleic, palmitic, and γ-linolenic acids (Borowy et al. 2017). Fatty acids are precursors for many volatile compounds responsible for the fresh, green, and fruity odour of fruits and vegetables. Different compounds belonging to several classes have been identified in borage leaves and flowers (Mhamdi et al. 2007a; Wannes et al. 2017). Two aliphatic hydrocarbons represented by nonadecane and tetracosane were the most abundant, followed by an alcohol ((Z)-3-hexenol), a keton (camphor), a phenol (carvacrol), several aldehydes and monoterpenic hydrocarbon classes in small amount. (E)-(E),2-4 decadienal has been identified as the main compound of the essential oils from borage stem and flower (Salem et al. 2014a, b) while benzenacetaldehyde, octanal, and nonanal are the major compounds in essential oil, flowers, and leaves, respectively (Zribi et al. 2019).

In spite of the high levels of fatty acids, studies focused attention also on the phenolic profile of borage and its antioxidant activity (Conforti et al. 2008; Mhamdi et al. 2010a, b; Zemmouri et al. 2014; Segovia et al. 2015). Tannins and anthocyanins were present in low amount in leaf extracts compared with total flavonoids (quercetin, isoquercetin, catechin-7-O-glucoside, naringenin O-hexoside, luteolin 7-O-glucoside, vitexin and isovitexin, luteolin

7,30,40-trimethyl ether, kaempferol 3,7,40-trimethyl ether, naringenin O-hexoside) and total flavonols. As the main class of flavonoids, anthocyanins are present also in borage flowers as pigments. They are responsible for the change in their colour from pink to blue, and their concentration increases during flower development. The major compound is represented by petunidin 3,5 diglucoside, followed by delphinidin 3,5 diglucoside (Salem et al. 2014a). Karimi et al. 2018 confirmed the presence of phenolic and flavonoid in borage flowers, and among them the salicylic acid and myricetin were the most abundant compounds.

Others antioxidant compounds such as rosmarinic, syringic, and sinapic acids were found both in borage leaves (Bandoniene and Murkovic 2002; Bandoniene et al. 2005) and seeds (Wettasinghe et al. 2001). A few sterols (β- and ɤ-sitosterol, campesterol, stigmasterol, cholesterol, ergosta-5,24-dien-3-ol), and a secoiridoid (oleuropein) with antioxidant properties were identified in leaves extracts too (Conforti et al. 2008; Zemmouri et al. 2014). Moreover, a new lignan glucoside (officinalioside), together with three megastigmane glucosides (actinidioionoside, roseoside, crotalionoside C), and one flavonoid galactoside (kaempferol 3-O-β-D-galactopyranoside), was isolated from the aerial parts of borage (Samy et al. 2015). A derivative of aspidospermine (1-acetyl-19,21-epoxy-15,16-dimethoxyaspidospermidine-17-ol) exerting an anti-amyloid activity by assisting the proper folding of the protein has been recently found in borage leaf extract (Kalhor and Ashrafian 2017).

In addition to the antioxidant compounds, a few amount of toxic pyrrolizidine alkaloids (lycopsamine and supinidine viridiflorate) were identified in leaves, seeds, and flowers of borage (Larson et al. 1983; Dodson and Stermitz 1986; Herrmann et al. 2002; Vacillotto et al. 2013). Concerning the mineral composition, borage showed a great amount of potassium, followed by sodium, calcium magnesium, and iron. Manganese, zinc, and copper were present in minor amounts (Medrano et al. 1992; Bianco et al. 1998; Volpe et al. 2015). Moreover, a variable percentage of mucilage, resin, and gum was found in leaves, stems, and flowers.

The quality and the ratio of borage tissues components change upon growing stages, as reported by several authors (Jamieson and Reid 1969; Sayanova et al. 1999; Peiretti et al. 2004; Aghofack-Nguemezi and Tatchago 2006; Mhamdi et al. 2007a, 2010b; Salem et al. 2014a). For example, nonadecane and tetracosane were predominant during growth period, then a decrease of hydrocarbons and aldehydes and a progressive increase in alcohols was observed. Moreover, the total fatty acids showed an initial increase followed by a decrease during senescence due to loss of membrane lipids. The evolution of the chemical composition was related to the stem elongation. Indeed, a progressive increase in fibre and lignin fraction and a decrease in raw protein was described (Medrano et al. 1992). Moreover, since the level of micronutrients, mineral and trace elements as well as the chemical composition of plant tissues are affected by the chemical and physical properties of soil, and by the environmental condition (Zribi et al. 2019), some differences were found from one study to another.

Current interest in borage cultivation is for its seeds that contain a high percentage of GLA in the oil. In mature seeds, it ranges between 170 and 280 g kg^{-1}. Palmitic, stearic, oleic, linoleic, linolenic, and erucic acids were also identified. As well as chemical composition in borage leaves or flowers, the fatty acid composition in borage seeds changes during time and growing conditions (Jaffel-Hamza et al. 2013; Peiretti et al. 2015). This phenomenon is particularly important in the definition of the best harvest time. A general increase in GLA concentration has been reported (Galwey and Shirlin 1990; Berti et al. 2002; Stähler et al. 2011; Gilbertson et al. 2014). Several phenolic acids have been found in borage seeds; their role is particularly important in preventing the oxidation of the oil and as a source of antioxidant in food or pharmaceutical formulation (Mhamdi et al. 2010a; Mohajer et al. 2016). In addition to rosmarinic, syringic, and sinapic acids content in defatted seeds, as reported above (Wettasinghe et al. 2001), ferulic acid represents the highest portion of total free phenolic compounds, followed by 2-hydroxy-4-methoxybenzoic, gallic, p-coumaric, and cinnamic acids (Zadernowski et al. 2002). Small quantities of caffeic acid were also identified. Similar results have been obtained by Mhamdi et al. (2007a, b), who also reported that borage seed oil is a good source of β-caryophyllene. Antioxidant activity of seeds extracts depends on the phenolic acid composition more than on the total phenolic concentration.

3.2.5 Borage Uses

Borage has been used since ancient times for culinary and medical purposes in different countries. Its leaves are used in salads, soups, or in different dishes and beverages (Río-Celestino et al. 2008; Zemmouri et al. 2014; Biscotti et al. 2015). Flowers sometimes can be served fresh or candied as edible decoration of cocktails and confectionery (Borowy et al. 2017). Since its antioxidant properties were demonstrated, several studies have focused on its application to food preservation such as in the preparation of gelatine films from fish (Gómez-Estaca et al. 2009; Giménez et al. 2011), preventing oxidation in sausages (de Ciriano et al. 2009) and oil (Bandoniene and Murkovic 2002), or extending lamb shelf life (Bellés et al. 2017, 2018). Besides its consumption as edible plant, many health effects have been attributed to the borage plant. They have been discussed by a great number of authors and published in scientific papers (Gupta et al. 2010; Pieszaki et al. 2012; Asadi-Samani et al. 2014; Tewari et al. 2019). It is recommended to alleviate and heal colds, bronchitis, and respiratory infections in general for its anti-inflammatory and balsamic properties. In naturopathy, borage is used for the regulation of metabolism and the hormonal system and is considered as a good remedy for PMS and menopause. Borage is used as a natural medicament to improve the intellective processes, activating and improving memory records (Pieszaki et al. 2012).

Borage uses are mostly related to the high content of GLA and its properties. GLA is a precursor of a prostaglandin in the human body, which is vital in many functions. Human organism is not capable of synthesizing it; therefore, its supplementation could be of value for preventing and/or treating various degenerative pathologies. GLA is generally prescribed as an anti-inflammatory, but it is also used for the treatment of multiple sclerosis, diabetes, arthritis, and dermatitis (Tollesson and Frithz 1993; Leventhal 1993; Gupta et al. 2010). Based on the traditional uses of borage, studies investigated its efficacy in gastrointestinal (colic, cramps, diarrhoea), respiratory (asthma, bronchitis), cardiovascular (cardiotonic, antihypertensive, and blood pressure), and urinary (diuretic and kidney/bladder disorders) disorders (Gilani et al. 2007). Moreover, Rezk et al. 2018 reported its beneficial effect in reducing hepatotoxicity and protecting the cells from the oxidative stress induced by radiation exposure. Recently, borage treatments showed a beneficial anxiolytic effect in rats and in patients with OCD (Sarris et al. 2012; Komaki et al. 2015).

The juice and fresh herb are used in cosmetics as rejuvenating or nutrients masks. The juice is responsible for inflammation alleviation and promotion of the regeneration of the skin. However, the ointment from the fresh herb is effective in the treatment of eczema and wounds. The most common use of oil in cosmetology is capsule; their regular intake positively affects the structure of hair and condition of nails. Due to its numerous properties, borage use as animal feed has been proposed (Peiretti et al. 2004). Besides its uses for human/animal health and food, it has recently been observed the inhibitive effect of aqueous borage extracts against the corrosion of mild steel, proposing its application in the mechanical field (Al-Moubaraki 2018). Recent studies proposed the potential use of borage as an affordable source of plant biostimulants (Bulgari et al. 2017, 2020; Franzoni et al. 2020). Indeed, results obtained from these works showed that aqueous extracts from borage leaves and flowers affected the primary and secondary metabolism of lettuce and rocket salads by increasing leaf pigments and photosynthetic activity both at harvest and during storage.

3.2.5.1 Evaluation of Borage Extracts as Potential Biostimulants

In recent times, biostimulants have been increasing for enhancing the sustainability of agriculture cropping systems. Biostimulants can improve the plants' nutrient use efficiency to reduce the consumption of fertilizers, stimulate plant growth and development (Kunicki et al. 2010; Calvo et al. 2014; Bulgari et al. 2015; Halpern et al. 2015; Le Mire et al. 2016), and counteract environmental stresses, eventually enhancing crop quality and yield (Ziosi et al. 2013; Van Oosten et al. 2017; Bulgari et al. 2019b). The significant increase in research papers focused on biostimulants and their economical relevance highlights the interest in this sector. Biostimulants are produced using several raw organic materials containing bioactive molecules. As reported above, borage plants are rich in antioxidants and potentially used as biostimulants. The antioxidant properties of borage extracts can mainly be ascribed to the presence of phenolics (Wettasinghe et al. 2001; Aliakbarlu and Tajik 2012). On the basis of this knowledge, it appears interesting to explore the use of borage as a possible source of biostimulants.

In a work on *Lactuca sativa* 'Longifolia', Bulgari et al. (2017) studied the efficacy of foliar treatments with raw aqueous extracts of leaves or flowers of *Borago officinalis* L. and tried to set up a protocol to assess their effects. The treatment conditions were water (control plants); 1 or 10 mL L^{-1} of borage leaf extracts (LE); and 1 or 10 mL L^{-1} of borage flower extracts (FE). Extracts were sprayed onto lettuce plants at the middle of the growing cycle and 1 day before harvest time. For the experiment, a holistic approach has been adopted, including both traditional and innovative investigation. At harvest, the levels of ethylene, photosynthetic pigments, nitrate, and primary (sucrose and total sugars) and secondary (total phenols and flavonoids) metabolites, including the activity and levels of phenylalanine ammonia lyase (PAL), were assessed. In addition, a preliminary study of the effects during postharvest was done. Borage extracts have been shown to enhance the primary metabolism of lettuce plants by increasing leaf pigment concentration and photosynthetic activity. Plant fresh weight increased upon treatments with 10 mL L^{-1} doses, as correctly estimated by multiview-angle images. Chlorophyll *a* fluorescence results showed that FEs were able to increase the number of active reaction centres per cross-section; a similar trend was observed for the performance index. Ethylene was threefold lower in FEs treatments. Nitrate and sugar concentrations did not change in a significant way in response to the different treatments tested. Total flavonoids, phenols, proteins, the in vitro PAL-specific activity, and the levels of PAL-like polypeptides were increased by treatment with all borage extracts. In particular, FEs were also able to prevent the degradation of photosynthetic pigments during storage.

Based on the observed effects on lettuce, a research was carried out on wild rocket plants (*Diplotaxis tenuifolia* L. 'Frastagliata') (Bulgari et al. 2020). The aim of the research was to investigate the role of borage extracts in affecting nitrogen and carbon metabolism. It was demonstrated by several works that biostimulant applications are effective in decreasing nitrate concentration in leafy vegetables, and they are able to increase antioxidants, with potential benefit for humans. It is important to remember that rocket is a species considered as a hyper-accumulator of nitrate (NO_3^-) (Di Gioia et al. 2013). In Europe, the limits for rocket commercialization range from 7000 NO_3^- [mg kg^{-1} FW] (harvest from October to March) to 6000 NO_3^- [mg kg^{-1} FW] (harvest from April to September) (European Reg. No. 1258/2011). In this experiment, borage extracts were obtained using the same procedure described by Bulgari et al. (2017). Extracts were properly diluted in water (10 mL L^{-1}) and then sprayed onto rocket leaves until runoff, 35 days after sowing and 1 day before harvest (45 days after sowing). To study the treatments effect, in vivo determinations (chlorophyll *a* fluorescence and chlorophyll content) were performed, as well as destructive analyses (nitrate concentration, sucrose, total sugars, chlorophyll, and carotenoids). In order to characterize the mechanism of action of extracts also at molecular level, a set of genes encoding for some of the key enzymes implicated in nitrate and carbon metabolism was selected and their expression was measured by qRT-PCR. Gene expression analyses were performed using gene-specific primers for nitrate reductase (*DtNR*), nitrite reductase (*DtNiR*), glutamine synthetase (*DtGS1*), glutamate synthase (*DtGLU*), nitrate transporter (*DtNTR*), isocitrate dehydrogenase (*DtIDH*), phosphoenolpyruvate carboxylase 2 and 4 (*DtPEPC2-like*, *DtPEPC4-like*), and Rubisco (*DtRuBisCO*). Interesting results concerned the increment of sucrose, coherent with a high value of Fv/Fm ratio, in addition to a significant reduction in nitrate and ABA than control, and an enhanced NR in vivo activity. Also, gene expression was influenced by borage extracts, with a more pronounced effect on N-related genes (Bulgari et al. 2020). Therefore, borage extracts seem to coordinate and optimize responses at a whole plant level on rocket and to improve the quality and nutraceutical characteristics of this leafy vegetable.

Franzoni et al. (2020) explored the possibility of using borage extracts also in abiotic stress conditions. Salinity is one of the major abiotic stresses, cause of yield losses and product quality decrease; the work aims to better understand the effect of salt stress on wild rocket (*Diplotaxis tenuifolia* L.) treated with a borage extract. In particular, the borage flower extracts diluted to 10 mL L^{-1} have been chosen as treatment because of the positive results obtained in previous experiments. The expression of some of the transcription factors (TFs) typically involved in salt stress response was studied within a 24 h period for linking the gene expression with treatments. Physiological parameters (chlorophyll, chlorophyll *a* fluorescence, carotenoids, phenols, and anthocyanin) were also analysed. Results obtained showed that salt stress induced a general increase in the expression levels of almost all TFs studied, whereas the treatment with the plant base extract only induced an increase at specific time points. Different pathways such as sugars metabolism, cuticular wax biosynthesis, and brassinosteroids signalling took part in plant responses. Nitrate concentrations were not affected by borage treatment, not under control or stress condition. This result was unexpected since, in contrast, Bulgari et al. (2020) observed an increase in NR in vivo activity and a marked reduction in nitrate concentration in rocket leaves treated with the same borage extract. This variability might be due to some differences in the two experimental plans (period of cultivation, number of treatments, and timing of the application).

In conclusion, borage extracts appear to indeed exert biostimulant effects on lettuce and rocket plants; future work will be required to further investigate on their efficacy in different conditions of cultivation and/or species. It will be also interesting to evaluate other extraction protocols in order to verify if it possible to enhance the extraction of the bioactive compounds responsible for the biostimulant activities.

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Simple Organic Acids as Plant Biostimulants

4

Ebrahim Hadavi and Noushin Ghazijahani

Abstract

Organic acids are the building blocks of plant metabolism. However, their role in plant metabolism has remained mostly unknown while their regulating role is becoming more evident. Interest in these chemicals is shaping in many disciplines in the agriculture sector, which is supported by ecological awareness and the search for more sustainable tools in agriculture that are more easily available. We have tried to review the related literature in this field to create a more solid picture of the knowledge that we have available today. In fact, the role of organic acids is far more diverse than we thought before; many organic acids help plants to accommodate different biotic and abiotic stresses and the others exert some levels of regulation effects that could be considered on par with known plant growth regulators. Whether we are in search of improvement in postharvest life, the bioactive content, absorption of minerals by roots, tolerance to biotic or abiotic stresses, or even to increase the yields of secondary metabolism, we can find a tool in the diverse toolbox of the organic acids.

Keywords

Abiotic stress · Biostimulants · Bioactive compounds · Organic Acids · Secondary metabolites

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_4

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DPPH	2,2-diphenyl-1-picrylhydrazyl free radical reaction test
GPX	Glutathione peroxidase
GR	Glutathione reductase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
IAA	indole acetic acid
MDA	Malon dialdehyde
NADP-ME	NADP–malic enzyme
PAL	Phenylalanine ammonia-lyase
POD	Peroxidase
SOD	Superoxide dismutase
TSS	Total soluble solids

4.1 Introduction

When we think about plant metabolism, sugars, fats, and proteins are the first to appear in our mind as the key groups. However, the importance of the set of substances that creates a crossroad between them, the “organic acids” that create the chain of connections and substrate needed to produce virtually everything in the plant metabolic network have remained relatively marginal. Starting from sugar breakdown, the “glycolysis,” the story of organic acids begins with pyruvic acid as the product that is converted to acetyl-CoA, the key substance that is a substrate for both “fatty acid synthesis” and fuels the Krebs or “citrate cycle,” which is practically an “all-organic acid” cycle in which carbon skeleton of almost every organic compound of primary and secondary metabolism, including proteins, is made.

During the past decade, the volume of research in this area has increased and this body of knowledge gives us a different perception of the role of organic acids in plant metabolism and now even we can use them in areas that were considered to be the exclusive domain of known plant growth regulators. The recent information may even invite us to rethink the frontiers we have defined before. Just as an example, it is demonstrated that acetic acid, the most simple of the organic acids, can trigger the jasmonate production pathway that induces drought tolerance and naturally other jasmonate-related responses in the plant. This role was solely listed in the functions list of the most famous plant auxin, the IAA, before. And even we may wonder about the role of IAA’s acetate group in the rest of the effects that are listed for it. In fact, the long list of actions attributed to some plant growth regulators could reflect, on the other side, the shortage of our knowledge regarding the mechanism (s) behind their action.

Anyway, the organic acids are already in the center of interest and ask for more serious consideration. They are practically used as biostimulants and usually create a remarkable response considering the applied dose. Therefore, we are witnessing some responses and of course patterns of response that resemble those of previously known plant growth regulators in some cases. In the current review, we will try to consolidate the knowledge that is gathered till now in different physiological and practical aspects and put a spotlight on organic acid's role in plant growth and development regulation.

4.2 Physiological Implications: Foliar Organic Acids as they Regulate Plant Growth and Development

4.2.1 Malic Acid on Nitrogen Metabolism

In mitochondria of plants, the malic acid is metabolized by the reaction of malic enzyme (Day and Hanson 1977). Malate is a common reserve anion that acts as a counter ion for K^+ and Ca^{++} in plant vacuole (Ting 1981), particularly in nitrate-dependent plants (Osmond and Laties 1969). It has been shown that supplying malate to the roots, either by addition to the external solution or by increasing its transport from the shoot in the phloem artificially, increased the net NO_3^- uptake rate. On the other hand, modifications of the NO_3^- reduction rate in the shoot lead to similar modifications of the net NO_3^- uptake rate by roots. Therefore, the hypothesis that the carboxylate anions produced during NO_3^- reduction in the shoot are translocated via the phloem to roots where they control NO_3^- uptake seems reasonable (Touraine et al. 1992). Sprayed malic acid promotes the release of nitrogen in the soil and accelerates the accumulation of nitrogen in tobacco seedlings while decreasing the share of leaf/root nitrogen reduction (Zhu et al. 2007). Later, this was used to lower ammonia poisoning in plants, and it was shown that α-ketoglutaric acid and oxaloacetic acid enhanced the ammonium assimilation process better than citric acid, pyruvic acid, malic acid, and fumaric acid (Xufeng and Jie 1993). In practice, spraying foliar malic acid in 2.2 mM concentration caused an increase in the leaf carbohydrate content and nitrogen percentage (El-Shanhorey et al. 2015). PEP carboxylase is the enzyme in root tissue responsible for de novo production of malate via the non-photosynthetic carbon fixation pathway in plant roots (Basra and Malik 1985). Its activity has been found to increase under Fe stress conditions, enabling the root to sustain proton efflux (Rabotti et al. 1995).

4.2.2 Root Exudation of Organic Acids

4.2.2.1 Effect on Soil Flora and Fauna

Plants release a diverse spectrum of organic compounds to the rhizosphere including but not limited to sugars and polysaccharides, amino acids, organic acids, fatty acids, growth factors, sterols, growth factors, enzymes, flavons, and nucleotides (Uren 2007). The exudated organic acids by plant roots are the main carbon source for rhizobacteria (Ohwaki and Sugahara 1997). Studies conducted to examine the exudation pattern of amino acids and sugars in four crop plants in response to foliar application of nitrogen and phosphorus revealed that the total concentration of amino acids increased while the sugar content in the exudates decreased after the treatment of the foliage with N, while P treatment inversed this response (Balasubramanian and Rangaswami 1969). Accordingly, the pattern of exudation of different pentose sugars was changed effectively under all foliar treatments; therefore, it seems possible that suitable foliar sprays could lead to the preferential suppression of root pathogens in the zone of root influence. Foliar sprays thus appear to affect plant metabolism and hence nutrient exudation by roots. Direct translocation of these chemicals to the roots or de novo synthesis in the roots may be the case (Jalali 1976). These are among evidence showing that when the plant receives N from sources other than root, the release of sugars that are used by fauna and flora of rhizosphere decreases. In fact, the N source is the most important gauge for plant-based on which the plant decides on investment in the rhizosphere, manifested as root growth and organic exudates. Even the positive response to foliar P might be interpreted as more need for N to support the metabolism of foliar received P. However, organic exudates play a diverse role in a wide spectrum of uptake strategies of plant roots. As an example for such a role, it is demonstrated that nitrogen derived from the atmosphere as well as the leghemoglobin content was positively correlated with citric acid in root exudates of alfalfa and facilitated biological N_2 fixation processes; This beneficial effect of citric acid was attributed to the improvement of P availability in the rhizosphere as well as the citric acid ability to satisfy a significant proportion of Fe needed for the synthesis of leghemoglobin and bacterial N_2-fixing enzyme through its chelating ability and formation of plant-available organic-Fe^{3+} complexes in the rhizosphere of legumes (Wang et al. 2020).

Most exudated organic acids are readily metabolized by rhizobacteria (Ohwaki and Sugahara 1997), which, on the other side, could act as a means for a plant to exert some influence on dependent bacteria. Studies on the nutritional requirements of rhizosphere microbial populations suggest that the number and activity of soil microorganisms rely greatly on simple organic compounds and mostly the organic acid exudation that affects the attractiveness of plant roots to plant-beneficial soil bacteria (Lopez-Bucio et al. 2000). Beneficial soil bacteria confer immunity against a wide range of foliar diseases by activating plant defenses, thereby reducing a plant's susceptibility to pathogen attack. However, there is growing evidence that it is more complicated than it seemed before. For example, a foliar pathogen (*Pseudomonas syringae*) induces the l-malic acid (the natural form that exists in plants) secretion from roots of Arabidopsis, which selectively signals and recruits the beneficial rhizobacterium *Bacillus subtilis* in a dose-dependent manner (Rudrappa et al. 2008). In other studies, it has been demonstrated that this is a two-way relationship, that is, the same bacteria (*Bacillus subtilis*) affects the synthesis and exudation of malic acid from roots via influence on the expression of the malic acid biosynthetic gene, malate synthase, and the transporter gene, aluminum-activated malate transporter (Rekha et al. 2018).

The intriguing idea that populations of rhizosphere organisms can be controlled by foliar applications of chemicals, which are either translocated to the roots and exuded into the rhizosphere intact or which change the pattern of exudation and in this manner affect the resistance of the plant, has excited plant pathologists and soil microbiologists (Hale and Moore 1980). This kind of effect is suggested to exist, and some studies point to results that support such hypothesis (Salahi et al. 2017). In another study, foliar citric acid positively affected the soil CO_2 efflux, showing an increase in respiration activity in the soil that was combined with an increased relative abundance of fungi, and they suggested that the soil fungi were much more affected than its bacteria (Soltaniband 2020). There are also effects on the population of invasive fungi as reported by Jalali and Suryanarayana (1970), who showed that exudation of amino acids, which promotes colonization of wheat root by an invasive fungus, could be restricted by various foliar treatments.

4.2.3 Nutrients Abortion

Organic acid excretion as anions by roots is a known strategy applied by the plant to mobilize and absorb cations and phosphorus from the rhizosphere (Gerke et al. 1994; Ryan et al. 2001). Therefore, it is not surprising that citric acid is revealed to be favorable for estimating plant-available P, Mg, Ca, K, and Fe in soils because it provides stronger correlations with the tree nutrition than the standard methods for determining exchangeable cations (Fäth et al. 2019).

The effects on nutrient absorption are manifested either directly, via the change in root organic acid content, or directly, under the effect of foliar treatments. Organic acids including citrate and malate can create soluble metal-organic complexes of great stability, making them more available for plant uptake (Struthers and Sieling 1950; Bradley and Sieling 1953). Later it became evident that the plant roots are the source of organic acids exudation to the rhizosphere (Dinkelaker et al. 1995) and calcicole plants (plants growing in alkaline soils) could exert large amounts of di and tricarboxylic organic acids during deficiency to absorb minerals, especially P and Fe, as their key mechanism enabling them to tolerate such soils (Dinkelaker et al. 1995; Tyler and Ström 1995; Lopez-Bucio et al. 2000; Arcand and Schneider 2006; Bais et al. 2006). It has been shown as part of natural plant response to Fe deficiency that the citrate concentration in roots increases while the aconitase activity reduces, being the enzyme that converts citrate to isocitrate, it creates more free citric acid output from the citrate cycle (McCluskey et al. 2004), which could be exudated. Jafari and Hadavi (2012) suggested an increase in mineral absorption by elevated exudation of organic acids in response to foliar organic acids as part of the mechanism responsible for improved growth parameters. Such increase in exudation of organic acids in response to foliar organic acids is confirmed by An et al. (2014). It is also shown that the active iron content of leaves increases by foliar spray of citric acid on hydroponically grown peanuts (Akhtar et al. 2018).

Differential A1 tolerance in maize cultivars has been partly explained by Al-induced release of citrate (as well as phosphate and malate) from the root into the rhizosphere. This Al-induced release of Al-chelating compounds could be an important adaptive mechanism, permitting the survival of plants in Al-toxic soils (Pellet et al. 1995). Experiments in nutrient solution revealed that the exudation of organic acids was generally diminished under the coupled stress of P deficiency and Al toxicity in comparison with either single stress, implying a possible antagonistic effect of the two stresses on organic acids exudation. Also, the exudation of organic acids was differentially induced by each stress in which oxalate and malate were specifically induced by P deficiency while citrate exudation was caused by Al toxicity (Dong et al. 2004). This implies that the pattern of response by root exudation is tailor-fit to the specific stimulus. And the effect of these exudates on mineral absorption is well known (Lopez-Bucio et al. 2000). In a study on rice plants grown in Cd polluted soil that were sprayed with 5.0 mM citric acid, the authors concluded that foliar citric acid efficiently inhibited Cd transport but promoted Mn mobilization in rice organs and that citric acid can regulate the transport of Cd and Mn in opposite directions by enhancing expression levels of related genes (Xue et al. 2021). El-Shanhorey and El-Sayed (2017), after foliar application of citric acid, concluded that foliar citric acid prevented the absorption of Cd from the root level.

4.2.4 Carbohydrate Partitioning

Changes in both carbohydrate concentration and allocation inside the plant are reported. Carbohydrate content was higher at two sprays of foliar 1 mM citric acid compared to a single spray, but the highest carbohydrate content in cotton was obtained with citric acid at 1.5 mM sprayed once (Ghourab 2000). Eidyan et al. (2014) showed that the middle concentration of 2.6 mM citric acid had interacted significantly with 0.5% (w/v) Fe to shift the photosynthetic carbon toward underground bulblets causing their size to increase in tuberose. Malic acid at 2.2 mM significantly increased bulb diameter and the number of new bulblets in the same plant (El-Shanhorey et al. 2015). Response to the combination of foliar citric acid and malic acid (3.9 and 5.6 mM each, respectively) was also reported in lilium in which a reduction in carbohydrate partitioning to aerial bulbils (formed on the leaf) was observed (Darandeh and Hadavi 2012).

Talebi et al. (2014) demonstrated that the root fresh and dry weight, as well as the root to shoot ratio, has increased by application of 1.5 mM citric acid or 2.2 mM malic acid. They concluded that each organic acid created a distinctive pattern of response that was in line with previous observation by Darandeh and Hadavi (2012). However, these changes were usually in favor of better adaptation of plants to environmental cues. An increase in root biomass and leaf area is also shown in strawberries (Soltaniband 2020). On the other hand, foliar spray of sugar beet with citric acid in nonsaline condition while increased the root weight compared to control but did not affect the root to shoot ratio. On the other hand, by an increase in salinity level both root weight and root to shoot ratio increased significantly (Hussein et al. 2020).

4.2.5 Protein Metabolism

Martinez-Pacheco et al. (2011) detected that citric acid affects the physiology of maize leaves, especially in the increase of soluble protein and proteolytic activity. Proteolysis is a requirement for the mobilization of stored proteins of seeds used in germination and for the efficient recycling of amino acids during senescence and apoptosis and into the housekeeping functions such as the removal of aberrant proteins and protein degradation as part of a homeostatic cycle of protein removal and renovation. In red alga *Galdieria sulphuraria*, specific growth rate, biomass concentration, and the highest concentration of protein were obtained in 5 mM malic acid added to the growth medium (Demirci et al. 2016).

Foliar application of citric acid or oxalic acid created a distinct pattern of protein electrophoretic bands compared to citric acid, which suggests that they induce the expression of different patterns of genes (Sadak and Orabi 2015).

4.2.6 Cell Membrane

The reducing power and pyruvate generated by the activity of NADP-ME may be used for respiration in cellular repair processes and as substrates for the synthesis of fatty acids for membrane repair. In their experiment, they observed that exogenous malic acid treatment caused the lowest content of MDA, which might be due to that malic acid has an essential function in preventing oxidative membrane damage as a transporter of reduction equivalents to the cytosol (Casati et al. 1999). A similar result was also found in faba bean seedlings conducted by Ameri and Kazemi (Ameri and Kazemi 2013).

4.2.7 Morphogenesis

Some reports are pointing to changes to rather qualitative aspects of plant morphogenesis like that of Ghazijahani et al. (2017, 2018), in which improved root formation in herbaceous cuttings was observed in cuttings taken from roses that were sprayed with solutions of citric acid and malic acid. Similarly, tulip bulbs leaf cuttings or bulb scales were dipped into a succinic acid solution, and as a result, a 17% and 50% root formation increase was observed, respectively (Mugge 1985). An increase in inflorescence count is reported by some studies (Mirzajani et al. 2015; Ali et al. 2020; Soltaniband 2020). Increase in branch number is also reported by the application of 1.2 mM citric acid in *Vicia faba* plants (Abdulla 2013). Similar results were observed by applying 5 mM citric acid on peppermint plant's shoot and root branch counts (Hamidi et al. 2014). The number of fruiting branches per plant increased by sprays of 1, 1.5, or 2 mM citric acid on the cotton plant (Ghourab 2000).

There are also reports regarding the quantitative aspects of morphogenesis like some reports indicating a change in leaf number (Abdulla 2013; El-Shanhorey et al. 2015; Radmanesh et al. 2015; Patel et al. 2017).

Salahi et al. (Salahi et al. 2017) reported that foliar Fe treatments containing either citric acid or glutamine caused a significant increase in the leaf trichome. Seed production was also positively correlated with the trichome index on the other side. As there are reports on the higher ectodesmata densities around the trichome bases, they deduced that the increase in trichome index could be a positive response to foliar treatment that may allow the leaf to further absorb the applied spray more efficiently. In the Bromeliaceae family, a relationship between the trichome density and foliar absorptive capacity is established (Benzing et al. 1985). Likewise, Fernández and Eichert (2009) concluded that the environmental conditions can affect the amount and composition of epicuticular waxes, which in turn may affect stomatal and trichome development and eventually influence the penetration rate of foliar solutes for the same plant species. They concluded a feedback mechanism for morphological changes in response to foliar sprays may exist (Salahi et al. 2017).

4.2.8 Photosynthetic Parameters

4.2.8.1 Foliar

It has been suggested that the direct effect of citrate is manifested through activation of plasma membrane iron-reductase enzyme in leaves and the chelating effect of citric acid on iron to help mobilize iron and cause an increase in the chlorophyll content (Camp et al. 1987). Citrate is the natural carrier of iron in xylem transport (Tiffin 1966). Foliar $FeSO_4^+$ citric acid treatment in peanut enhanced greater recovery of chlorosis and leaves contained more chlorophyll (Singh and Dayal 1992). Tagliavini et al. (1995) showed that 10.4 mM and 31 mM citric acid increased the chlorophyll content and performance of the kiwifruit. Since then, foliar citric acid in combination with Fe sources has been used to recover plants from iron chlorosis in a variety of annual, biannual, and perennial plants (Tagliavini et al. 2000; Rombolà et al. 2001; Abadía et al. 2002; Álvarez-Fernández et al. 2004; Gheshlaghi and Tafazoli 2005; Amri and Shahsavar 2009; Eidyan et al. 2014; Salahi et al. 2017).

However, there are increasing reports demonstrating positive effects of organic acids when applied without added iron. The chlorophyll content of cotton plants increased by 1.5 mM citric acid treatments (Ghourab 2000). Likewise, in the berseem plant, foliar 5 mM succinic acid led to significantly higher transpiration and chlorophyll contents (Kang et al. 2010). In lilium plants, foliar 5.5 mM malic acid spray increased chlorophyll content and SPAD reading better than citric acid-containing sprays (Darandeh and Hadavi 2012). This was confirmed also in tuberose (El-Shanhorey et al. 2015) and later in the study by El-Shanhorey and Saker (2018) and El-Shanhorey and Barakat (2020) by use of 2 or 4 mM foliar malic acid. Ali et al. (2016) reported that spraying citric acid concentrations of up to 20.8 mM on vine plants caused increased chlorophyll content (a, b, and total). Leaf SPAD value was also increased by foliar spray of citric acid on hydroponically grown peanuts (Akhtar et al. 2018). Total chlorophyll content of rain-fed figs augmented significantly by both foliar 1 and 1.5 mM citric acid application while ascorbic acid was ineffective (Mohamed and El-Berry 2019). It has been demonstrated that foliar citric acid (600 μM) or salicylic acid (100 μM) or their combination increased carotenoid and chlorophyll content and stomatal conductance and aperture size, improved the CO_2 availability inside the cells, and enhanced its capacity to bind with rubisco by elevating the carbonic anhydrase activity, which ultimately resulted in an enhanced photosynthetic rate of *Brassica juncea* plants (Faraz et al. 2020).

In many cases, the response to organic acid has been more pronounced when the plant has been subject to abiotic stress, which belongs that their role in the activation of stress response pathways could be important as well. Foliar application of 1.3 and 2.6 mM citric acid caused a significant increase in SPAD value of those *Senecio cineraria* plants subjected to Cd-polluted medium while in unstressed plants no difference was noticed (El-Shanhorey and El-Sayed 2017). Spinach plants under any level of Cr pollution stress had higher photosynthetic parameters by an increase in foliar citric acid concentration. The 2 mM citric acid-treated plants had even better performance in total chlorophyll and carotenoid contents, photosynthesis and transpiration rates, stomatal conductance, and water use efficiency compared to the plants irrigated with Cr-free water but did not receive foliar citric acid (Maqbool et al. 2018). An identical pattern of effect on the same traits was reported by foliar sprays of 2.5 and 5 mM citric acid on castor beans grown in Pb-polluted soil (Mallhi et al. 2019). Green bean plants responded in the same manner to 4 and 8 mM foliar citric acid by an increase in chlorophyll and carotenoid contents (Rafati Alashti et al. 2020). This is confirmed on red bean plants treated with foliar application of citric acid (2.5 or 5 mM), which showed a dose-dependent improvement in the chlorophyll and carotenoid contents that became more noticeable by the increase in pollution level (Mahdavian 2021). In the same study, the anthocyanin content was also increased significantly in response to applied citric acid. It is suggested that the effect of citric acid and malic acid on the production of carotenoid compounds could be due to their conversion to pyruvate, which serves as a precursor for isopentenyl pyrophosphate (IPP), the carotenoid precursor (Chen et al. 2009).

Other modes of use were also tried with promising effects; soaking seeds with 2.5 mM succinic acid improved leaf photosynthetic characteristics of maize (stomatal conductance, intracellular CO_2), prolonged the high photosynthesis period, enhanced the photosynthetic ability in the afternoon, and the water use efficiency was ameliorated. Higher concentrations had adverse effects on plant performance (Ma et al. 2007).

4.2.8.2 Added OA to Medium

Adding 5 mM citric acid added to Ni-polluted hydroponic medium of *Mentha piperita* (L.) increased the SPAD value, chlorophyll content (a, b, and total), and carotenoid content (Khair et al. 2020). Similar results were reported before on rice (Khatun et al. 2019) and oil palm seedlings (Hidayah et al. 2020). Also, the photosynthesis rate was enhanced significantly in Al-stressed oil palm seedlings subjected to 0.13 and 0.26 mM citric acid in their growth medium (Hidayah et al. 2020). Net photosynthetic rate, stomatal conductance, maximal photochemical efficiency (Fv/Fm), and effective PSII quantum yield were monitored 10 days after initial treatment by Cd or malic acid, or both. The addition of 100 μM Cd alone caused a significant and gradual decrease in all measured photosynthetic parameters while a dose-dependent response to malic acid caused a partial stepwise and significant recovery in all parameters (Guo et al. 2017).

4.2.9 Antioxidative Capacity and Stress Tolerance

4.2.9.1 Foliar OA

The application of oxalic acid has been shown to effectively improve the antioxidant activity in cucumber (Peng et al. 2000) and sunflower (Malenčić et al. 2004). In the halophyte plant, *Leymus chinensis*, the endogenous concentration of citric acid in the leaves and roots increases dramatically under saline stress and alkaline stress reaching a peak between 3 and 6 h after the start of stress treatment; When citric acid is applied exogenously, the response of plants was similar to that of exogenous proline, which is a known component of the plant stress response (Sun and Hong 2010). Antioxidant activity as measured by DPPH activity is demonstrated to increase substantially by applied citric acid in a positive dose-dependent pattern (Mahdavian 2021). Similarly, pepper fruits treated by pre-harvest foliar citric acid contained substantially higher levels of PPO activity and DPPH radical scavenging activity (Mekawi et al. 2019).

In a study conducted on fig trees during two consecutive years, it was observed that 1 and 1.5 mM citric acid foliar sprays decreased the level of proline significantly (Mohamed and El-Berry 2019), which indicates that they have ameliorated the stress effects. They also found that citric acid significantly increased the specific leaf area. This implies that plants had used less biomass for construction of the same leaf area as of control treatment possibly by production on thinner leaves. Decreasing the specific leaf area is a well-documented adaptation strategy of plants in drought conditions (Marron et al. 2003). A similar decreasing effect by foliar malic acid (2 or 4 mM) was noted on the proline content of Cd-stressed *Salvia splendens* plants, and the effect was even more notable in unstressed plants (El-Shanhorey and Barakat 2020). Green bean plants responded to foliar citric acid sprays of 4 and 8 mM by a decrease in CAT and GST enzyme activities as well as in MDA content (Rafati Alashti et al. 2020). Also, there are a handful of literature available that demonstrate that the level of enzymes related to the antioxidative capacity of plant changes in benefit of higher resilience for plant especially under stress conditions in response to exogenous organic acids (Li et al. 2008; Zamani et al. 2011; Kazemi et al. 2011; Sabzi et al. 2012; Ameri and Kazemi 2013; Ashtari et al. 2013; Farshid et al. 2013; Kant et al. 2013; Begri et al. 2014; Jamshidi et al. 2014; Huang et al. 2016; Guo et al.

2017; Maqbool et al. 2018; Mallhi et al. 2019; Mekawi et al. 2019; Kundu et al. 2021; Rafati Alashti et al. 2020; Khair et al. 2020).

4.2.9.2 Adding OA Medium

The addition of organic acids to the medium has been effective also in support of the antioxidative machinery of the plants. Total antioxidative capacity was improved by the addition of 100 μM malic acid to hydroponic medium in the course of 10 days after treatment while the addition of 100 μM Cd caused an initial significant increase in the middle of the period, but then back to initial value in the tenth day. The addition of malic acid to Cd-contaminated medium caused the increase in total antioxidative capacity to increase irreversibly twofold in 200 μM malic acid-treated plants. In the same way, superoxide radical content, CAT and APX activity, H_2O_2, and MDA decreased significantly in response to applied malic acid while the activity of antioxidative enzymes including SOD, POD, GR, GPX, GST, GSH, and GSSG increased significantly. The study of the expression of antioxidant enzyme-related genes in leaves confirmed that malic acid regulated the activities and the gene expression of antioxidative enzymes (Guo et al. 2017). Likewise, adding 5 mM citric acid added to Ni polluted hydroponic medium decreased electrolytes leakage and MDA and H_2O_2 contents in both roots and shoots of *Mentha piperita* while the activity of antioxidative enzymes including SOD, POD, CAT, and APX both in root and leaf increased significantly in a consistent manner (Khair et al. 2020). Similar results have been also reported before on the rice plant's response to citric acid in a Cr-polluted medium in which an increase in glutathione and proline contents and increase in GR activity in shoot and root were also reported (Khatun et al. 2019).

4.2.10 Plant Defense

A study on the mechanisms of resistance to *Helicoverpa armigera* in resistant genotypes of chickpea revealed that the inhibition of larval growth was dependent on a water-soluble substance present on the surface of the leaves, which was determined to be accumulated oxalic acid (Yoshida et al. 1995). In oilseed rape, it is observed that a 20 mM oxalic treatment of the lowermost leaf of flowering plants induced systemic disease resistance in the three next youngest leaves. Six hours after the treatment, expression of this resistance was detected and continued for at least 5 weeks. The resistance also became detectable after 24 h following the use of as little as 500 μM oxalic acid. They verified that the resistance was not due to the translocation of fungi toxic concentrations of oxalic acid. In the stem treatments, the signal traveled both acropetally and basipetally. Interestingly, the local resistance in the oxalic acid-sprayed leaves was only detectable only at certain time intervals (12 and 48 but not 24 h after treatment) and both induced by at very low (250 μM) or high ($\geq$40 mM) oxalic acid concentrations (Toal and Jones 1999).

The plant pathogen *Sclerotinia* is a member of a group of at least 12 fungal plant pathogens that generate and secrete millimolar concentrations of oxalate into their surroundings; Cessna et al. (2000) suggested that suppression of the oxidative burst of the host plant and inhibition of H_2O_2 production by oxalate help the pathogen to survive in plant tissue. Heller & Witt-Geiges (Heller and Witt-Geiges 2013) suggested that the pathogen uses oxalic acid to quench calcium ions released during cell wall breakdown to protect growing fungus hyphae from toxic calcium concentrations in the infection area; ironically, it is the same pathogen for which Toal and Jones (1999) demonstrated that oxalic acid sprays on plant act in favor of plant against the pathogen. Long before, it was shown that the plasma membrane of the resistant cultivars of white bean appeared to be more tolerant to damage induced by exogenous oxalic acid than that of the susceptible cultivar (Tu 1989).

4.2.11 Miscellaneous

Spinach plants under irrigation with different ratios of Cr polluted tannery wastewater when receiving increasing levels of foliar citric acid (0, 1, or 2 mM) responded by significant reductions in leaf and root electrolyte leakages, on one hand, and increase in leaf SOD, POD, and CAT enzymatic activities, on the other hand (Maqbool et al. 2018). The function of malic acid in Ni tolerance and detoxification was investigated using natural variation among Arabidopsis accessions. It was concluded that it participates in metal chelation. Specifically, intracellular malic acid modulated the Ni-induced production of reactive oxygen species, which is essential for Ni detoxification (Agrawal et al. 2012). However, when Khatun et al. (2019) measured the phytochelatins content of the root that acts as chelators and is important for heavy metal detoxification, but no change in its concentration in response to added citric acid to the growth medium was noted.

There is a report concluding that IAA is a key signaling molecule that triggers an increase of malic acid against Al toxicity in wheat (Liu et al. 2017). Succinic acid (4.2 mM) also has been tested on cabbage plants, causing them to reach their highest leaf area (Yadav et al. 2000). The inductive effect of citrate on the production of citrate by microorganisms was previously reported (Cantino and Goldstein 1967), which was confirmed later (An et al. 2014; Hu et al. 2016); this may describe at least part of the results due to application of exogenous citric acid and a similar concept also may apply to other organic acids as well, which remains to be studied.

4.3 Agricultural Implications

4.3.1 Yield Components

The yield component differs among agricultural plants based on the part that is considered to be the product. Therefore, we opted to classify the effect based on the organ that is affected so that the researchers can find an easier clue when focusing on a particular plant organ or characteristics that they deem to be important in the production of the subject plant. From 2002 to 2021, a handful of studies have used a mixture of organic acids (ascorbic acid and citric acid with a ratio of 2:1) for their positive effect on plant physiology with promising results (Rezk 2005; Mohamed 2020; Nowar and Vvedenskiy 2020). However, in studies where both organic acids are used with different concentrations it has been shown that the citric acid effect alone has been dominant while that of ascorbic acid has been synergistic or marginal (Ebrahim et al. 2019; Ali et al. 2020).

4.3.1.1 Shoot

Basil plants treated with 5.2 mM citric acid produced significantly more shoot fresh and dry weight (Jaafari and Hadavi 2012). All foliar spray combinations of 15.6 mM and 5.2 mM malic acid, or citric acid or each alone, produced dill plants with significantly higher shoot dry weight (Jafari and Hadavi 2012). Likewise, when 5 mM citric acid was applied as a foliar spray, it caused an increase in basil's shoot fresh weight and shoot dry weight (Maleki et al. 2013). Two foliar sprays of 1.2 mM citric acid increased the height of broad bean (*Vicia faba* L.) plants (Abdulla 2013). Applying 5 mM citric acid to peppermint plants increased branch count and stem fresh weight (Hamidi et al. 2014). The height of tuberose plants increased by 2.2 mM malic acid sprays (El-Shanhorey, Shehata and Soffar, 2015). Garlic plants became taller in response to foliar sprays of low concentrations of (0.26 and 0.52 mM) citric acid (Patel et al. 2017). A significant increase in plant height and shoot dry weight of summer savory (*Satureja hortensis* L.) was reported by foliar sprays of citric acid (Radmanesh et al. 2015) and even by very low concentrations of 52 μM and 104 μM (Al-Zubaidi et al. 2020). The shoot dry weight of peppermint (*Mentha piperita* L.) was significantly increased by foliar citric acid sprays (Pourhadi et al. 2018). A significant increase in all vegetative traits of *Artemisia abrotanum* by foliar application of citric acid was reported (EL-Zefzafy et al. 2016). An increase in plant height was noted in all five cultivars of canola under study by the use of foliar sprays containing 1 mM citric acid (Ali et al. 2020). Also, 2 or 4 mM foliar malic acid increased the height of *Salvia splendens* plants as well as branch weight and branch count per plant (El-Shanhorey and Barakat 2020).

4.3.1.2 Leaf

Treatment with 5.2 mM citric acid caused the leaf area to increase significantly (Jaafari and Hadavi 2012). Leaf area per plant was superior by the use of 1.2 mM citric acid in broad bean (Abdulla 2013). Tuberose (*Polianthes tuberosa*) plants treated by foliar 2.2 mM malic acid showed increased leaf number, leaf dry weight, and leaf area (El-Shanhorey et al. 2015). An increase in leaf area and dry weight was noted in apricot tree sprayed by foliar citric acid (Al-Aa'reji and Perot 2017) and *Senecio cineraria* (El-Shanhorey and El-Sayed 2017). Plane trees sprayed with 8 mM citric acid + Fe responded by a significant increase in leaf dry and fresh weight (Salahi et al. 2017). Leaf area increased in response to foliar 0.6 mM citric acid in *Brassica juncea* (Faraz et al. 2020) and on strawberries (Soltaniband 2020). However, in rain-fed figs, Mohamed & El-Berry (Mohamed and El-Berry 2019) noted that citric acid did not affect the leaf area of rain-fed fig trees while the leaf area index increased significantly.

4.3.1.3 Leaf Count

The leaf count increased in garlic plants sprayed by 0.26 and 0.52 mM citric acid (Patel et al. 2017). Abdulla (2013) observed that a couple of 1.2 mM citric acid sprays created more leaf in broad bean. An increase in leaf count of summer savory (*Satureja hortensis* L.) has also been noted by 4 mM foliar citric acid (Radmanesh et al. 2015).

4.3.1.4 Florescence Count

A consistent increase in inflorescence count is reported across five cultivars of canola under study by use of 1 mM citric acid foliar sprays confirmed in two subsequent cultivation seasons. (Ali et al. 2020). In strawberries, also an increase in yield in the protected culture system by foliar citric acid was evident but coincided with an increase of nonmarketable fruits (Soltaniband 2020).

4.3.1.5 Seed

Cottonseed yield increased by foliar sprays of 1 mM citric acid (Ghourab 2000). Foliar 5 mM succinic acid application at flower initiation stage produced significantly higher seed yield in the berseem plant (Kang et al. 2010). The citric acid (10 mM) applied before the appearance of the flag leaf on two hybrids of maize induced an increase in the grain yield (Martinez-Pacheco et al. 2011). Pod number, green pod yield per plant, green pod productivity, fresh seed yield per plant and productivity, pod length, the weight of 100 seeds, percentage of dry matter on seeds, and total soluble solids were highest when 1.2 mM citric acid was sprayed on broad bean (*Vicia faba* L.) plants (Abdulla 2013). Spraying 3.3 mM oxalic acid on wheat caused a significant increase in yield components and grain yield per plant (El-Shabrawi et al. 2015). Yields of seed, pod, and shell of canola increased by foliar citric acid of 1.25 mM (Ebrahim et al. 2019). Foliar potassium citrate sprays increased the grain yield of rice (Kundu et al. 2021).

4.3.1.6 Oil Production

In cotton, the seed oil yield had reached to maximum by 1 mM foliar citric acid treatment (Ghourab 2000). Later it was shown that canola oil yield, as well as its acidity number, had more than doubled by foliar application of 1.25 mM citric acid (Ebrahim et al. 2019). A substantial increase in both seed and oil yields of five cultivars of canola was reached by use of the same range of concentration (1 mM) of foliar citric acid sprays, and the result was also confirmed in the next test season. Similar but less persistent effects were noted by ascorbic acid (Ali et al. 2020).

4.3.1.7 Protein Content

Adding 5 mM citric acid to Ni-polluted hydroponic medium of *Mentha piperita* increased the root and shoot protein content (Khair et al. 2020). Similar results were reported before by similar treatments with citric acid and Cr on rice (Khatun et al. 2019). An increase in seed protein content of canola by foliar application of 1.25 mM citric acid has been reported (Ebrahim et al. 2019).

4.3.1.8 Root

The foliar citric acid of 5 mM increased root fresh weight and root dry weight of basil (Maleki et al. 2013). The root fresh and dry weight increased by application of either 1.5 mM citric acid or 2.2 mM malic acid (Talebi et al. 2014). Applying higher (5 mM) concentrations of foliar citric acid to peppermint plants increased root branching and volume up to 26% and 48%, respectively (Hamidi et al. 2014). Root biomass of rice was also increased by foliar potassium citrate sprays (Kundu et al. 2021). Root fresh and dry weight and volume are reported to increase by very low concentrations (0.05 and 0.01 mM) of foliar citric acid in chamomile (Al-Zubaidi et al. 2020).

4.3.1.9 Bulb

When garlic plants were sprayed with 0.26 and 0.52 mM citric acid, bulbs size and count were increased significantly, yielding a near 30% increase in yield as compared with control plants (Patel et al. 2017). Similar results were obtained in tuberose by 2.2 mM malic acid sprays (El-Shanhorey et al. 2015). An increase in tuberose bulb size had been also reported by Eidyan et al. (2014).

4.3.1.10 Fruit

A wide spectrum of concentrations of citric acid (2.6, 5.2, 10.4, and 20.8 mM) was sprayed on vine plants by Ali et al. (2016), who noticed that the berry setting percentage and yield per vine increased in a linear and dose-dependent manner. Foliar sprays of citric acid (2.6, 5.2, and 7.8 mM) on the apricot tree also significantly increased fruit weight and yield per tree in two consecutive test seasons (Al-Aa'reji and Perot 2017). Similar results were reported by Mohamed and El-Berry (2019) after spraying 1 and 1.5 mM citric acid on the foliage of rain-fed "Sultani" fig by noting a rise in the fruit yield. This was mostly driven by increased fruit count while the fruit size remained unchanged. However, in strawberry, the weight of the fruits was also increased by foliar 10 mM citric acid (Mandour et al. 2019).

Flowering

When tuberose plants had been sprayed with 2.2 mM malic acid, the number of flowers per spike increased significantly, as well as the flowering duration, flower dry weight, spike dry weight, and the length of the rachis (El-Shanhorey et al. 2015). The number of florets per spike, spike length, and flower dry weight of *Salvia splendens* plants was also increased either without or under Cd stress as a response to foliar sprays of 2 or 4 mM malic acid. Two years later, similar results were obtained with the same plant and malic acid treatments but under Ni stress. However, in the latter study, the pattern of response to malic acid concentrations was different by observing the maximum improvement in flower parameters created by the middle concentration of 1.8 mM malic acid (El-Shanhorey and Saker 2018).

4.3.2 Stress Tolerance

4.3.2.1 Abiotic Stress

Salt and Drought Stress

Acetic acid as the most simple organic acid has come to the center of interest for drought researches. There is a natural acetate synthesis from glycolysis that stimulates the jasmonate signaling pathway to trigger drought tolerance. Also, foliar application of 20–30 mM acetic acid has successfully enhanced drought tolerance in a variety of plants including *Arabidopsis thaliana*, rapeseed, maize, wheat, and rice. In fact, it acts as an epigenetic regulation and hormone signaling tool in plants (Kim et al. 2017). Foliar application of 20 mM acetic acid improved the growth of mung bean plants, which were under the stress of diluted seawater and ameliorated the toxic effects of seawater. This was attributed to enhanced levels of photosynthetic rate and pigments, improved water status and increased uptake of K^+, reduced accumulation of Na^+, improved water use efficiency, enhanced accumulations of proline, higher total free amino acids and soluble sugars, increased catalase activity, and higher levels of phenolics and flavonoids content (Rahman et al. 2019). In another study on soybean, similar results were obtained, and they concluded that "acetic acid treatment enabled soybean plants to positively regulate photosynthetic ability, water balance, mineral homeostasis and antioxidant responses," which makes it a cost-effective and easily accessible tool for the management of soybean growth and productivity in drought-prone areas (Rahman et al. 2020).

Enhancement of drought tolerance of bean plants (*Phaseolus vulgaris* L.) that were treated with citric acid as a foliar application stress conditions is reported. Water status of bean plants under drought stress conditions was improved by 8 mM citric acid applied before the exposition to drought, indicated by higher RWC of leaves compared to control plants (El-Tohamy et al. 2013). The exogenous application of citric acid or its mixture with algal extract depressed the negative effect of salt stress on sugar beet through its role in improving growth and balancing the sodium concentration and content and the other nutrients in sugar beet plants (Hussein et al. 2020). In pistachio nut, foliar sprays of malic acid (1 and 3 mM) improved the water balance in the plant in rain-fed condition, as marked by the increased relative water content of leaves and a drop in sunburn damage and hollow nuts that are known to be connected to water status in the plant (Saboory et al. 2014).

Growth, relative growth rate, and CO_2 assimilation rate of halophyte plant, *Leymus chinensis,* were reduced by both salinity and alkalinity stress, but on the other side, the endogenous citric acid concentrations increased. When 250 μM citric acid was exogenously applied to the culture medium, the plant growth was significantly improved and internal citric acid concentration increased as well as the activities of antioxidant enzymes (Sun and Hong 2010).

Seed priming with malic acid is reported to alleviate the effects of salt stress (Ameri and Kazemi 2013). Also, citric acid applied during germination of evening primrose (*Oenothera biennis* L.) caused a substantial increase (near 2.5-fold) in the ferric-reducing antioxidant power (FRAP) of leaf extract, which indicates its effect on activation on the antioxidative system of the plant (Asadi-Kavan et al. 2020).

Adding citric acid applied to the growth medium in the hydroponic system improved the plant physiology and enhanced the antioxidant enzymes to alleviate oxidative damage and electrolyte leakage (Amir et al. 2020).

Heat Stress

Foliar application of citric acid or oxalic acid alleviated the harmful effect of high-temperature stress due to late sowing on many metabolic and physiological processes of the wheat plant that was reflected in increasing seed yield quality. Oxalic acid was superior in terms of tolerance capacity. In terms of stress tolerance, oxalic acid showed better indices, whereas the quality of the yielded wheat responded better by citric acid rather than oxalic acid (Sadak and Orabi 2015). Hu et al. (2016) found that external application of citric acid induced the accumulation of endogenous citric acid levels in leaf tissue that coincided with alleviation of the detrimental effects of heat stress on tall fescue. They attributed this beneficial effect to observed protective effects on peroxidation-linked membrane deterioration, free radical scavenging, maintenance of membrane stability, an increase in root activity, and activation of antioxidant response and HSP genes.

Chilling Stress

Maize seedlings were sprayed one time by 26 mM malic acid 10 days after seeding and after 24 h were subjected to chilling temperatures of 10 °C; after 5 days under cold stress, they had 48% more shoot fresh weight compared to control treatment (Jang and Kuk 2018).

Nutrients Toxicity

Addition to Growth Medium

A handful of studies h ave confirmed the beneficial effect of citric acid addition to medium to alleviate heavy metals toxicity by strengthening the antioxidative system of the plants (Hassan et al. 2016; Kaur et al. 2017, 2018; Lu et al. 2013; Song et al. 2018a). Recently, there are reports of similar effects by succinic acid as well (Duan et al. 2019; Song et al. 2018b). Adding citric acid to the irrigation water increased P availability for organically grown tomato plants (Båth and Otabbong 2013). Guo et al. (2017) observed that the addition of 100 μM malic acid to the hydroponic medium of *Miscanthus sacchariflorus* slightly improved all growth indices while the addition of 100 μM Cd caused at least 20% reduction in all growth indices. By the addition of 100 μM malic acid to the contaminated medium, the plants recovered roughly 10% of lost growth, and the addition of 200 μM increased the recovery to around 20%. Regular application of the citric acid to Cr-polluted soil proved to be a good chelator in enhancing concentration and uptake in sunflower by increasing Cr mobility and availability in soil. On the other hand, it also alleviated the toxic effects of Cr on morpho-physiological and biochemical characteristics of sunflower (Farid et al. 2017). Similar results for Cr (Ali et al. 2018), Pb, and Cd were reported before

(Chen et al. 2003). Application of citric acid to Cr contaminated medium of *B. napus* significantly increased plant growth, biomass, and chlorophyll content by enhancing antioxidant enzyme activity, and it also increased Cr uptake by the plant (Afshan et al. 2015). Adding citric acid (5 mM) to a hydroponic medium containing high levels of Pb and Hg in *Typha latifolia* plants enhanced the uptake and accumulation of Pb and Hg in plant tissue. (Amir et al. 2020). In the same way, 5 mM citric acid added to the hydroponic medium of *Mentha piperita* increased the Ni uptake and accumulation as well as all measured growth indices of the plants (Khair et al. 2020). All vegetative parameters of rice plants grown in a high Cr medium were also improved substantially by citric acid added to the growth medium (Khatun et al. 2019). Oil palm seedling's tolerance to Al stress was enhanced by 0.13 and 0.26 mM citric acid added to the growth medium as well (Hidayah et al. 2020).

However, the consequence of adding to growth medium is that citric acid chelates metal elements (Yeh et al. 2012), and so in many cases, it even could increase their uptake to plant (Afshan et al. 2015; Freitas et al. 2013; Najeeb et al. 2011), making this method a candidate for phytoextraction of heavy metals (Freitas et al. 2013; Yeh et al. 2012). Application of citric acid to the growth medium of switchgrass resulted in the highest level of Al and iron Fe in plant foliage, resulting in severe phytotoxic effects. Total Pb phytoextraction was significantly increased as well (Aderholt et al. 2017). The fact that the heavy metal concentration in the plant is increased by citric acid added to the growth medium suggests that it is playing a double role, both enhancing the metal absorption due to its chelating properties, on one side, while alleviating heavy metal toxicity by its chelating properties (Khatun et al. 2019) or by its biostimulative properties suggested by several studies showing the similar effect by foliar application of citric acid (El-Shanhorey and El-Sayed 2017; El-Shanhorey and Emam 2020; Faraz et al. 2020; Mahdavian 2021; Maqbool et al. 2018; Meers and Tack 2004; Xue et al. 2021).

Foliar Application

The potential of foliar treatments including organic acids for enhanced phytoextraction of heavy metals from contaminated soil was tested by Meers and Tack (2004); however, contrary to what was expected at that time, a reduction in heavy metal absorption by organic acids was observed. This finding created a basis for future researches in this area; in Cd-stressed mustard (*Brassica juncea*) plants, foliar citric acid sprays enhanced all measured growth indices in nonstressed plants and practically alleviated the Cd stress in subjected plants. Combined salicylic acid and citric acid sprays even did better by fully protecting the plants from Cd stress (Faraz et al. 2020). Leaves of rice plants grown in Cd-polluted soil were sprayed at anthesis by 5.0 mM citric acid, and consequently, the Cd content of grain and rachis decreased significantly (Xue et al. 2021). Foliar application of 1.3 and 2.6 mM citric acid caused a dose-dependent reduction in Cd content of roots, stems, and leaves of high-Cd stressed *Senecio cineraria* plants (El-Shanhorey and El-Sayed 2017). In the same study, all measured growth parameters (leaf count, leaf area, leaf dry weight, tiller count, stem height, stem diameter, stem dry weight, root length, and root dry weight) were higher in 2.6 mM citric acid-treated plants in contaminated soil than those plants growing in noncontaminated medium and without any treatment. Similarly, another experiment using Cr-polluted tannery wastewater on spinach plants under foliar treatment of 0, 1, or 2 mM citric acid and different wastewater ratios revealed that in all measured features (shoot length, shoot dry weight, and root dry weight) the 2 mM citric acid-treated plants had better situation compared to the plants that did not receive foliar citric acid and were irrigated with clean water (Maqbool et al. 2018). In the latter study, however, the Cr levels in citric acid-treated plants were not lower than those plants grown with clean water, even though citric acid still caused a significant reduction in Cr content of root and shoot in each contamination level, which is interpreted as reduced uptake of Cr. Also, the Cr content of both shoots and roots of red bean plant grown in Cr-polluted mediums was negatively and significantly affected by foliar citric acid applications (Mahdavian 2021). Likewise, in Ni-polluted soil, foliar citric acid caused a significant increase in all vegetative parameters, chlorophyll content, carbohydrate percentage, while significantly decreased the Ni content in leaves and roots (El-Shanhorey and Emam 2020). A similar result was reported also by foliar malic acid on Cd- or Ni-stressed *Salvia splendens* plants (El-Shanhorey and Saker 2018; El-Shanhorey and Barakat 2020). In another study, 2.5 and 5 mM foliar citric acid caused a dose-dependent improvement in all measured traits of castor bean plant exposed to any level of Pb in soil (Mallhi et al. 2019); however, in dire contrast to reports on Ni, Cr, and Cd phytoextraction tests, in this study, foliar citric acid caused a consistent and dose-dependent increase in Pb uptake and concentration in plant root and shoot. Further studies may be needed to reveal the nature of this observation.

In two separate studies, malic acid (2 or 4 mM) was studied for the possible effect on Cd or Ni uptake and tolerance in n *Salvia splendens* plants. A remarkable (50%) increase in leaf area was noted in the plants irrigated with clean water by foliar malic acid supplication. This effect was also present in Cd- or Ni-stressed plants, causing them to keep comparable leaf areas to control even in the highest contamination level. All the shoot and root and flowering figures were also enhanced by the foliar treatment. The concentration of Cd or Ni in the leaves and stems of plants irrigated with the highest contamination level in the water, but sprayed with malic acid remained on par with control plants without any foliar treatment (El-Shanhorey and Barakat 2020).

Other Abiotic Stress

The 4 mM malic acid +0.7% Fe foliar treatment on the plane tree caused the minimum leaf necrosis among a variety of organic acid-iron combinations and the control treatment (Salahi et al. 2017). Malic acid (26 mM) sprays before paraquat herbicide treatments could detoxify it partially, and when applied at the same time, could fully detoxify the herbicide effects on the plant (Jang and Kuk 2018).

4.3.2.2 Biotic Stress

Exogenous organic acid spraying may act as a kind of elicitation to induce physiological changes and stimulate defense or stress-induced responses in plants (Baenas et al. 2014). It is reported that oxalic acid application could increase the defense-related enzymes in mango fruit during storage (Zheng et al. 2007a, b). Oxalic acid was isolated from leaf sheath extract of rice and was identified as a sucking inhibitor against the brown planthopper (Yoshihara et al. 1980; Génard et al. 2001). In a study on sunflower plants, wilting and inter-veinal necrosis after infection with *Sclerotinia sclerotiorum* was connected to oxalic acid, where it was shown that it moves systemically to the leaves where it apparently accumulates to a "critical" level and elicits the wilt syndrome (Noyes and Hancock 1981). In cucumber leaves treated by oxalic acid, the activities of SOD or POD were enhanced and also it induced resistance against downy mildew caused by *Pseudoperonospora cubensis* (Li et al. 2008). Maximum-induced resistance to *Rhizoctonia solani* infection in rice was reached when a foliar spray of oxalic acid (1 mM) was applied 3 days before inoculation with the fungus (Jayaraj et al. 2010). In winter wheat (*Triticum aestivum*), treatment with oxalic acid protected it against *Septoria tritici,* and the infected square of flag leaves, ears, and stems reduced, and the yield loss was limited (Zhuk et al. 2014). Preharvest spraying of 5 mM oxalic acid on kiwifruit enhanced the disease resistance of the fruit, inhibiting the blue mold rot and mycotoxin accumulation by *Penicillium expansum* (Zhu et al. 2016). In addition, tomato plants treated with 3 mM oxalic acid showed resistance to *Botrytis cinerea,* and the expression of oxalate oxidase gene and *Germin* reached 3- and up to 18-fold higher levels at 24 h after inoculation, respectively (Sun et al. 2019).

The tolerance to powdery mildew was improved in dill by a combination of 15.6 mM citric acid and 5.2 mM malic acid foliar spray (Jafari and Hadavi 2012). Whether added to in vitro medium or applied as a foliar spray, citric acid was able to inhibit the mycelial growth and thus disease severity of the *Cercospora beticola* on sugar beet in a dose-dependent manner (El-Fawy 2018). Both the severity and incidence rate of gray mold disease in strawberry fruits were reduced by 10 mM foliar citric acid (Mandour et al. 2019). However, in some studies, higher concentrations of citric acid have been used for known broad-spectrum bactericidal and fungicidal activity (Kandel et al. 2019). Also, preharvest foliar application with relatively high concentrations of citric acid (15 and 30 mM) and salicylic acid (8 mM) improved substantially the resistance of pepper fruits to *Botrytis cinerea* (Mekawi et al. 2019).

The foliar malic acid in 1 and 3 mM concentration also reduced the brown kernel incidence in pistachio nut, which is an index for fungal infestation and aflatoxin presence in pistachios (Saboory et al. 2014).

4.3.3 Postharvest Quality

4.3.3.1 Oxalic Acid

Zheng et al. confirmed the positive effect of oxalic acid in mango, litchi, and peach (Zheng and Tian 2006; Ding et al. 2007; Zheng et al. 2007a, b). A preharvest application also yielded similar results on kiwi (Zhu et al. 2016). Also, 5 mM oxalic acid reduced ethylene production, respiration rate, and activity of exo-polygalacturonase enzyme while keeping higher fruit firmness and pectin esterase activity in mango fruit during ripening and cold storage period (Razzaq et al. 2015). Both preharvest and postharvest methods of application of oxalic acid proved to be beneficial in peach fruit's quality preserving after harvest (Razavi and Hajilou 2016; Razavi et al. 2017). Postharvest application of oxalic acid (5 mM) delayed jujube fruit senescence and reduced ethylene production and increased fruit resistance against blue mold (Wang et al. 2009). Also, 2, 4, or 6 mM oxalic acid has been tested successfully to alleviate chilling stress and increase the concentration of bioactive molecules in pomegranate (Sayyari et al. 2010). In sweet cherry, 1 mM oxalic acid delayed the postharvest ripening process and maintained quality attributes for much longer periods with higher content of bioactive compounds and antioxidant activity as compared with control fruits (Valero et al. 2011). Similar results were obtained by dip application of 5 mM oxalic acid on plum (Wu et al. 2011). Oxalic acid increased the activity of anti-browning-related enzymes POD and PPO to keep total phenol content higher and increase reducing power in banana fruit (Huang et al. 2013). The 3 mM oxalic acid treatment extended the shelf life of tomatoes by 4 days as compared to control while salicylic acid was even more effective (Kant et al. 2013). A combination of low O_2-modified atmosphere packaging and dipping in 5 mM oxalic acid caused a significant reduction in leaf edge browning, respiration rate, weight loss, and electrolyte leakage, preserving the visual quality of fresh-cut lettuce until 8 days at 8 °C (Pace et al. 2020).

4.3.3.2 Citric Acid

After preharvest foliar 10 mM citric acid or calcium citrate sprays, the strawberries' firmness and vitamin C content increased significantly during postharvest storage of fruits and the share of decayed fruits and fruit weight loss decreased substantially while TSS and total acidity remained unaffected (Mandour et al. 2019). In another study on peach fruits that were sprayed with 53 mM citric acid after harvest, it was observed that the fruits kept their firmness, malic acid, citric acid, and titration acid and were less decayed and had lower total soluble solids. Besides, higher contents of bioactive compounds (chlorogenic acid, neochlorogenic acid, catechin, and L-epicatechin) were maintained in fruits treated with citric acid during the storage period (Yang et al. 2019).

4.3.3.3 Malic Acid (L-Malic Acid)

Preharvest 25 mM malic acid treatment significantly decreased the incidence and index of pear skin browning during storage that was attributed to the maintenance of membrane integrity and increasing of activity of some antioxidant enzymes in treated fruit (Wen et al. 2009). Also, malic acid proved to induce resistance in pear fruits to *Penicillium miscellaneous* and significantly decreased lesion diameter of the treated fruit inoculated with the fungus (Gao et al. 2009). Postharvest 80 mM malic acid treatment alleviated the occurrence of chilling injury symptoms and delays skin browning of banana fruit under low-temperature. Malic acid retarded the rapid decline of total phenolics and chlorophyll fluorescence (Fv/Fm) and chlorophyll content and caused low levels of superoxide anion generation and H_2O_2 content, on one side, and on the other side it enhanced antioxidant activities including PPO and POD activities as well as relatively high antioxidant activities marked by a reduction in MDA accumulation (Huang et al. 2016).

4.3.3.4 Other Combinations

Numerous studies have been carried out showing the potential of acetic acid to be used for control of postharvest diseases in the form of vapor, dip, etc., for a variety of fruits and vegetables (Sholberg and Gaunce 1995, 1996; Liu et al. 2002; Ochoa-Velasco and Guerrero-Beltrán 2014; Hassenberg et al. 2018).

While adding organic acids as an ingredient of vase solutions has a long history, the positive effect of pure citric acid and malic acid incorporated into vase solution has been shown to cause the prolongation of vase life of a variety of cut flowers including roses (Sabzi et al. 2012; Ashtari et al. 2013; Farshid et al. 2013), chrysanthemums (Zamani et al. 2011), carnations (Kazemi et al. 2012; Begri et al. 2014), and gerbera (Jamshidi et al. 2014). It has been demonstrated that malic acid increases chlorophyll and carbohydrate content while decreasing ACC-oxidase activity and delays the senescence and peroxidation of lipids causing vase life extension of cut *Eustoma grandiflorum* flowers (Danaee 2015). Spraying the citric acid on lilium at the preharvest stage (7.8 mM) also caused the mean vase life of cut flowers to increase from 8 in control treatment to 14 days (Darandeh and Hadavi 2012). The positive effect of preharvest citric acid on tuberose vase life was studied before (Eidyan et al. 2014). Fruit TSS, total sugars, firmness anthocyanin content, and vitamin C content during storage were significantly improved while fruit weight loss, acidity, and physiological and pathological disorders were substantially decreased by application of 1, 2, or 3 mM citric acid together with salicylic acid, ascorbic acid, and putrescine (Aly et al. 2019).

Succinic acid (5 mM) also improved the quality of stored strawberries compared to control, and when it was combined with ultraviolet C, the outcome on postharvest quality of fruits was deemed much better (Kim et al. 2010).

4.3.4 Secondary Metabolism and Essential Oil Production by Plants

Use of 5.2 mM citric acid alone or in combinations with salicylic acid and malic acid increased the essential oil production of sweet basil (Jaafari and Hadavi 2012) and dill (Jafari and Hadavi 2012). The composition of essential oil also was changed in response to different combinations of foliar citric acid and malic acid (Jaafari et al. 2015). A twofold increase in essential oil content of summer savory (*Satureja hortensis*) was reported by foliar sprays of 8 mM citric acid (Radmanesh et al. 2015). A 30% increase by use of foliar 5 mM citric acid is noted in the essential oil content of thyme (Miri et al. 2015). A significant increase in essential oil content and yield by foliar application of citric acid is also reported in *Artemisia abrotanum* (EL-Zefzafy et al. 2016).

The preharvest foliar citric acid (15 and 30 mM) improved substantially the level of PPO activity and total content of phenolic compounds after 5 days from inoculating the pepper fruits to *Botrytis cinerea* (Mekawi et al. 2019). It is also reported that foliar citric acid caused an increase in the wood-ripening coefficient (Ali et al. 2016). Wood formation is a process involving elevated phenol metabolism, so this is considered in accordance with the findings of Mekawi et al. (2019). Phenolic content was also higher in cotton when 3 mM citric acid was sprayed once (Ghourab 2000). Similarly, 3 days after rice plants were treated with 1 mM foliar oxalic acid, a twofold increase in phenolic content in leaf sheaths was recorded together with a significant increase in activities of PAL and POD starting from 2 days after treatment (Jayaraj et al. 2010). An increase in anthocyanin content in strawberries is also noted (Soltaniband 2020).

The addition of 100 mM pyruvate into the culture medium of *Chlorella zofingiensis* enhanced the yield of astaxanthin. Also, citrate and malic acid had similar stimulatory effects on the formation of astaxanthin (Chen et al. 2009).

4.3.5 Quality Indices of Product

Color development of grape cultivar “Yaghooti” was improved by preharvest foliar sprays of citric acid (Mahnam and Hadavi 2010). Mung bean sprouts, sprayed with both malic acid and oxalic acid, showed significant degradation of phytic acid, which is considered a nondesired content for the food products; besides, a significant increase in measured bioactive contents of ascorbic acid and phenols was noted (Jin et al. 2016). The positive effect of malic acid sprays on organoleptic features of pistachio nut was reported (Saboory et al. 2014). Improvement in fruit flavor of “Thompson Navel” orange after storage by preharvest foliar sprays containing citric acid was reported as well (Mollapur et al. 2016).

Ali et al. (2016) sprayed citric acid (2.6, 5.2, 10.4, and 20.8 mM) on vine trees and reported that while the cluster count per vine remained unchanged but other qualitative features of the cluster (millerandage, weight, length, and shoulder) and berry (weight, length, and diameter) improved. All the qualitative features of the fruit, that is, TSS, TSS/acid ratio, and reducing sugars percentage were also improved while the titratable acidity was reduced. A single foliar spray on grape by various organic acids (including 8 or 12 mM benzoic acid, 11 or 17 mM oxalic acid, and 5 or 7.5 mM citric acid) enhanced the contents of total phenolic compounds and total anthocyanin content (Kok and Bal 2019). Similarly, in “Red Spur” apple, foliar citric acid improved the quality and nutraceutical properties of the fruit (Allahveran et al. 2018). In fig trees, a significant increase in TSS value of fruits is reported by application of 1.5 mM citric acid, which was still significantly more than that of 1 mM citric acid that itself had a significantly more TSS compared to control treatment (Mohamed and El-Berry 2019). Foliar 10 mM citric improved strawberry fruits weight, firmness, TSS, and vitamin C even more by foliar calcium citrate while total acidity of fruits remained unchanged (Mandour et al. 2019).

Also, 5 mM oxalic acid sprays at preharvest enhanced the postharvest quality and disease resistance of the kiwi fruits (Zhu et al. 2016). In sweet cherry cultivars that were treated with 2 mM oxalic acid in 98, 112, and 126 days after full blossom, fruit size at harvest increased, and they had better color and firmness (Martinez-Espla et al. 2014). Likewise, in artichokes that were sprayed with 2 mM oxalic acid during growth, the postharvest senescence was delayed and the reported health-beneficial properties of artichokes consumption improved (Martínez-Esplá et al. 2017). In a later study, they confirmed consistent results on plum as well, and the ripening process was delayed both on-tree and during cold storage, which was manifested by lower firmness and acidity losses and reduced ethylene production. Antioxidant compounds (phenolics, anthocyanins, and carotenoids), as well as the activity of antioxidant enzymes, were higher in oxalic acid-treated fruits both at harvest and during 50 days of cold storage (Martínez-Esplá et al. 2019).

4.3.6 Mineral Acquisition

Even in a hydroponic culture system in which interactions of plant with flora and fauna of the growth medium was minimal, it has been demonstrated that S and B nutrients absorption responded to foliar treatments radically while the rest of the nutrients were absorbed similarly, possibly because lake of any important effect created from differential root exudates that could be generated by foliar sprays (Ghazijahani et al. 2014).

A combined spray of N, P, K, Zn, Fe, Mn, S, B plus 5.2 mM citric acid was much more effective compared to when the elements were applied alone on yield and fruit quality of pear trees (Mansour et al. 2008). Similarly, all barley growth traits improved when sprayed by 200 ppm of micronutrient mixture (Fe + Zn + Mn) for each element plus 8 mM citric acid (Shendy et al. 2016). Accordingly, when maize plants were sprayed with a foliar combination of micronutrient enriched citric acid (containing 15% citric acid, 2% Fe, 2% Mn, and 2% Zn) with different concentrations from 0 to 0.5%, They noticed that 0.3% concentration of this combination scored the best result almost in all measured growth indices and cause the maximum of phosphorus and potassium uptake by plants (El-Yazal 2019). In fact, the addition of sole organic acids to soil could affect mineral acquisition radically. For instance, the concentration of Mn, Fe, Co, Ni, and Cr in leaves of *Noccaea caerulescens* and *Thlaspi arvense* increased between 1.4- and 14-fold in response to the addition of citric acid (20 mM kg^{-1}) to soil (do Nascimento et al. 2020). However, recent findings reveal that spraying citric acid alone can affect mineral acquisition. Vine trees sprayed in a large spectrum of concentrations (from 2.6 to 20.8 mM) demonstrated a linear increase in leaf N, P, K, and Mg contents by applied citric acid concentration (Ali et al. 2016). Correspondingly, roots of rice plants exposed to foliar potassium nitrate sprays exhibited a significant increase in uptake of N, P, K, and S (Kundu et al. 2021).

Snap bean (*Phaseolus vulgaris*) plants were sprayed with 8 mM citric acid combined with different levels of farmyard manure (25%, 50%, 75%, and 100% of the recommended rate) in sandy soil conditions. The citric acid +50% farmyard manure treatment increased almost all studied parameters including both vegetative growth or pod yield (Ahmed 2020).

4.3.7 Other

Application of malic acid at different times on leaf potted tobacco plant has been investigated before, and it was found that earlier applications (among 20, 40, and 60 days after transplanting) were more effective in increasing the content and proportion of nonvolatile organic acids (Xu et al. 2008). The flower display period in an ornamental plant "gazania" is increased by the application of all concentrations of citric acid or malic acid (Talebi et al. 2014). In mung bean sprouts, under 0.6 mM foliar malic spraying showed improved sprout growth (Jin et al. 2016).

4.4 Conclusion

As can be seen from the body of study conducted, the organic acid application could create a solid base for the management of plant metabolism and response in a wide scope of situations. However, by reviewing available knowledge we could have some conclusions that might help us to decide regarding future studies in this field.

Spraying the organic acids on plant foliage seems to be the preferred strategy concerning input economy and the variety of responses it creates. However, in some cases, the foliar approach remains the only solution. As an example, if we aim for phytoextraction then adding to medium is the preferred way, while when we aim to increase the plant tolerance to heavy metals then the foliar approach is the preferred option.

As is raised by some experiments, using salts of organic acids could be a wiser strategy giving us the possibility to exert a dual effect, one from the organic acid group and the other from the cation of our choice for nutrition like Fe or for postharvest improvements and firmness like Ca.

In most studies, at least two sprays have been needed for a full response even though in some cases a single spray has been enough. It seems that a regular spray with an interval of 2–4 weeks could be considered optimum depending on the plant and the desired physiological changes in plants. However, it seems reasonable to find the optimum interval for each case, and then a conclusion could be made with a better insight.

We have to remember that the effect resulted from much higher concentrations of organic acids like the fungicide or herbicide effects is mostly considered direct and by using lower concentrations the response is likely to be a secondary result of a change in plant metabolism in response to our organic acid, which in such case is acting as an elicitor or a biostimulant. Even in lower concentrations, an optimum middle response has been noted, which necessitates further studies to validate optimum doses for each response and/or plant. This observation supports considering a growth regulation function for them like many growth regulators which act in a bell-shaped curve in low concentrations. However, in order for the data to be better and easier interpreted, it is highly advised to stick to the molar system for concentrations so we can have easier comparison among different studies as well as to be able to compare the effectiveness of different organic acids based on equal molar concentrations.

The use of foliar sprays for ameliorating soil-related problems seems a valid and promising strategy, especially in trees where the soil amendment practices are both hard and albeit expensive. Organic acids could affect the rhizosphere surrounding the tree root system with cheap foliar sprays that would not be an easy task by every other means.

Many of today's foliar formulations that are used in agriculture contain different organic acids as an ingredient. The extent of effect from these organic acids however remains hidden. Therefore, working with pure organic acids might be a better idea to first create a library of response for each one and then to predict and try their interactions in the next stage. This way we can know our tools better and understand that to which extent the action of more complex formulations relies on their organic acid content.

Organic acids can create specific responses based on the applied dose that is hard to reach by more complex formulations, making them a good candidate for solving the puzzle of plant growth and development with the minimum cost. This might explain why most of the research in this area has been conducted in the less developed areas of the world. Moving toward finding the response curves and developing models to predict the plant response could create a strategy and a manual of application for different situations with a "constructive" approach instead of a "trial and error" one. Considering the environmental-friendly aspect of these compounds, there seems a better prospect for them in long term considering the current awareness regarding ecological issues.

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5 Animal-Derived Hydrolyzed Protein and Its Biostimulant Effects

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Abstract

Animal-derived hydrolyzed protein has a long history of usage as plant growth enhancer and biostimulant. Worldwide, hydrolyzed protein products represent the third biostimulant category by market size after seaweed extracts and humic substances. Hydrolyzed protein can be produced from either animal by-products, and nonanimal raw materials and by-products (e.g., plants, bacteria, fungi). Indeed, hydrolyzed protein derived from animal by-products was the first discovered and commercialized, actively contributing to the birth of the term and concept of "biostimulant." The first part of this chapter will provide an overview of the regulatory status of hydrolyzed protein, starting with a general excursus on the different regulatory frameworks outside EU. Then the recent approval of fertilizer regulation CE 1009/2019 (that comprises for the first time the biostimulants, and thus hydrolyzed protein) will be taken into consideration, together with other important regulations (EC 1069/2009 and EC 142/2011), that contain the definition of hydrolyzed protein, the raw materials from which it can be produced, and their transformation methods. Afterward, a detailed description of the most used production methods, focusing mainly on chemical hydrolysis and enzymatic hydrolysis, will be provided. A paragraph that describes the protein chemistry of both the raw materials and the hydrolyzed protein, and an excursus on the main analytical methods used for its characterization, will be

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_5

included. The second part of the chapter will analyze hydrolyzed protein effects on plants. The effects on whole plant level will be grouped in major categories, according to the claims cited in the biostimulant definition included in fertilizer regulation CE 1009/2019 (i.e., nutrient use efficiency, tolerance to abiotic stress, quality traits, availability of confined nutrients in soil or rhizosphere). The final paragraph will give an overview of the state-of-the-art of scientific knowledge on the action mechanisms of these products.

Keywords

Biostimulants · Hydrolyzed protein · Fertilizers · Animal by-products · Plant productivity · Plant resilience

5.1 Regulatory Status of Animal-Derived Hydrolyzed Protein, Production Methods, and Characteristics

5.1.1 International Regulatory Status

Biostimulants are a relatively new category of inputs in the agricultural market [e.g., the use of hydrolyzed protein (HP) as plant biostimulant began in the 1970s, in Italy and Spain, and subsequently and rapidly spread in many other countries]. This is why HPs' regulatory framework is still very fragmented at the international level, with very few countries having a specific category for biostimulants in their legislations. In all the other countries, companies wanting to place on the market HPs are forced to follow legislation frameworks belonging to other product categories (e.g., fertilizers, plant protection products, complexing agents, plant growth regulators). This leads to a very uncertain panorama, impeding the definition of clear boundaries between the category of biostimulants and other agricultural inputs (causing confusion especially in the end users), and frequently involving unnecessary high registration costs and requirements. The main options available for the registration of HPs in non-EU countries are summarized below.

- Nitrogen fertilizers: HPs contain a certain amount of organic nitrogen, present in amino acids, so they could be registered as fertilizers. This option is possible in all countries, albeit with some limitations related to animal origin, such as in some countries of the Middle East region. The complexity of the registration dossier varies considerably (from "registration not requested," as happens in India for the so-called "bioproducts," to the request for a complete dossier containing physicochemical characterization, eco-toxicological profile, and agronomic efficacy demonstration).

- Complexing agents: HPs are able to complex nutrients, and in some countries, they can only be registered when formulated in a mixture with a minimum quantity of meso- or microelements, as, for example, occurs in China.
- Plant protection products: In many countries (e.g., Brazil, the United States), it is possible to register HPs in the same category of hormonal substances, within the specific category of plant protection products. In this case, the dossier is always very complex and the registration costs quite high.

Recently, some countries are studying or applying specific regulations for biostimulants. In India, a new specific legislation is being studied for those products (bioproducts) that currently do not fall within the specifications provided for fertilizers (Fertiliser Control Order) or for pesticides (Insecticide Act) and that include all biostimulants (HPs, seaweed extracts, humic acids, etc.). In the United States, there is the possibility of registering HPs as fertilizers or within the category plant regulators, after notification to the Environmental Protection Agency (EPA). The possibility of creating an autonomous category of biostimulants is also recently being discussed. Indeed, 2018 Farm Bill contained for the first time a definition of biostimulants as “substance or micro-organism that, when applied to seeds, plants, or the rhizosphere, stimulates natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, or crop quality and yield” (Agriculture and Nutrition Act of 2018 2018). In 2019, the USDA produced a report on plant biostimulants that proposes two alternative definitions. The first recites that “a plant biostimulant is a naturally occurring substance, its synthetically derived equivalent, or a microbe that is used for the purpose of stimulating natural processes in plants or in the soil in order to, among other things: improve nutrient and/or water use efficiency by plants, help plants tolerate abiotic stress, or improve characteristics of the soil as a medium for plant growth. The characteristics may be physical, chemical, and/or biological. The plant biostimulant may be used either by itself or in combination with other substances or microbes for this purpose.” The alternative definition states that “a plant biostimulant is a substance(s), microorganism(s), or mixtures thereof, that, when applied to seeds, plants, the rhizosphere, soil or other growth media, act to support a plant’s natural nutrition processes independently of the biostimulant’s nutrient content. The plant biostimulant thereby improves nutrient availability, uptake or use efficiency, tolerance to abiotic stress, and consequent growth, development, quality or yield.” By 2021, EPA is expected to publish a guideline on the labeling of products with biostimulant claims. In China, as mentioned, HPs are registered only as fertilizers in association with microelements, but there is increasing attention to the new European regulatory model. Canada considers HPs to fall in the category of “supplements” defined as “any substance or mixture of substances other than a fertilizer, that is manufactured, sold or represented for use in improving the physical condition of soils or to aid plant growth or crop yields” (Caradonia et al. 2019). To be registered, the dossier must contain information regarding the safety, merit, and value of the supplement (Caradonia et al. 2019). In Brazil, HPs are comprised in the category of biofertilizers, which are defined as “a product containing an active ingredient or an organic agent, free of pesticide substances, able to act directly or indirectly on all or part of cultivated plants, raising their productivity, without regard to their hormonal or stimulant value." The requirements for the registration are quite complex and include a company profile and a description of the production of a quality control process used to guarantee product safety for their proposed use (Caradonia et al. 2019).

5.1.2 European Regulatory Status

The European Community (EC) was the first to address and solve in a structured way the regulatory problem of biostimulants, which previously were regulated on a national basis. Indeed, in June 2019 the Official Journal of European Union published the new Regulation (EC) No. 1009/2019 on fertilizing products (The European Parliament and the Council of the European Union 2019), which for the first time uniforms biostimulant legislation among EU countries. The regulation will be applied starting from July 2022. One of the aims of the Regulation, which repeals Regulation (EC) No. 2003/2003 and amends Regulation (EC) Nos. 1069/2009 and (EC) 1107/2009, was to provide a unique legal framework for plant biostimulants across all EU countries. This would overcome the barriers to free internal market, and the uncertainties for operators and controlling authorities posed by the highly fragmented and diverse panorama of pre-existent national laws (Caradonia et al. 2019), which forced companies to register the same product multiple times in order to be able to sell it throughout the EC territory. The regulation classifies fertilizer products into seven categories according to their function, which are called product functional categories (PFCs). The PFC number 6 of the Regulation (EC) No. 1009/2019 establishes a claim-based biostimulant definition and recites that "a plant biostimulant shall be an EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content, with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency; (b) tolerance to abiotic stress; (c) quality traits; (d) availability of confined nutrients in soil or rhizosphere." PFC 6 also designates specific requisites for this category of products and denotes specific limits for contaminant content (i.e., cadmium, hexavalent chromium, lead, mercury, nickel, and inorganic arsenic), pathogenic microorganisms (*Salmonella* spp., *Escherichia coli,* or *Enterococcaceae*), copper, and zinc. The Regulation furthermore specifies that a biostimulant product "shall have the effects that are claimed on the label for the plants specified thereon" (Ricci et al. 2019), which implies that the demonstration of agronomic efficacy will be a prerequisite for placing a CE-marked biostimulant product on the market (Madende and Hayes 2020). For this reason, the European Committee of Standardization (CEN) is developing a series of standards laying down the scientific requisites of validity of agronomic efficacy testing. Plant biostimulants can be manufactured starting from one or more of the component material categories (CMCs) described in the regulation. The regulation identifies 11 CMCs: virgin material substances and mixtures; plants, plant parts, or plant extracts; compost; fresh crop digestate; digestate other than fresh crop digestate;

food industry by-products; microorganisms; nutrient polymers; polymers other than nutrient polymers; derived products within the meaning of Regulation (EC) No. 1069/2009; and by-products within the meaning of Directive 2008/98/EC. A microbial plant biostimulant can be constituted only by CMC 7 materials, while for nonmicrobial biostimulants the allowed CMCs are not specified in the Regulation. Hydrolyzed protein from animal by-products is included in CMC 10, which recites that "an EU fertilising product may contain derived products within the meaning of Regulation (EC) No 1069/2009 having reached the end point in the manufacturing chain as determined in accordance with that Regulation, and which are listed in the following table and as specified therein." The Regulation further specifies that the table will be established by delegated acts, which at the moment of writing this publication have not been published yet. Regulation (EC) No. 1069/2009, referred to in the description of CMC 10, sets the legal framework for animal by-products (ABPs) and derived products not intended for human consumption and contains the definition of derived products as ". . . products obtained from one or more treatments, transformations or steps of processing of animal by-products." ABPs are divided into three categories according to the level of risk they pose to public and animal health. Only materials belonging to category 3, which are by-products either resulted fit for human consumption but excluded for commercial reasons, or that do not show any kind of human and animal transmissible disease – certified by veterinary inspection – and thus do not constitute a health risk of any kind, are allowed to be used for the production of hydrolyzed protein. Hydrolyzed protein is defined in Regulation (EC) No. 142/2011 (The European Comission 2011) as "polypeptides, peptides and amino acids, and mixtures thereof, obtained by the hydrolysis of animal by-products." Annex X, Section 5-D of Regulation (EC) No. 142/2011 rules down specific requirements for this derived product and recites that "hydrolysed protein must be produced using a production process involving appropriate measures to minimise contamination. Hydrolysed protein derived from ruminants shall have a molecular weight below 10.000 Dalton." This paragraph describes also specific transformation methods for hydrolyzed proteins entirely or partly derived from ruminants' hides and skins: ". . . shall be produced in a processing plant dedicated only to hydrolysed protein production, using a process involving the preparation of raw Category 3 material by brining, liming and intensive washing followed by exposure of the material to: (a) a pH of more than 11 for more than three hours at a temperature of more than 80 °C and subsequently by heat treatment at more than 140 °C for 30 min at more than 3,6 bar; or (b) a pH of 1 to 2, followed by a pH of more than 11, followed by heat treatment at 140 °C for 30 min at 3 bar." Fertilizers in general, obtained from category 3 ABPs, can be produced by using one of the production methods described in Regulation (EC) No. 142/2011, Annex IV, Section III.

5.1.3 Production Processes

The process used to produce HPs is the hydrolysis reaction, which involves the cleavage of peptide bonds in an aqueous media, to obtain a mixture of peptides and amino acids (Moreno-Hernández et al. 2020). Diverse industrial processes can be used to obtain HPs. They can be classified according to the agent used to hydrolyze the protein, which can be acids, alkali, enzymes or high temperatures, or their combination (Corte et al. 2014). It has to be noticed that, despite individual choices of each manufacturer regarding the production process, HPs derived from category 3 ruminant by-products need to undergo a specific sterilization step ($pH > 11$, $T > 140$ °C, $p > 0.36$ MPa, HP molecular weight < 10 kDa) to avoid the risk of transmissible spongiform encephalopathies (EC 2001; Corte et al. 2014). The hydrolysis method chosen depends on raw material characteristics; indeed, materials containing keratin are usually subjected to alkaline or acid treatment (Hou et al. 2017), while collagen-based raw materials can be processed using either chemical or enzymatic hydrolysis. Downstream processes can consist in a salt removal step (Hou et al. 2017; Aliashkevich et al. 2018), an evaporation phase, and/or spray drying to obtain powdery products (Pasupuleti and Demain 2010).

5.1.3.1 Acid or Alkaline Hydrolysis

The first report of the acid hydrolysis of a protein was done by the French chemist H. Braconnot in 1820 (Hill 1965). While the complete hydrolysis of protein in experimental setups occurs in 24 h, at 110 °C with 6 mol/L HCl, shorter reaction times are needed to produce peptides (Hill 1965). Important factors in acid hydrolysis are the concentration and type of acid (e.g., HCl or H_2SO_4), temperature (e.g., 120–140 °C), pressure (32–45 psi), reaction time (2–8 h), and protein concentration in the raw material (50–65%) (Pasupuleti and Demain 2010). The resulting HP is typically composed by a high degree of free amino acids and dipeptides (Hill 1965; Pasupuleti and Demain 2010), with different peptide bonds showing diverse susceptibility to acid cleavage due to electrostatic and steric effects (Hill 1965). Generally, tryptophan indole ring is disrupted, with the production of several of degradation products; glutamine and asparagine are converted to glutamic acid and aspartic acid; however, the magnitude of degradation/conversion of amino acids varies greatly depending on process duration and raw material (Hill 1965). Alkaline agents such as calcium, sodium, or potassium hydroxide are able to completely hydrolyze proteins at high temperature (e.g., 105 °C) and long reaction times (e.g., 20 h); however, usually lower temperatures (e.g., 25–55 °C) and reaction times are used to produce HPs (Hou et al. 2017; Aliashkevich et al. 2018). During the process several aminoacids decompose: serine to glycine and alanine; threonine to glycine, alanine, and α-aminobutyric acid; arginine gives ornithine, citrulline, and ammonia; cysteine and cystine yield alanine, hydrogen sulfide, ammonia, and pyruvic acid (Hill 1965). Alkaline hydrolysis allows a 100% recovery rate of tryptophan (Pasupuleti and Demain 2010) and leads to the racemization of some amino acids from the L form to the D form (Hill 1965). A recent paper indicates that a certain degree of amino acid racemization occurs in all HPs, irrespective of their production process (Corte et al. 2014); however, the process is favored in the presence of acid, alkali, or high temperatures (Marcone et al. 2020). D-amino acids have long been regarded as useless or even harmful to plants; however, there is a growing evidence for them to play important and still underresearched roles in plant growth and development (Hill et al. 2011; Aliashkevich et al. 2018; Hener et al. 2018; Kolukisaoglu 2020).

5.1.3.2 Enzymatic Hydrolysis by Cell-Free Proteases

In the enzymatic hydrolysis of proteins, the agent used for the cleavage of peptide bonds is a protease, which can be naturally present in the substrate (endogenous), or added from commercially available sources (exogenous) (Aliashkevich et al. 2018). Commercially available proteases can have a microbial (e.g., Alcalase, Flavourzyme), animal (e.g., pepsin, trypsin), or plant origin (e.g., papanin, bromelain) (Moreno-Hernández et al. 2020). They can cleave peptide bonds in terminal regions (exopeptidases) or within the peptide chain (endopeptidases) (Hou et al. 2017). Proteases exhibit a wide range of action in terms of substrate selectivity, optimum pH, and temperature conditions. Indeed, these enzymes can be classified either according to the nature of their catalytic residues (e.g., aspartic proteases, cysteine proteases, serine proteases, metallo proteases, threonine proteases, glutamic proteases, and asparagine peptidases) or optimum pH conditions (i.e., acid, neutral, and alkaline proteases) (Oda 2012; Moreno-Hernández et al. 2020). The typical hydrolysis process involves a preliminary phase in which the raw material can be heated, and acid or alkali are added to reach the appropriate conditions for the chosen enzyme or mixture of enzymes (Pasupuleti and Demain 2010). The subsequent step begins when the enzyme is added and binds to the substrate beginning to release peptides and amino acids in solution (Anzani 2018). The reaction kinetics follows an initial rapid increasing phase due to substrate excess, and as the reaction proceeds, the rate slows down to a plateau due to changes in pH, fewer bonds available, substrate, and product inhibition (Aliashkevich et al. 2018). Hydrolysis times can vary from 1 h to more than 100 h (Pasupuleti and Demain 2010); in case of long reaction times, it is required to prevent microbial contamination either adding bacteriostatic/ bactericidal preservatives (Pasupuleti and Demain 2010; Hou et al. 2017), or using other types of treatments (e.g., UV light). Another option would be to use enzymes that can work at higher temperatures and acidic pH (Pasupuleti and Demain 2010). Once the required degree of hydrolysis is obtained, the reaction is terminated by desaturating the enzyme at high temperature (e.g., 90 °C for 10 min) (Aliashkevich et al. 2018). Usually, enzymatic hydrolysis produces HPs with a low degree of free amino acids, while the peptide fraction composition is somehow predictable (Colla et al. 2015) if enzymes exhibiting a high degree of specificity are used.

5.1.4 Physicochemical Characteristics and Analysis Methods

5.1.4.1 Raw Material Protein Chemistry

Amino acids are the monomers that, linked via peptide bonds in genetically predefined sequences, constitute the primary protein structure (Bischoff and Schlüter 2012). Proteinogenic amino acids can have acidic, basic, hydrophilic, or hydrophobic characteristics depending on the side chain R group (Bischoff and Schlüter 2012). In most polypeptides, the typical peptide bonds are formed from the α-amino group and α-carboxyl group of adjacent amino acids (Hou et al. 2017). Short chains of amino acid residues (between 2 and 50) are called oligopeptides, while peptides with more than 50 amino acids are called polypeptides (Ouellette and Rawn 2018). Proteins are composed by one or more high-molecular-weight polypeptides and exhibit four orders of structure. The primary protein structure refers to the sequence of amino acids in the polypeptidic chain. Secondary structure is the regular repetition of conformations of the peptide backbone such as α-helices and β-sheets (Ouellette and Rawn 2018). The spatial three-dimensional arrangement of secondary structure elements results in the formation of the tertiary structure, or fold, of a protein that is held together by noncovalent interactions (hydrogen bonds, ionic interactions, van der Waals forces, and hydrophobic packing), disulfide bonds, and metal ion coordination (EMBL-EBI European Bioinformatics Institute n.d.). Some proteins form assemblies with other molecules that origin the quaternary structure (EMBL-EBI European Bioinformatics Institute n.d.). As can be seen in Table 5.1, the main proteins present in ABPs used for HPs production are collagen, keratin, and hemoglobin. Collagen is the most abundant structural protein in animals (Shoulders and Raines 2009). It is located in connective tissues such as skin, bones, tendons, and cartilage, forming elongated fibrils whose main function is to provide

Table 5.1 Examples of raw materials of animal origin used to produce hydrolyzed protein for biostimulant uses

Example of raw material type	Raw material main protein type	References
Meat meal	Collagen	Ertani et al. (2009); Cavani et al. (2017)
Bone meal	Keratin	Veselá and Friedrich (2009)
Fleshing	Collagen	Corte et al. (2014); Baroccio et al. (2017)
Feather meal	Keratin	Hill et al. (2011); Gurav and Jadhav (2013); Tejada et al. (2018); Popko et al. (2018)
Blood meal	Hemoglobin	Polo et al. (2006); Marfà et al. (2009); Cristiano et al. (2018)
Whey	β-Lactoglobulin, α-lactoalbumin	Caballero et al. (2020)
Wool	Keratin	Gousterova et al. (2005); Zoccola et al. (2015)
Hairs		Galarza et al. (2010); Wang et al. (2019)
Fish meal	Collagen	Madende and Hayes (2020)
Shavings and trimmings	Collagen	Migliore et al. (2013); Corte et al. (2014)

mechanical stability to the body (Bella 2016). Collagen primary structure shows a repetitive sequence of amino acids with units of glycine-proline-X or glycine-Y-hydroxyproline, with X and Y being various amino acid residues (Anzani 2018). The three-dimensional conformation of collagen is attained by three parallel polypeptide strains coiled together in a left-handed, polyproline II-type (PPII) triple helical conformation (Shoulders and Raines 2009; Bella 2016). Single triple helices (tropocollagen) further assemble in a hierarchical manner to form the macroscopic fibers and setups contained in tissue, bone, and basement membranes (Bella 2016). Keratin is, after collagen, the second most abundant biopolymer in animal tissues (McKittrick et al. 2012). It composes the tegument of vertebrates, forming, for example, the epidermis, the wool, and the horns of mammals, as well as feathers, claws, and beaks of birds and reptiles, and assures a variety of functions, such as protection, defense, and predation (Wang et al. 2016). It shares with collagen a number of features, that is, they both have α-helix polypeptide chains with a well-defined amino acid sequence; and both contain a high amount of the smaller amino acid residues, glycine and alanine. However, while in keratin two polypeptide chains (α -keratin) twist together to form a coiled coil, in collagen, three α-helices twist together and assemble to form the collagen fibril. Another difference is that keratin is a "dead" tissue that is not vascularized, while collagen forms in the extracellular matrix (McKittrick et al. 2012). Keratin polypeptide chains contain high amounts of cysteine that have a thiol group (-SH), which produces a strong, covalent disulfide bond with other intra- or intermolecular cysteine residues. Intermolecular cystine bonds, peptide and hydrogen bonds, cause the high stability of keratin, giving it strength and toughness, but also partial insolubility (Zoccola et al. 2015). Keratin filaments are composed by distinct regions, that is, crystalline fibrils forming helices, embedded in an amorphous polymeric matrix, and the terminal domains of the filaments. While the crystalline region is insoluble in water, the amorphous matrix can swell after the absorption of water (McKittrick et al. 2012). Red blood cells in human and other vertebrates are called erythrocytes. In their mature state, these cells are packed with hemoglobin, whose function is to transport oxygen throughout the body (Ouellette and Rawn 2018). Hemoglobin quaternary structure is composed by two α chains and two β chains, each linked to a molecule of heme. The units are held together by hydrophobic interactions, hydrogen bonds, and salt bridges (Ouellette and Rawn 2018).

5.1.4.2 Chemical Characteristics of Hydrolyzed Protein

It has to be underlined that since different hydrolysis methods and parameters lead to very different products in terms of amino acid composition; the tables shown in this paragraph have to be considered only as a display of example compositions of HPs and are not intended to be considered as a comprehensive review of all the HPs present in the market. One of the distinctive features of HPs that more often appears in scientific literature is total amino acid content. This is to be considered as a descriptive feature that could be used for the identification of the raw material of origin (Baroccio et al. 2017). Total amino acid content can vary a lot depending on the physical form of the product, from almost 85% in solid powders, to 40–50% in concentrated liquids, to 30–20% and below in more diluted or multicomponent products. The most distinctive feature of HPs of animal origin is their high content of total glycine and proline, which are often the prevailing amino acids (Baroccio et al. 2017). Levels of alanine and glutamic acid are also high (Table 5.2). HPs from raw materials rich in keratin contain considerable amounts of aspartic acid and leucine, generally higher than those present in HPs from collagen. In a hydrolysate obtained from porcine blood, the two most represented amino acids are leucine and aspartic acid (Table 5.2).

Free amino acid content represents the amount of amino acids present in free form in the final product. It is an important indicator of the amino acids directly interacting with plant organs after the application of HPs. Raw material and hydrolysis methods, but also the specific hydrolysis parameters chosen (e.g., duration and temperature), hugely influence the total quantity and relative abundance of free amino acids. Due to this, even HPs derived from the same raw materials and produced with the same hydrolysis method (i.e., chemical or enzymatic) can have dramatically different percentages of free amino acids. This is clearly visible in Table 5.3, where it can be noticed how the content of free amino acids of HPs produced by chemical hydrolysis from bovine hides can range from 5 to 22.3%, while that of HPs from hair vary from 4.7 to 30%. Fewer data were available for HPs from meat meal and feather, so those reported in Table 5.3 have to be considered only as example compositions. Glycine is the most represented free amino acid in HPs from bovine hides (1.8–7.5%) and meat meal (0.86%), the second most abundant in HPs from feather (1.01%), and the third in HPs from hair (1.6–6.0%). Alanine is the most abundant free amino acid in HPs obtained from keratinous raw materials (1.57% in HPs from feather, 1.6–6.0% in HPs from hair), and the second most abundant in HPs from collagen (1.0–3.8 in HPs from bovine hides and 0.77% in HPs from meat meal). Proline is another amino acid highly present in free form, being the third for abundance in HPs from bovine hides, meat meal and feather (0.4–2.5%, 0.25%, and 0.58%), and the fourth in HPs from hair (0.4–3.6%).

5.1.4.3 Analytical Methods

There are currently no official analytical methods to characterize biostimulants, but work is underway by standardization bodies to fill this gap. The following are examples of some of the most used methods to characterize hydrolyzed proteins.

Degree of Hydrolysis

The degree of hydrolysis can be considered as an index of how much the hydrolysis reaction affected the original protein structure by producing peptides of various lengths and single amino acids. Various methods can be used; that is, the pH stat method, which is based on the number of protons released during hydrolysis; or titration methods (that are based on the measurement of amino acids generated by hydrolysis), which make use of trinitrobenzenesulfonic acid (TNBS), o-phthaldialdehyde (OPA), or formaldehyde. From these, two are the most widely used: the titration methods using OPA or formaldehyde. In the first one, the degree of hydrolysis is calculated on the basis of the ratio between α-amino nitrogen content and organic nitrogen. The α-amino nitrogen content is evaluated by

Table 5.2 Content of amino acids (g/100 g protein) of selected HPs

Type of HP	Hydrolyzed collagen	Hydrolyzed Collagen	Hydrolyzed keratin	Hydrolyzed keratin	Hydrolyzed keratin	Hydrolyzed keratin	Hydrolyzed collagen	Hydrolyzed keratin	Hydrolyzed hemoglobin	Hydrolyzed collagen
Raw material	Bovine hides	Meat meal	Feather	Hair	Feather	Feather	Fish by-products	Feather	Porcine blood	Meat meal
Type of hydrolysis	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Enzymatic	Enzymatic	Enzymatic	Enzymatic
Alanine	11.0–13.0	9.4	9.9	10.0–15.0	4.7	5.5	7.5	5.0	8.1	16.6
Arginine	0.05–2.5	3.1	5.0	0.04–1.5	5.9	6.5	4.18	7.1	6.2	5.0
Aspartic acid	3.5–6.2	7.7	6.5	3.5–8.7	8.2	8.5	9.0	10.9	11.7	12.8
Cysteine	nd	nd	nd	nd	2.9	4.0	1.27	nd	2.7	nd
Glutamic acid	12.0–13.5	15.4	13.5	19.0–22.0	9.4	11.5	14.4	11.8	8.5	5.6
Glycine	21.5–22.0	13.7	10.6	8.0–9.0	8.2	9.5	8.66	8.4	4.8	4.7
Histidine	0.5–0.9	2.1	0.9	0.5–0.8	1.2	2.0	2.53	1.6	7.5	10.3
Hydroxylysine	0.6–1.1	0.3	udl	udl	nd	nd	nd	nd	nd	nd
Hydroxyproline	5.7–7.2	4.1	udl	0.8–1.0	nd	nd	nd	nd	nd	nd
Isoleucine	1.4–2.0	3.6	5.5	2.2–3.2	3.5	4.0	5.44	6.1	0.2	1.9
Leucine	4.8–5.2	6.4	9.1	9.5–11.0	8.2	8.5	8.39	9.1	13.0	6.7
Lysine	4.3–4.8	6.0	2.1	3.5–4.5	1.2	2.5	4.52	2.9	8.5	5.4
Methionine	0.9–1.2	2.5	0.7	0.6–0.8	0.2	1.0	2.87	1.1	0.8	4.7
Ornithine	4.5–6.5	3.7	2.8	5.5–6.5	nd	nd	nd	nd	nd	nd
Phenylalanine	2.5–3.0	3.5	5.1	3.0–4.0	4.1	4.5	4.08	5.5	7.0	3.4
Proline	11.5–12.5	7.9	11.9	10.0–12.0	10.0	10.0	6.18	9.5	3.3	4.4
Sarcosine	udl	0.3	udl	udl	nd	nd	nd	nd	nd	nd
Serine	0.1–0.5	0.2	1.4	0.03–0.6	8.8	9.5	5.48	10.9	4.6	5.9
Taurine	udl	0.5	0.02	udl	nd	nd	nd	nd	nd	nd
Threonine	0.04–0.3	0.2	0.5	0.04–0.4	2.9	3.5	5.86	4.2	2.9	3.3
Tyrosine	1.8–2.2	3.4	3.9	4.0–5.0	2.9	3.0	1.52	1.1	2.3	3.5
Tryptophan	nd	nd	nd	nd	0.1	0.05	1.12	nd	udl	udl

(continued)

Table 5.2 (continued)

Type of HP	Hydrolyzed collagen	Hydrolyzed Collagen	Hydrolyzed keratin	Hydrolyzed keratin	Hydrolyzed keratin	Hydrolyzed keratin	Hydrolyzed collagen	Hydrolyzed keratin	Hydrolyzed hemoglobin	Hydrolyzed collagen
Valine	3.5–4.5	5.4	10.3	7.0–8.5	7.1	7.5	6.84	8.9	8.0	3.0
Total amino acids (g/100 g product)	40.6	50.0	53.0	40.6	17.0	20.0	nd	nd	84.8[a]	33.1
References	SICIT data	SICIT data	SICIT data	SICIT data	Popko et al. 2018	Popko et al. 2018	Lapena et al. 2018	Tejada et al. 2018	Polo et al. 2006	Ertani et al. 2009

udl under detection limit, *nd* not determined

[a] Expressed on product dry weight

Table 5.3 Free amino acid content (g/100 g product) of selected HPs (Source: SICIT data)

Type of HP	Hydrolyzed collagen	Hydrolyzed collagen	Hydrolyzed keratin	Hydrolyzed keratin
Raw material	Bovine hides	Meat meal	Feather	Hair
Type of hydrolysis	Chemical	Chemical	Chemical	Chemical
Alanine	1.0–3.8	0.77	1.57	1.6–6.0
Arginine	0.01–0.03	0.05	0.16	<0.01–0.05
Aspartic acid	0.13–0.8[a]	0.11	0.32	0.2–1.0
Glutamic acid	0.15–2.0	0.02	0.02	0.5–4.5
Glycine	1.8–7.5	0.86	1.01	1.2–3.6
Histidine	0.01–0.06	0.02	udl	0.02–0.2
Hydroxylysine	0.16–0.3	0.10	0.09	0.08–0.2
Hydroxyproline	0.30–1.4	0.11	udl	<0.01–0.1
Isoleucine	0.01–0.07	0.03	0.04	0.04–0.4
Leucine	0.09–0.6	0.09	0.28	0.1–3.3
Lysine	0.07–0.6	0.04	0.02	0.03–0.7
Methionine	0.02–0.15	0.02	0.02	<0.01–0.15
Ornithine	0.15–1.2	0.09	0.09	0.2–1.8
Phenylalanine	0.15–0.6	0.11	0.26	0.1–1.0
Proline	0.4–2.5	0.25	0.58	0.4–3.6
Sarcosine	nd	0.14	nd	nd
Serine	0.03–0.06	0.03	0.19	0.03–0.1
Taurine	nd	0.18	0.05	nd
Threonine	0.01	0.01	udl	udl
Tyrosine	0.05–0.3	0.07	0.21	0.1–1.7
Valine	0.5–0.3	0.06	0.11	0.1–1.6
Sum of free amino acids	5.0–22.3	3.2	5	4.7–30

udl under detection limit, *nd* not determined

[a] Range representing free amino acid content variation due to different hydrolysis parameters

spectrophotometry using o-faldialdehyde (OPA) as a derivatizing agent. The second method is based on the Schiff reaction occurring between the formaldehyde and the free amino groups. Formaldehyde is added to inactivate the free amino groups, thus allowing the subsequent potentiometric titration of the carboxylic groups with sodium hydroxide. By convention, the final titration pH is assumed to be 8.5. It has to be stated that all these methods are based on the hypothesis that all N-terminal amino acids have a similar response factor, but this can lead to inaccuracies in the final data.

Racemization Degree

The analytical methods used for the separation of chiral amino acids include liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC), and capillary electrophoresis (CE). The most common method consists in the separation with the HPLC technique, and this can be done using either a direct or an indirect approach. Indirect separation of a racemic mixture is obtained by derivatization with a chiral agent before analysis, while direct separation can be achieved in two ways: (a) with a chiral additive in the mobile phase by separating in forward or reverse phase or (b) using a chiral stationary phase. The separation of chiral amino acids using CE is currently in a phase of strong development because it offers some advantages over other techniques. These are low injection volume, allowing small solvent consumption; lower costs owing to the fact that it is not necessary to buy very expensive chiral columns since the chiral agent is added to the buffer; and the very high reproducibility of the runs because the chiral agent is restored at each run.

Molecular Weight Distribution

The analysis method for the molecular weight distribution of hydrolyzed protein is a method based on the gel filtration technique, in which the molecules, flowing in a chromatographic column, are separated according to their size, or rather according to their hydrodynamic volume. The higher is the molecular size in solution, the lower is the retention time of the analyte. Usually, gel filtration is applied to the determination of the molecular weight distribution (MWD) of synthetic polymers, but it found also an application in the analysis of protein hydrolysates.

Determination of Free and Total Amino Acids

The method involves the extraction of free amino acids with 0.1 N hydrochloric acid, while total amino acids are obtained after hydrolysis with 6 N hydrochloric acid containing an antioxidant agent. The quantification phase can be done using HPLC equipped with a fluorescence detector on a reversed-phase column through a pre-column derivatization with o-phthaldialdehyde (OPA) for the primary amino acids and 9-fluorenylmethylchloroformate (FMOC) for the secondary amino acids. The amino acids can alternatively be quantified by ion exchange chromatography and determined after postcolumn derivatization with ninhydrin, using photometric detection at 570 nm, and at 440 nm for proline and hydroxyproline. Both these procedures give satisfactory results, but for an accurate investigation, the degradation of some amino acids must be taken into account.

Determination of Nitrogen

Total nitrogen can be determined by dry combustion or using the Kjeldahl method. Dry combustion was originally developed as a manual method by Dumas. Its application has been significantly improved through the use of automated instrumentation and is applicable to all forms of nitrogen. The method is based on the complete combustion of the sample in the presence of oxygen. In order to obtain a complete oxidation, the sample is transported by a helium flow through a catalyst. The nitrogen oxides that are formed pass into a second reduction column, obtaining nitrogen and water. The nitrogen is separated in a chromatographic column and detected with a conductivity detector. The Kjeldahl method consists in the determination of total nitrogen intended as the sum: ammoniacal nitrogen plus organic nitrogen. With this method, the nitrogen contributions due to inorganic components (nitrates, nitrites) and the organic nitrogen contributions of heterocyclic compounds (compounds containing N–N or N–O bonds and hydrazine and hydroxylamine) are not determined. Nitrogen is transformed into ammonium ions with the use of concentrated sulfuric acid. The use of a catalyzing agent increases the boiling point of the acid and accelerates the process. The mixture obtained with digestion is alkalinized with sodium hydroxide before distillation to release ammonia. Ammonia is steam-distilled in a receiving solution of boric acid. The pH of the target acid solution increases with the addition of ammonia. The nitrogen and protein content is then determined by titration of the boric solution.

Determination of Carbon

Total carbon can be determined by dry combustion or using the Springer–Klee method. The dry combustion method is based on the complete combustion of the sample in the presence of oxygen. In order to obtain a complete oxidation, the sample is transported by a helium flow through a catalyst. The nitrogen oxides that are formed pass into a second reduction column obtaining nitrogen, carbon dioxide, and water. Carbon dioxide is separated in a chromatographic column and detected with an IR detector. In order to obtain the organic carbon value, the analysis is repeated by pretreating the sample in the muffle at 550 °C. The Springer–Klee method is based on the oxidation of organic carbon to carbon dioxide with a solution of potassium dichromate in the presence of sulfuric acid. The unreacted dichromate is quantified by oxidimetric titration with ferrous sulfate. The reaction between organic carbon and dichromate gets quantitative by heating the mixture to 160 °C. The presence of higher manganese oxides, ferrous compounds, and chlorides can have an effect on the accuracy of the analysis results.

Determination of Micro- and Macronutrients

The nutrient elements are determined using the ICP-OES technique, after acid-hydrogen peroxide or perchloric acid mineralization in microwave. To compensate for the matrix effect, an internal standard based on yttrium is added. The reference methods are EPA3015a and EPA6010b.

5.2 The Effects of Animal-Derived Hydrolyzed Protein on Crop Performance

5.2.1 Plant Growth, Development, and Quality Traits

The life cycle of a plant begins with germination, which represents the onset of the vegetative growth. In the initial phase, the growth of the plantlet relies on mobilization of stored reserves and after the emergence from the soil surface on photosynthetic activity. The vegetative growth of a plant that is typically indeterminate and characterized by reiterated formation of lateral organs is the result of both cell division, cell expansion, and differentiation processes. The juvenile stage of the life cycle is followed by the development of the reproductive organs; the transition from the vegetative to the reproductive phase is regulated by internal factors as well as environmental cues such as temperature and photoperiod. Although plant growth and development are genetically determined processes, they are characterized by an accentuated plasticity and can be modulated by several external factors. In fact, plants being sessile organisms are extremely susceptible to environmental changes and possess a sophisticated system to perceive and respond to chemical and physical stimuli.

Several natural substances defined as biostimulants can favorably affect plant growth and development when supplied either by foliar application or soil drenching (Calvo et al. 2014; Colla et al. 2015; du Jardin 2015; Yakhin et al. 2017). Biostimulants, according to the definition of the European Union, exert their positive effects on plant performance independently from the product's nutrient content (The European Parliament and the Council of the European Union 2019). Therefore, a biostimulant does not act as a fertilizer but rather as an agent able to stimulate nutrient availability and acquisition, tolerance to abiotic stress, and consequently plant growth.

There are many examples in the scientific literature of the growth stimulation capacity of animal derived hydrolyzed protein obtained from various raw materials such as animal feathers, meat, bovine hairs, and epithelial tissues and in general collagen-derived material, erythrocytes, and fish by-products (Colla et al. 2017; Callegaro et al. 2019; Madende and Hayes 2020). Depending on the raw material and type of industrial process applied, the HP can contain mineral and organic component in various amounts; however, the common characteristic is the high concentration of organic N in the form of free amino acids and peptides. To point out the effects of HPs on plant growth and development, it is therefore crucial to exclude that such effects can be caused by a fertilizing action of the product assuring that the HP is utilized at very low doses and that its effects are not justified simply by a nutritional effect. Another important issue when monitoring the effects of a HP as a biostimulant is the choice a proper negative control. Comparing the growth of plants treated with the HP with that of plants not receiving any treatment can be sometimes misleading; an alternative way could be to treat the control plants with N supplied in an inorganic form (NH_4^+ or NO_3^-) at the same total amount present in the HP (Santi et al. 2017).

On these premises, we have based this survey on data presented in manuscripts selected from the recent scientific literature that report the effects of low doses of animal derived HPs and provide a detailed description of the product (e.g., composition and total N content) and the methods used in the experiments carried out either in controlled conditions or in open field.

In many works reporting experiments done under laboratory conditions, the plants are grown hydroponically in a nutrient solution supplemented with the HP. The hydroponic culture is particularly useful for testing the effects of the product on the first stages of plant growth after germination, paying particular attention to the changes in the root apparatus. Such systems generally assure a good reproducibility of the results due to the precise control on the dose of the HP and on the other components of the nutrient solution (Ambrosini et al. 2021).

The effects of low doses of HP produced from bovine hides or in general animal connective tissue on maize seedling grown under hydroponically condition are documented in several papers (Ertani et al. 2009, 2013, 2018; Santi et al. 2017). In such studies, the most pronounced stimulatory effects of the HPs were observed in the root (Ertani et al. 2009, 2013, 2018; Santi et al. 2017; Ambrosini et al. 2021), although in some experiments also an increase in shoot biomass was reported but to a lesser extent (Ertani et al. 2009, 2013). The promotion of growth appeared in some cases independent from a direct nutritional effects of the HP since the total N concentration in the plant tissues did not show significant differences when compared with those of control plants (Ertani et al. 2013; Santi et al. 2017). Interestingly, root morphological changes that include an increased development of lateral roots compared to the primary and seminal roots and a stimulatory effects on root hairs were also observed in maize as a consequence of the HP treatment (Ertani et al. 2013; Santi et al. 2017). The changes in root architecture caused by low HP doses (total N in the order of few mg/L) suggest a signaling effect that might be ascribed to the peptide component (Santi et al. 2017). It is well known that endogenous signaling peptides exert a regulatory role on root growth and architecture (Lay and Takahashi 2018; Chapman et al. 2020; Wang et al. 2020). The specific role of small peptides compared with free amino acids, the other important bioactive component of HPs, has been explored by Santi et al. (Santi et al. 2017). In this work, the effects on maize seedlings of a bovine collagen-derived HP were compared with those caused by a mixture of free amino acid mimicking the composition of the HP and containing the same total N amount. The HP displayed a higher promoting effect on root growth compared with the amino acid mixture and also with a nutrient solution containing the same amount of total N supplied as $NH_4H_2PO_4$ (Santi et al. 2017).

Overall, this type of experiments has clearly demonstrated that the application of HPs promotes the root biomass and in particular the production of lateral root and root hairs, enhancing the root capacity to explore the soil solution and therefore favoring the uptake of mineral nutrient during the first stages of seedling growth that can represent an advantage for the plant to emerge quickly from the soil.

Positive effects of drench application of HPs on shoot biomass were also observed, as is the case of lettuce plants treated with a fish-derived HP (Xu and Mou 2017). The plants were grown in a climatic chamber in pots filled with sandy loam soil and supplemented with a nutrient solution containing a low dose of the HP (total N in the order of tens of milligrams) for three times during the cultivation period. Lettuce plants treated with the HP showed an increased leaf number per plant, increased stem diameter, and shoot fresh and dry biomass. The treatment also enhanced the leaf relative water content and succulence without changes in specific leaf area (Xu and Mou 2017).

An interesting application for improving plant growth is the use of gelatin capsules placed near the seeds at the time of sowing (Wilson et al. 2015, 2018). Gelatin is a mixture of amino acids and peptides obtained from the hydrolysis of collagen-containing animal tissues. Hard gelatin capsules represent a novel way to deliver the biostimulant to the seeds for greenhouse production. The application of two gelatin capsules each containing 7.1 mg of N increased the shoot growth of six crops: cucumber, pepper, broccoli, tomato, arugula, and field corn (Wilson et al. 2018). The seeds were not embedded in the gelatin, but the capsules were placed adjacent to the seed in order to avoid inhibition of germination. After 28 days of greenhouse cultivation, all the crops exhibited significant increases in shoot dry weight and total leaf area compared to plants obtained from nontreated seeds. In cucumber, the effects of gelatin capsules were compared with those produced by other types of hydrolyzed collagen as well as urea and amino acid mixtures that simulated the same composition as gelatin, providing in all the treatment equal amounts of N. The biomass (dry weight) of cucumber plants treated with hydrolyzed collagen was higher as compared with that plants treated with urea or amino acids.

Animal-derived HPs are effective also in favoring the reproductive organs as illustrated in a study carried out on two cultivars of an ornamental plant, snapdragon. The plants were cultivated in pots under greenhouse conditions and treated with different doses of a product obtained by the enzymatic hydrolysis of proteins from erythrocytes (Cristiano et al. 2018). The HP was applied weekly as either foliar spray and root drenching treatments starting 45 days after transplanting until flower bud differentiation. In both cultivars, the treatments increased the number of flowers and the dry weight of flowers per plant besides stimulating the growth of leaf and stems.

Several studies carried out under field conditions demonstrated that the beneficial action of animal-derived HP is not restricted to plants grown in climatic chamber or under greenhouse conditions. Tejada et al. (2018) studied the effects of an HP obtained by enzymatic hydrolysis from chicken feathers and containing 15.7 ± 1.9 g kg^{-1} of N on maize yield over two consecutive seasons at two total doses 10.8 L ha^{-1} and 21.6 L ha^{-1}, respectively. The plants were fertilized with N and P following the common cultivation practice for the area; the controls did not receive the HP treatment. Foliar application significantly increased grain protein content and yield by 26% and 14%, respectively, at the higher dose for both seasons. Treated plants also exhibited an increase concentration of macro- and micronutrients.

Another interesting open field study investigated the beneficial effects of animal-derived HPs on tomato fruit yield (Polo and Mata 2018). This work describes the results of a field experiment carried out on gold cherry tomato cultivated under low stress condition. The plants were treated with Pepton obtained by the enzymatic hydrolysis of animal proteins and containing more than 16% of free amino acids and peptides with an average molecular weight around 2000–3000 Da. The HP was applied starting at 15 days after transplant and every 2 weeks thereafter for a total of 5 applications, two by foliar spray and three by irrigation at different application rates (2, 3, or 4 kg/ha). All groups of plants (control and Pepton-treated) received a regular fertilization program for tomato, and the contribution of Pepton application in terms of total nitrogen was very limited compared with the normal fertilization program. The peptone treatments promoted vegetative growth and also fruit development; both fruit diameter and yield presented a linear increase with increased application doses of Pepton. The authors estimated that for the highest application dose the tomato yield was increased by 27% as compared with the control treatment.

Few studies have evaluated the effects of animal-derived HPs on product quality after long-term application. Maize plants grown under field conditions and treated by foliar spraying with an HP obtained from chicken feathers showed a higher concentration of macro- and micronutrients, as well as higher level of proteins in both leaves and grains compared to untreated plants (Tejada et al. 2018). Improvements in the nutritional and nutraceutical quality of bean seeds were observed in a field experiment conducted on a Mexican Black cultivar of *Phaseolus vulgaris* L. after foliar application of an amino acid-based biostimulant containing 20% free amino acids, 5% organic N, and microelements (Kocira et al. 2020). The application increased the protein and fiber content and improved the nutritional value of bean seeds by stimulating the synthesis of antioxidant compounds (Kocira et al. 2020). In another open field study, *Vitis vinifera* L. cv. Corvina vines were sprayed with a casein hydrolysate containing 0.60% w/w of organic N three times every 10 days from fruit set until bunch closure. The application of HPs improved plant performance (i.e., yield, cluster weight, and number per vine) and berry quality (Boselli et al. 2019). The treatment significantly increased total soluble solid and anthocyanin content in the berries (Boselli et al. 2019). A positive effect of HP application on the nutritional value of the fruits was also observed in *Malus domestica* cv. Annurca (Graziani et al. 2020). A peptone- based product containing 16% of free amino acids, 85% of total amino acids, and 13% (w/w) of total N was applied to the leaves starting from fruit set every 10 days at a dose of 150 g/50 L. The product had a positive effect on the quality of Annurca fruits, inducing a significant increase in the content of total polyphenols in the flesh. The effects of HPs on secondary metabolism were also observed in maize leaves after 15 days of treatment with an HP obtained from bovine epithelial tissues (Ertani et al. 2018). The treated leaves contained higher amounts of some phenolic acids such as caffeic and coumaric acids compared to the control leaves (Ertani et al. 2018).

The exemplificative works described demonstrated that HPs derived from diverse animal tissues possess the capacity to stimulate both vegetative and reproductive growth in a wide range of crops, also under unstressed conditions. Furthermore, it seems that the method of application and the dosage of the HP can be adjusted in dependence of the effects to be obtained on different organs of the plant. Particularly interesting is the possibility to alter by HP application, the morphology of an organ (e.g., roots). This aspect deserves in the future further study in order to use animal-derived HPs to shape the adaptability of a crop to environmental changes (e.g., soil water shortage).

5.2.2 Response to Stresses

Plants must often face adverse environmental conditions such as drought, floods, high or low temperatures, nutrient shortages or imbalances, and high light intensity radiation that may result in crop yield loss (Wang et al. 2004; Wani et al. 2016). These abiotic stresses are on the rise, worsened by climate changes (Boyer 1982; Rahmstorf and Coumou 2011; Rosenzweig et al. 2014), and it is crucial to have at our disposal environment-friendly tools, such as plant biostimulants, to improve crop performances, especially for stress resilience (Suhag 2016).

Moreover, it is important to recollect that improving abiotic stress tolerance is one of the characteristics that the European Union identifies as mandatory to recognize a certain product as biostimulant (The European Parliament and the Council of the European Union 2019), and is therefore crucial not only to assess a product's efficacy, but also its modes of action, or better, mechanisms of action.

Although there is a vast literature on HPs, just few works focus on animal-derived ones, and even a smaller number focus on their capacity to improve stress tolerance in crop plants. In this paragraph is discussed the role of animal-derived HPs as general abiotic stress mitigators, with the exception of the nutrient deficiency case, which will be included in Sect. 2.3. A very recent work from Ambrosini et al. (Ambrosini et al. 2021) assessed the capacity of a collagen-derived HP to mitigate drought and hypoxic stress in maize plants (Ambrosini et al. 2021). Plants were grown hydroponically and treated either with the HP or with inorganic nitrogen, the former chosen as a proper control to balance the extra nitrogen added with the HP to the solution. In order to mimic hypoxic stress, plants were grown in vases that were not provided with tubes to insufflate air in the solution. While roots treated with inorganic nitrogen grew shorter in the air-depleted vases than those grown with air, the HP treatment was sufficient to overcome hypoxic stress; in fact, plants grown hypoxically with the HP maintained root growth levels comparable to those of the control plants grown under normal oxygen regimen treated with inorganic nitrogen. The same product was also able to mitigate drought stress, simulated adding 15% w/w PEG (polyethylene glycol) to the nutrient solution, which conversely to drought stress leads plants to grow longer roots, covering a wider absorption area (Ji et al. 2014). The HP application further increased root length and surface area of PEG-treated plants compared to those stressed but treated with inorganic nitrogen, therefore providing evidence for the assumption that the water stress did not alter the positive effects of the HP, and that during prolonged drought periods plants HP-treated could better acquire water and nutrients compared to the control ones. The mode of action of this HP was elucidated at a transcriptomic level by Santi et al., who found that its drench application in similar hydroponic conditions to maize plants caused an altered expression of genes involved in metabolism, transport, and signal transduction of gibberellin, cytokinins, and auxin, highlighting capacity to perturb hormone activity (Santi et al. 2017), which can be ascribed responsible for the plant-improved responses to stresses (Ambrosini et al. 2021).

Another HP, obtained from trimmings and shavings of bovine hides through thermobaric hydrolysis, was found to be effective in maize seedlings and in grapevine as a multistress mitigator for the former (Trevisan et al. 2019) and as a drought mitigator for the latter (Meggio et al. 2020). In the work of Trevisan et al. (2019), this HP was tested to enhance tolerance to hypoxia, salt stress, nutrient deficiency, and combinations of the three. Comparing the stressed plants treated with the HP to those that did not receive it, the authors observed that the former had longer roots in all cases; however, for what concerns hypoxic and salt stress, the molecular mechanisms underlying the improved responses for these two stresses are yet to be clarified (Trevisan et al. 2019). A different study on the same product (Meggio et al. 2020) reported a beneficial effect of the drench treatment on grapevines subjected to water stress, which had a wider leaf area, higher SPAD values, an increased shoot biomass, and bigger berries compared to stressed control plants (Meggio et al. 2020).

Another example of HP applied to mitigate water stress in grapevine comes from Boselli et al. (Boselli et al. 2019), who exploited a dairy mix-based casein HP. This HP, when sprayed to grapevine leaves, significantly increased the yield and reduced conductance index IG in treated vines, thus ameliorating the tolerance to water stress reducing stomatal conductance and hence the transpiration demand (Del Campo et al. 2004).

The two last works that will be mentioned refer to an HP used to mitigate water stress and nutrient deficiency stress in tomato plants (Casadesús et al. 2019, 2020). It is particularly interesting since the authors suggested that the HP might have a hormonal-like effect on treated plants, and collected evidence to support their hypothesis detecting higher levels of indole-3-acetic acid, jasmonate, and cytokinins and gibberellins in their active forms to counteract water stress (Casadesús et al. 2019) or salicylic acid to respond to cold and nutrient deficiency stress (Casadesús et al. 2020).

5.2.3 Nutrient Uptake and Assimilation

The effects on the uptake and assimilation of macronutrient and micronutrient by plant in response to the treatment with animal-derived HPs were evaluated in several works with a vast heterogeneity in the experimental conditions and in the aspects on plant nutrition focused on. The main difficulty to analyze and identify the effects of these biostimulants on plant nutrition is linked to the dose of treatment and the mode of application. In fact, in literature we can find examples of the application of HP as fertilizers compared to commonly used inorganic fertilizers (Popko et al. 2015; Testani et al. 2017; Vita et al. 2018). In particular, the soil application of protein hydrolysate of keratin increased the content of some macronutrients (N, P, K, and Mg) and micronutrients (Mn and Zn) in rapeseeds grown in pots (Popko et al. 2015). In the case of two durum-wheat genotypes in field (Vita et al. 2018), the effects of the fertilization with two different HPs were analyzed at level of changes in protein and transcript levels in leaf tissues, with particular attention to processes involved into N uptake and assimilation. The study underlined that the effects on gene expression of HPs were genotype-dependent in particular when it was considered the processes linked to nitrate uptake (NO_3^-, low-affinity nitrate transporters, *NRT1.2*) and N assimilation (glutamate synthase, GLU) (Vita et al. 2018). In the case of fertilization of sweet pepper with HPs, no significant differences in N content in plant tissues were observed in comparison to non-treated plants (Testani et al. 2017). The foliar fertilization of maize (10.8 and 21.6 L ha^{-1}) with a biostimulant obtained by the enzymatic hydrolysis of chicken feathers increased the N in leaves and grain in comparison to control plants fertilized only with conventional NPK (Tejada et al. 2018). A similar trend was observed in two subsequent seasons. Furthermore, the same HP treatment, in particular at the second dose, increased the content in leaf and grain of the other macro- (P, K, S, Ca, and Mg) and micronutrients (Fe, Cu, Mn, Zn, and Ni) in both seasons (Tejada et al. 2018).

Moving to the application of HPs as a true biostimulant (according to the definition of Regulation EU 2019/1009: “certain substances, mixtures and micro-organisms, referred to as plant biostimulants, are not as such inputs of nutrients, but nevertheless stimulate plants’ natural nutrition processes”), the effects on plant nutrition were evaluated mainly in consideration of N uptake and assimilation also exploring sometimes the involved molecular mechanisms (Mladenova et al. 1998; Ertani et al. 2009, 2013; Santi et al. 2017; Trevisan et al. 2017, 2019; Meggio et al. 2020). The effect of leaf treatment (7 g L^{-1}) with an animal-derived organic biostimulant on maize plants concerning the biochemical aspects of NO_3^- assimilation underlined that this product improved the plant responses under salt stress in terms of NO_3^- assimilation rates in roots and activity of nitrate reductase (NR) in leaves (Mladenova et al. 1998). In addition, the biostimulant increased the total activity of glutamate dehydrogenase (GDH) in leaf tissues putatively involved in the ammonium (NH_4^+) reassimilation under stress conditions (Mladenova et al. 1998). Further investigations were carried out in maize concerning the effects on N metabolism of different HPs. The treatment of maize seedlings in hydroponics (24, 240, and 2400 μg C L^{-1}) with a biostimulant produced by enzymatic hydrolysis of animal-connective tissue increased the activity in root and leaves of enzymes linked to N metabolism as nitrate reductase (NR) and glutamine synthase (GS) (Ertani et al. 2009). In particular, in leaves, it was observed a higher level of the plastidial GS2 isoform that seems to be involved into the assimilation of ammonia (NH_4^+) produced by the reduction of NO_3^- (Ertani et al. 2009). The effects of the same product on maize plants hydroponically grown (0.01 and 0.1 mL L^{-1} of biostimulant) were analyzed in depth, studying also the changes at transcriptional levels of genes coding for enzymes playing a role in N assimilation and the tricarboxylic acid cycle (TCA) (Ertani et al. 2013). The treatment did not cause differences in N total concentration in plant tissues. Anyway, an increase in protein and a decrease in NO_3^-, PO_4^{3-}, and SO_3^{2-} concentration respectively was recorded in response to the biostimulant application both in roots and leaves (Ertani et al. 2013). The same authors justified the decrease of these ions with their faster metabolic conversion by the enzymatic activities involved in N (NR; nitrite reductase, NiR; GS; GOGAT) and C metabolism (malate dehydrogenase, MDH; isocitrate dehydrogenase, IDH; citrate synthase, CS) supported by the enhanced transcriptional levels of the corresponding genes (Ertani et al. 2013). Focusing on genes encoding the components involved in the uptake of different N forms, it was reported that the soil application of an HP derived by the hydrolysis of bovine hides previously tanned with wet-blue technology (11.76 and 23.52 mg *per* pot) caused a positive modulation in lateral roots of transcripts encoding the high-affinity NO_3^- transporters (*ZmNRT2.2*, *GRMZM2G010251_T01*; *ZmNRT2.3*, *GRMZM2G163866_T01*) and of the ZmNRT3.1A gene (*GRMZM2G179294_T01*) (Trevisan et al. 2017), which is believed to be essential for the functional activity of NO_3^- high-affinity transport system (HATS) (Nacry et al. 2013). The effects on putative NO_3^- low-affinity transporter genes belonging to the PTR family are not so clear with members positively and other negatively affected by the treatment (Trevisan et al. 2017). These results suggested a positive stimulation of the high-affinity NO_3^- HATS that can justify the observed increased in total N and protein concentration in root and in particular in leaves of plants of another maize genotype grown in pot in the presence of two different quantities (2.1 and 4.2 mg/kg N) of the same biostimulant (Ertani et al. 2018). The root maize transcriptional response to the same biostimulant (applied in dose equal to 5% of total N of the nutrient solution) was further investigated focusing on the genes encoding the components of both the HATS and low-affinity NO_3^- transport system (LATS) when plants were subjected in hydroponics to single and combined stresses as hypoxic condition, salt, and nutritional stresses (Trevisan et al. 2019). The treatment with this HP mainly affected expression of genes involved in the HATS in responses to the abovementioned stresses (Trevisan et al. 2019). The authors justified the results on the basis of the major involvement of the HATS in influencing the N use efficiency (NUE) under limiting condition (Trevisan et al. 2019). Different results were obtained with another HP produced by the hydrolysis of cow connective tissue on the expression of genes involved in the uptake of inorganic N forms in maize roots (Santi et al. 2017). In this work, the effects of the HP on root transcriptome were compared to those obtained with the treatment in hydroponics of the same N dose (11.3 mgN L^{-1}) applied as reduced inorganic N form (NH_4^+) and as a mixture of free amino acids identical in composition and concentration to the amino acids present in the HP. Only transcripts involved in LATS were modulated by HP if compared to NH_4^+ treatment. In particular, *ZmNRT1.2* (*GRMZM2G137421_T01*) was downregulated whilst the *ZmNRT1.4B* (*GRMZM2G476069_T01*) was specifically induced by the HP (Santi et al. 2017). The same transcriptional comparison underlined a repression of a putative NH_4^+ transporter (AMT2; *GRMZM2G335218_T01*). The transcriptional behavior of these genes is in line with the total N concentration in roots showing no significant differences between the treatments (Santi et al. 2017). The observed differences on the expression of maize genes involved in root in N uptake could be ascribed to the differences in experimental conditions (e.g., maize genotypes, dose, hydroponics vs. pots, control conditions, time of the treatment, and stress conditions). Anyway, the modulation of gene expression agrees with the results concerning N concentration in plant tissues recorded in responses to application of these different HPs (Santi et al. 2017; Trevisan et al. 2017, 2019; Ertani et al. 2018). Beyond the molecular aspects of the N metabolism, the treatment with different HP can modify the N content in tissues of different plant species. It was in fact observed an increase in N concentration in roots and shoots of snapdragon plants when leaves were treated with two doses of an HP (0.1 and 0.2 g L^{-1}) produced by enzymatic hydrolysis of proteins from erythrocytes in comparison to non-treated plants (Cristiano et al. 2018). Moreover, an increase in total N amount in cucumber plants was observed when seeds were treated with hydrolyzed collagen compared with the low-molecular-weight gelatin hydrolysate, a mixture of amino acids or urea supplied in equal amount (Wilson et al. 2018).

As far as the effects HPs on the processes involved in micronutrient acquisition by plant roots are concerned, Fe was the most investigated element. The root treatment (0.1 mL L^{-1} and 0.2 mL L^{-1}) in hydroponics of tomato plants with a product of amino acids from animal origin increased the activity of the Fe^{III}-chelate reductase (FCR) of roots only on lime nutrient solution without any differences in leaf Fe content (Cerdán et al. 2013). The foliar application of the same product (0.5 mL L^{-1} and 0.7 mL L^{-1}) positively and negatively affected the leaf FCR activity under normal and lime nutrient solutions, respectively, whilst no significant differences in this enzymatic activity in roots were observed in both nutritional conditions (Cerdán et al. 2013). In addition, the treatments slightly decreased the leaf Fe content (Cerdán et al. 2013). Anyway, the treatment with different HPs of maize plants grown both in hydroponics (Santi et al. 2017) and soil (Trevisan et al. 2017) caused changes in the expression of *ZmYS1* (GRMZM2G156599_T01) involved in the uptake of complexes between phytosiderophores (PS) and metals as Fe (Schaaf et al. 2003). In addition, the first work (Santi et al. 2017) underlined a positive modulation due to HP of a transcript encoding a nicotianamine aminotransferase (NAAT; *GRMZM2G096958_T01*) and involved in PS biosynthesis (Jin et al. 2015) that correlates with the increased concentration in response to the same treatment of metals as Cu, Mn, and Zn in roots. The authors observed only a tendency to increase in response to the HP application for the Fe concentration that was supplied in a very available form, as complex with an inorganic chelator. In the second work (Trevisan et al. 2017), it was observed a general positive regulation of genes encoding the nicotianamina synthase playing a role in the production of the precursor of the PS, the nicotianamine (Zhou et al. 2013). Altogether, these transcriptional analyses suggested that the HP can improve the acquisition of metal micronutrients positively affecting the synthesis and release of natural chelating agents, the PS, a process at the basis of strategy II of Fe acquisition typical of gramineous species (Kobayashi and Nishizawa 2012). Recently, experiments carried out in hydroponics with maize using the HP produced by the hydrolysis of cow connective tissue previously studied by Santi et al. (Santi et al. 2017) showed that this biostimulant mixed to $FeCl_3$ confers a faster recovery from deprivation as compared with treatments with $FeCl_3$ or FeEDTA as suggested by higher leaf SPAD index and Fe content (Ambrosini et al. 2021). These effects agreed with modulation of transcripts involved into Fe acquisition in roots such as *ZmTOM1* and *ZmIRT1* observed in response to the treatment with this HP. Interestingly, the same authors reported that this HP can interact with Fe by circular dichroism analysis hypothesizing a role as Fe chelating agents for this biostimulant (Ambrosini et al. 2021).

As already described, the treatment with HP is able to improve not only the content of N and Fe, but also that of other macro- and micronutrients in maize. In particular, the application in field for two seasons of two commercial products consisting in highly concentrated mixture of amino acids and short peptides derived from the hydrolysis of feathers (1 L ha^{-1} for the first product and 1 and 1.25 L ha^{-1} for the second one) increased the yield and the content in Cu, Na, Ca, and Mo in grain in comparison to non-treated samples (Popko et al. 2018). An increase in the content of Mg and some micronutrients (Cu, Fe, and Mn) content both in roots and shoots of maize seedlings grown and treated with an HP derived by animal-connective tissue in hydroponics was reported (Ertani et al. 2013). Similar results were obtained for maize roots when seedlings were treated in hydroponics with another HP (Santi et al. 2017). In this work, as previously mentioned, it was reported an increase in content of K, Cu, Mn, and Zn. It can be assumed that the HPs, despite being applied in some experiments in very low doses, can supply additional quantities of nutrients present in their formulation (Popko et al. 2018). Anyway, the results of transcriptional analyses carried out in roots (Santi et al. 2017; Trevisan et al. 2017) suggested the these products can improve the acquisition of nutrient-modulating genes involved in their acquisition by roots from the soil. Besides the modulation of genes involved in strategy II Fe acquisition previously discussed, the two works evidenced that the HP can modulate the expression of transcripts involved in the transport of macro- and micronutrients (Santi et al. 2017; Trevisan et al. 2017).

5.2.4 Impact on Soil Properties

The literature concerning the impact of biostimulants on the soil environment is, in general, scarce and, narrowing to animal-derived HPs, a minimal number of papers deal with this subject. In addition, research on this topic has been carried out using amounts of HP (in the range of tons per hectare) more compatible with their use as nutrients than as biostimulants (kilos per hectare).

The direct application of HPs to the soil entails the evaluation that their use does not cause negative effect on this fragile environment. With a multidisciplinary laboratory approach, Corte et al. (Corte et al. 2014) studied the safety and efficacy as nitrogen (N)-fertilizer of seven HP representative of different raw materials and processes (chemical or enzymatic hydrolysis). They did not find negative effects on soil microbiota, yeast, and plants, concluding that HP can be safely used in conventional and organic farming. Furthermore, based on mineralization tests carried out in laboratory conditions, the authors observed a nutritional effect on soil microorganisms at relatively high concentrations and the stimulating effect on carbon cycling at lower application rates. The importance of N mineralization is related to the general assumption that in the most spread agricultural ecosystems plants take up mainly NH_4^+ and NO_3^-, rather than amino acids or other N organic forms (Marschner 1995), which apparently only play a role in extremely N-poor and cold ecosystems where N mineralization from soil organic matter is very limited (Chapin et al. 1993). On the other hand, it has been ascertained that plants can also uptake organic N forms (Näsholm et al. 2009) and indeed several plant amino acid and peptide transporters have been identified in heterologous systems but their functions in planta have not yet been characterized (Nacry et al. 2013).

It has been shown that in the soil the rate of mineralization of protein, peptides, and amino acids is inversely related to the molecular weight with no significant difference between the two latter compounds (Jones and Kielland 2012). Wilkinson et al. (2014), analyzing the mineralization of amino acids and peptides in four grassland soils, showed that the removal of amino acids and peptides from soil solution by the microbial community can be extremely rapid (in the span of few minutes). Moreover, only in one soil the mineralization of alanine was faster than that of trialanine. The authors concluded that peptides can be taken up directly by microbes via peptide transporters rather than being cleaved externally by extracellular enzymes, and then transported into cells as free amino acids. This supports the view that short-chain length peptides can be a significant source of N and C for microbes (Farrell et al. 2011).

In an attempt to evaluate the N mineralization dynamic of liquid feather hydrolysates obtained by hydrothermal treatment, Nurdiawati et al. (2019) found that the soil type strongly influenced the net N mineralization more than the fertilizer treatments; the net N mineralization for the two hydrolysate-amended soils was in the range of 34–51% after 30 day of incubation. The temperature of hydrothermal treatment seemed to exert an influence on the rate of mineralization as the product obtained at higher temperature—possibly containing shorter peptides – mineralized faster than ones produced by a lower-temperature treatment. On the other hand, this was true only for one kind of soil, leaving open the possibility that the hydrolysis done at the highest temperature could determine the production of microbial toxic compounds. Biostimulants can also be applied to soil for restoration purposes, a matter that is of particular interest in semiarid regions. Many degraded soils are often characterized by low organic matter content and low microbial activity, which eventually hampers the establishment of plant cover. Tejada et al. (2011) applied to a Xerollic Calciorthid soil four biostimulants, among which one was obtained from poultry feathers after hydrolyses. The biostimulants were applied annually at 4.7 t organic matter (OM) ha^{-1} for a 3-year period to evaluate their efficiency in soil restoration. The application of biostimulants had a positive effect on soil biological properties measured as enzyme activities and caused a change in the soil microbial community. Furthermore, soil enzyme activities and bacterial and fungal biomass were highest in soil amended with the biostimulants with a higher percentage of peptides under 0.3 kDa (>60%)—as those obtained from poultry feathers—possibly because the compounds with lower molecular weight can be more easily absorbed by soil microorganisms.

5.2.5 Action Mechanisms of Hydrolyzed Protein: Experimental Evidences and Hypotheses

Free amino acids and small peptides, the bioactive components of animal-derived HPs, represent the organic form of N that plants can absorb from the external medium and then distribute to different organs through the activity of amino acid and peptide transporter proteins (Yang et al. 2020). Amino acids and peptides provide the building blocks for organic matter, but they also act as signaling molecules in developmental processes and stress responses. A well-known example of an amino acid with signaling activity is glutamate, which plays a signaling role in a variety of processes such as seed germination, root development, and pollen germination when it interacts with its specific receptors (Qiu et al. 2020). In recent years, the role of peptides as hormonal signaling molecules in plants has been intensively studied, leading to the identification of several families of extracellular short peptides and peptide receptors involved in cell-to-cell communication and long-distance signaling (Ghorbani 2014; Breiden and Simon 2016; Takahashi and Shinozaki 2019). These signaling peptides have been shown to regulate numerous developmental processes including shoot and root growth and architecture, reproduction, and responses to abiotic stresses (Hirakawa and Sawa 2019; Hsiao and Yamada 2021). Considering that animal-derived HPs exhibit biostimulatory activity independent of their nutritional effects, we can speculate that in plant cells they may interfere with amino acid and peptide signaling. In the case of the free amino acid component of the HPs, they could affect cellular amino acid homeostasis and consequently downstream signaling transduction and biosynthetic pathways. Regarding the possible effects of the peptide component of animal derived HP, several considerations can be made. The profile of the short peptides present in animal-derived HPs depends largely on the protein composition of the original raw material (e.g., collagen, keratin). It has yet to be studied whether some of these peptides show similarities in terms of amino acid sequence or secondary structure to the endogenous ones. HP peptides could compete for the binding site of some receptors with the endogenous ones, therefore inhibiting or activating the receptor in a deregulated manner. In this regard, it has been shown that a number of short synthetically produced peptides of random sequence when expressed individually in *Arabidopsis thaliana* were able to affect several physiological processes such as photosynthesis and flowering (Bao et al. 2017). Recently, it has been shown that plant regulatory peptides can also be synthesized from microRNA (miRNA-encoded peptides) genes and that they regulate the abundance of miRNA transcripts (Couzigou et al. 2015). miRNAs are small single-stranded noncoding RNAs that regulate gene expression by acting as key regulators of a variety of developmental processes. Thus, it would be interesting to evaluate whether HP-derived peptides might influence the expression of plant miRNAs. Together with these intriguing hypotheses, there might be a more general and undifferentiated plant response to take into account when exogenous amino acids and peptides are provided by the application of animal-derived HPs. Unfortunately, to date, these are just speculations, and more studies are needed to elucidate what has been proposed so far.

5.3 Conclusions

The HPs are one of the most ancient products used as biostimulants in agriculture. Notwithstanding the diversity of the matrices of raw materials, processes, and hence the final composition, the analysis of scientific literature allows to identify some common effects shared among the products independently from the plant species and experimental conditions. These effects are summarized in Fig. 5.1. Above all, the most striking effect often recorded as result of HP application is an increased

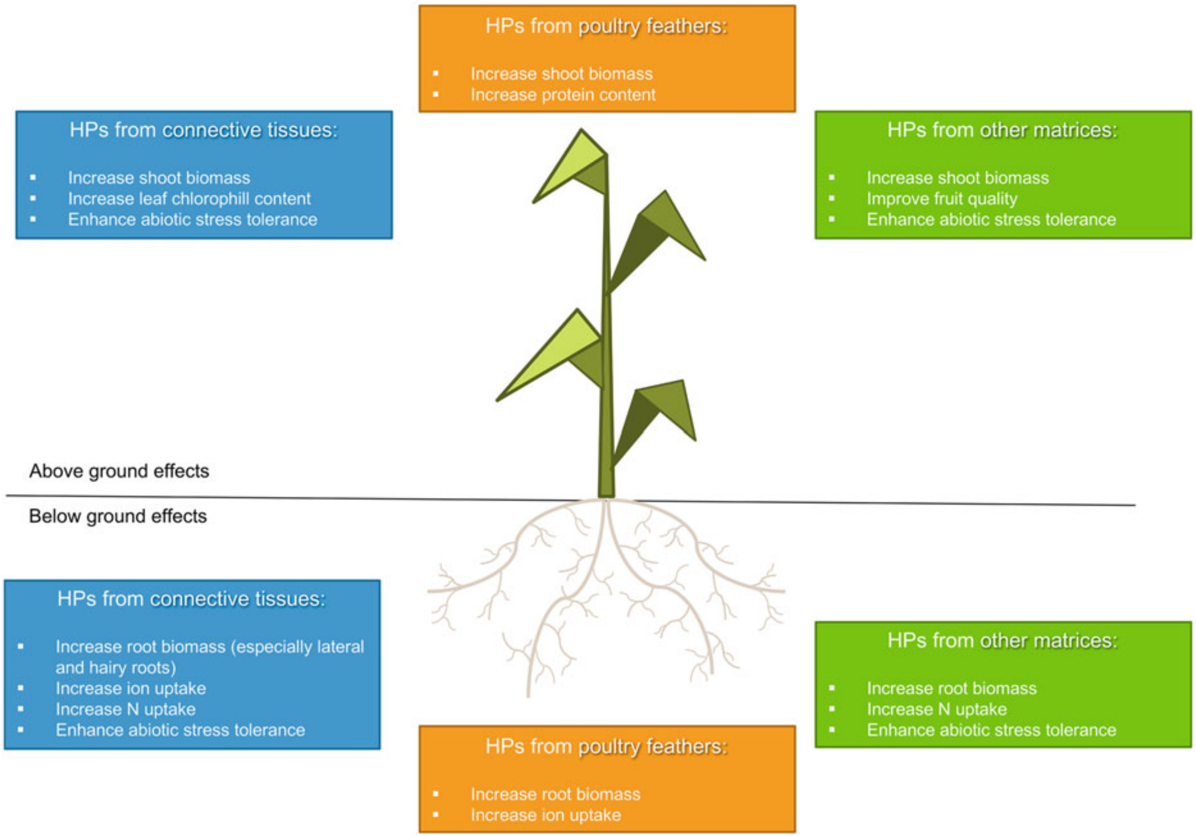

Fig. 5.1 Main morpho-physiological shared effects of different HPs classes on shoots and roots. Features listed in boxes are taken from the available indexed literature. Other matrices: casein, erythrocytes, peptone, PEPTON 85/16®

plant biomass. Together with the peculiar effects on the nutrient transporter modulation, which differ depending on the HP given to the plant, the increased root biomass, directly linked to an increased absorptive surface, could be ascribed as a common feature responsible for the enhanced nutrient uptake observed in many of the studies presented in literature. Another interesting characteristic that has been found after the application of HPs is an enhanced tolerance to abiotic stress. Although different products can be used as stress protectants, evidence suggests to look for mechanisms of actions rather than a general common pathway that can explain all the effects observed. Although from an observational and qualitative point of view the data produced to date clearly show the beneficial effects of animal-derived HPs, a more rigorous and multidisciplinary approach must be developed to tackle and overcome the methodological biases encountered so far. Together with the physiological analysis, it is crucial to characterize the HPs with chemical and biophysical techniques and to exploit molecular biology tools to shed a light on the underlying mechanisms of action.

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Protein Hydrolysates as Biostimulants of Plant Growth and Development

6

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Abstract

For the actual climate crisis, resilient agriculture is required to guarantee access to enough food, in quality and quantity, for a growing population. To face these challenges, innovative agricultural practices under organic or biological concepts for a sustainable crop production are required. To achieve the goal of sustainable agriculture, it is necessary to incorporate new models, agricultural supplies, and biotechnologies to enhance crop productivity. Plant biostimulants emerge as an innovative option to conventional chemical plant nutrition schemes. Active molecules in these compounds trigger complex physiological and metabolic responses in plants, enhancing plant performance and stress adaptation traits that ultimately result in an increased yield. Biostimulants based on protein hydrolysates (PH) are particularly relevant in the concept of plant stimulation. PH-based biostimulants are produced from different protein by-products and wastes by enzymatic processing, and the mixture of oligopeptides released during these proteolytic events is the main active compound associated with the stimulatory effects observed in different crops. This chapter describes the fundamentals in the technologies used in PH-based biostimulant productions, enzymatic processing, and recent advances in biostimulant research and development, as

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_6

well as the incorporation of new phenomics and transcriptomic technologies to elucidate the mode of action of these biostimulants in a concept of rational design.

Keywords

Bioactive peptides · Proteolysis · Phenomics · Rational design · Ecofriendly · Sustainability

6.1 Introduction

Since the past decade, serious questions are being raised about the overuse of agrochemicals in agricultural systems and their adverse effects on the environment and soil biochemistry. An increase in soil salinization, toxicity, and loss of soil microbial diversity are the most common problems around this production concept that ultimately result in poor crop productivity (Ganguly et al. 2021). This concern has led to an increasing interest in new agroecological alternatives for crop nutrition and management, especially under a climate change scenario. As a result of different research efforts, plant biostimulants emerge as an innovative option to chemicals in agriculture; additionally, it has been proven that the use of these compounds also promotes interesting changes in crop physiology, enhancing environmental resilience, yield, and quality (du Jardin 2015; Colla et al. 2017). Biostimulants differ in their chemical nature, stimulatory mechanism, and efficiency; particularly protein hydrolysates (PH) are considered one of the most complex in composition, as well as in the mode of action, triggering intricate plant responses at the cellular level. Conceptually, PH-based biostimulants must be produced from any protein source (food, waste, or by-product); however, some technical issues arise during its manufacturing, characterization, and testing (Moreno-Hernández et al. 2020). Given the increasing use of PH-based biostimulants in food production, regulation of the market is necessary to provide accurate pieces of evidence of experimental biostimulants and their primary function (Ricci et al. 2019; EBIC 2021). Recently, numerous PHs are recognized as plant biostimulants by improving specific traits in crops, and others are under continuous evaluation by phenomics and omic-based approaches. This chapter highlights some fundamentals involved in the science of PH-based biostimulants, focusing on the technology for its production and evaluation for agricultural purposes.

6.2 Proteins from By-Products for Hydrolysates Production

By-products represent an excellent source of valuable compounds for organic agriculture. Some of these resources are especially rich in protein content that might be recovered, isolated, and bioconverted into add-value products. Annually, large volumes of waste effluents and solid by-products are produced worldwide, only 54 billion pounds correspond animal-derived, and similar volume to cereal-processing waste, under-utilized foods, and unoptimal edible horticultural fruits (one-third of food for human consumption), unfortunately only a small part of these resources are properly exploited, and transformed into commodities (Martínez-Alvarez et al. 2015; FAO 2021). Table 6.1 indicates the most representative by-products produced by agriculture, livestock, and seafood industries as well as its protein component and summarizes protein recovery principles applied for waste processing. Solid or effluent by-products from vegetable or animal sources are complex matrices with a differentiated composition, not only in protein content but also other macromolecules, including other aggregated proteins, fatty acids, starch, fibers, gums, and polyphenols, rendering difficult its extraction and utilization for PH production. Protein solubility is a key feature for successful protein recovery in general; highly soluble proteins are extracted-solubilized easily by washing process or maceration with low ionic strength solutions (<0.05 M NaCl, pH 4.5–5.5) that include sarcoplasmic fraction in minced meal and green leaves proteins (chloroplastic, cytosolic protein) accounting high recovery yields (70–80%) by combining centrifugation process (Kim et al. 2005; Tamayo Tenorio et al. 2016). Similar approaches have been used in aqueous two-phase partitioning (ATP) for a proper resolution of whole blood proteins (hemoglobin, plasmin, albumin) employing polyethylene glycol/sal combination, recovering around 85% of albumin from blood suspension in the aqueous phase (Rito-Palomares et al. 1998). In contrast, extraction of stromal, myofibrillar, and structural proteins requires the combination of several extractions and fractionation principles. In meat wastes, both terrestrial and aquatic organisms, myofibrillar contractile proteins (myosin-actin complex) represent over 70% (w/w) of total protein content, its separation requires consecutive washing protocols with concentrated chaotropic salt solutions (0.3–0.6 M NaCl or KCl) improving protein extractability in different meat systems (Dara et al. 2021).

Particularly, fish processing waste represents an important source of proteins with biotechnological potential, typically 60–70% of the fish weight is discarded in the form of frames, heads, tails, and guts, representing a suitable resource for myofibrillar, collagen, and elastin protein isolated manufacturing. The pH-shifts process improves the separation of myofibrillar proteins from collagen-enriched structures, by employing isoelectric solubilization/precipitation (ISP). ISP-disruption induces selective solubilization in conditions away from isoelectric point (pI) of proteins, and precipitation near to protein pI (pH 5.5 for myofibrillar proteins) increasing over 90% the concentration of crude hydrolyzable protein (Matak et al. 2015). After separation of collagen-containing tissues (bones, skin, cartilage) from other proteins, insoluble collagen might be extracted by a combination of acid-saline-enzymatic conditions to increase collagen solubilization, the amount of protein recovered vary according to cross-linking grade in collagen molecule, source, and method for processing. Generally, higher collagen yields (80-84%) are obtained by pepsin-solubilized methods in comparison with acid-assisted extraction (Ahmed et al. 2020). Solubility problems are observed in keratin, which is the main component in chicken feathers and horn wastes. Sinkiewicz et al. (2017) describe a method for the preparation of soluble feather keratin, coupling ether pre-treatment (defatting),

Table 6.1 Proteins from by-products and extraction-recovery approaches

Industry	By-products source	Proteins components	Extraction, concentration, and recovery process	References
Agrifoods	Alfalfa Legume seeds Meals Processing wastes Soybean paste Wet/dry-milling	Chloroplastic green proteins Cytoplasmic Germins Globulins Prolamins	Alcoholic-solubilization Centrifugation Filtering Hydrolysis-assisted extraction Hydrothermal extraction pH-shifts Surfactant solubilization Ultrasonic-assisted extraction	Tamayo Tenorio et al. (2016); Tapia-Hernández et al. (2019); Rahman et al. (2020); Rico et al. (2020)
Livestock	Blood Feathers Feet Gut Heads Hooves Leather Wastewater Whey	Albumin Collagen Gelatin Hemoglobin Immunoglobulins Keratins Peptides Whey proteins	Centrifugation Dialysis Extraction by reducing agents Hydrothermal alkaline extraction Hydrothermal hydrolysis Microwave irradiation Superheat process Two/three-phase partitioning Ultrafiltration	Rito-Palomares et al. (1998); Sinkiewicz et al. (2017); Chilakamarry et al. (2021)
Seafood	Carcass Fishmeal Gut Heat Skin Tails Wastewater	Actin Collagen Elastin Myoglobin Myosin Sarcoplasmic Tropomyosin	Acid solubilization Chitonsan flocculation Electro-flocculation Extrusion-hydro-extraction Floating Freeze-drying Isoelectric solubilization/ precipitation	Ahmed et al. (2020); Liu et al. (2020); Dara et al. (2021); Venugopal and Sasidharan (2021)

(continued)

Table 6.1 (continued)

Industry	By-products source	Proteins components	Extraction, concentration, and recovery process	References
			Salting in/out Sedimentation Ultra-nano filtration/ fractionation Ultrasound	

and chemical alkaline-hydrolysis reaction in presence of reducing agents (2-mercaptoethanol, sodium *m*-bisulfite, sodium bisulfite, or dithiothreitol) obtaining over 80% of keratin yield. Microbial fermentation, microwave irradiation, and superheat processing have been also discussed in detail for keratin extraction from different natural resources (Chilakamarry et al. 2021).

Agrofood wastes offer extraordinary potential as sources of protein substrates in PH manufacturing. Waste from wet-milling, soy paste, and legumes are excellent sources of albumins, globulins, glutenins, and prolamin proteins, the last one represents around 80% of crude protein content. Prolamins extraction protocols include alcoholic solubilization of cereal meal in 70% ethanol aqueous solution in continuous stirring system, after centrifugation the supernatant containing prolamins is recovered and concentrated by lyophilization. This approach is employed practically for different cereal meals, including barley, sorghum, wheat, and corn, with minor modifications to obtain between 60 and 80% of prolamin protein (Tapia-Hernández et al. 2019). Recently, ultrasonic and hydrothermal processing have been proposed to improve protein/peptide extraction from soy, legume, algal material as an alternative to chemical-based processes (Rahman et al. 2020; Rico et al. 2020).

6.3 Fundamentals in Protein Hydrolysates Production

Protein hydrolysates (PH) are considered as mixtures of polypeptides, oligopeptides, and amino acids released by partial hydrolysis of proteins (Schaafsma 2009). Due to the importance of peptides and amino acids as basic building blocks of proteins and their multiple physiological functions in the plant, the selection of protein source is a key factor to obtain PH-based biostimulant with attractive functions (Popko et al. 2018; Moreno-Hernández et al. 2020). In addition, since amino acid synthesis is a highly energy-consuming process, its presence in PH allows plants to save energy and increase the metabolic rate or *de-novo* reconstruction (Popko et al. 2014).

6.3.1 Amino Acid Content and Profile Analysis

Amino acids are one of the main bioactive ingredients in PHs applied as biostimulants, the accurate determination of its content is important. Generally, protein substrates are hydrolyzed at acidic conditions to their constituent amino acids and consequently are separated by chromatographic techniques, mainly Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Once the amino acids are separated, its detection and quantification involve the reaction of the amine portion with a derivatizing reagent (Klampfl 2005). The selection of hydrolysis conditions of samples and the kind of derivatization (pre- or post-column) is an important aspect to be considered to obtain reliable results of amino acid composition. For instance, the most common method for protein hydrolysates involves the usage of strong monoprotic acid at high concentration (HCl 6 M) in combination with high temperature (110 °C) under vacuum for 20–24 h; however, this method might lead to loss of Ser, Thr, and Tyr (Jajić et al. 2013). Regarding derivatization reagents, the most common is *o*-phthaldialdehyde (OPA), which reacts with primary amines producing a fluorophore that is able to be excited at 302–395 nm and detected at 420–650 nm (Roth and Hampaĭ 1973). However, the OPA reagent is only suitable to detect primary amines, therefore, other reagents such as 9-Fluorenylmethyl chloroformate (FMOC) is utilized for the derivatization of secondary amino acids, including Hyp and Pro (Turnell and Cooper 1982). The main issue of derivatives reagents like OPA is their limited stability and so must be prepared routinely before each run to avoid interferences (Halket et al. 2005). More recently, the application of high-performance liquid chromatography (HPLC), coupled with tandem mass spectrometry (LC–MS/MS) using hydrophilic columns, offers a suitable method that doesn't need the usage of unstable derivatives reagents and has been used for successful resolution of amino acids profile for animal and plant protein matrices (Kambhampati et al. 2019).

Amino acids are crucial for plant biochemistry, participating as anti-stress (Hyp, Pro) and chelating agents (Cys, Glu, Gly, His, Lys), as well as stimulating chlorophyll synthesis (Ala, Lys, Ser), seed germination (Asp, Glu, Lys, Met, Phe, Thr), and signaling process in hormone metabolism. (Ala, Pro) (Paleckiene et al. 2007; Popko et al. 2018); however, some consideration must be taken into account during protein source processing in order to guarantee amino acid integrity and functionality. In practice, chemical (strong acids or alkalis) and enzymatic methods are used for hydrolysate production, and both strongly affect the amino acidic composition of the final product (Colla et al. 2015). For instance, acid hydrolysis is a low-cost process, but causes the destruction of Trp and a partial loss of Met. Alkaline hydrolysis (using NaOH or KOH) has the advantage of low cost and full recovery of Trp, but can generate the loss of most amino acids (Hou et al. 2017). Also, chemical hydrolysis combining high temperatures (121–137 °C) and acid or alkaline treatment, is considered a drastic process that results in the conversion from L-forms to D-form of amino acids, and the hydrolyzed product is composed by free amino acids and to a lesser extent by soluble peptides, limiting their metabolism and causing other toxic effects in plants (Cerdán et al. 2008). On the other hand, enzymatic hydrolysis can be performed under mild conditions with precise control of the degree of hydrolysis, minimizing side reactions and the presence of toxic chemicals in the products; thus, the final PH contains higher peptides: free amino acids ratio, and proportion of L-amino acids (proteinogenics) in comparison with those obtained by chemical hydrolysis (Colla et al. 2017; Álvarez-Viñas et al. 2020).

Related to the protein source (animal or vegetal) and amino acid composition of PH-based biostimulants, it has been observed that animal protein hydrolysates from collagen possess elevated concentrations of Gly and Pro, whereas in legume derived PH, Asp and Glu acids are predominant (Ertani et al. 2014; Colla et al. 2015, 2017). In this sense, comparing several protein sources applied as plant biostimulants, it has been observed that Gly concentration of fish and chicken hydrolysates per 100 g of protein is higher than that of alfalfa but less than that of animal gelatin, although this parameter must be strongly influenced by the source (Table 6.2). Legume-derived PH biostimulant Trainer® (Italpollina S.P.A., Italy) comprises mainly amino acids and soluble peptides (75% of free amino acids and peptides), 22% of carbohydrates, and 3% of mineral nutrients (Di Mola et al. 2020; Lucini et al. 2020). The foliar application of Trainer® in spinach and lamb's lettuce (sprayed four times at 21, 27, 33, and 39 days after sowing, at a concentration of 4 mL/L), induced a major improved N uptake/use efficiency compared to untreated plants (Di Mola et al. 2020). Feather keratins have low bio-availability, and it is deficient in amino acids His, Lys, Met, and Trp (Callegaro et al. 2019), while collagen has high assimilation by its significant amounts of Gly, Pro, and Lys (Colla et al. 2015) with distinctive assimilation/functions in plant metabolism and development. The deficiency of some amino acids could limit the biostimulant activity for some PH.

6.3.2 Hydrolysis Degree

Protein hydrolysates are composed of a complex mixture of free amino acids and peptides of different chain lengths. Generally, it is assumed that the degree which indicates that a protein substrate has been hydrolyzed is proportional to the number of peptide bonds broken and, consequently, to the average size and molecular mass of the peptides present. In this regard, the number of peptide bonds broken as a proportion of the total number of peptide bonds present is defined as the percentage degree of hydrolysis (DH) (Rutherfurd 2010).

Protein enzymatic hydrolysis is frequently preferred over chemical hydrolysis for several reasons. For instance, it preserves the nutritional quality of the amino acids better than chemical hydrolysis. In addition, the choice of the enzyme allows the control of protein breakdown at desired DH and drives the hydrolysis toward the desired hydrolysis products (Friedman 1996; Wouters et al. 2016). Although, a high percentage of PHs used as biostimulants are produced by chemical hydrolysis of proteins from animal origin and their production processes are considered harmful to the environment. In this sense, due to enzymatic hydrolysis being ecologically safe, this kind of PH is gaining acceptance for organic agriculture, being the preferred

Table 6.2 Amino acids and the general composition of PH-based biostimulants from different sources

Source	Hydrolysis principle	General composition Amino acid content (five major concentrations, %)		References
Vegetal				
Sunflower seed meal (by-product from biodiesel process); liquid product	Enzymatic hydrolysis (Alcalase-Flavourzyme)	Glu (14.3); Arg (11.8); Leu (8.7); Val (5.9); Asp (5.5)	Protein content: 67.7% Organic matter: 75.8% Majority of peptides comprised low MW (<25 kDa)	Ugolini et al. (2015)
Alfalfa protein hydrolysate; liquid formulation Trainer®; commercial liquid product, derived from legume seeds	Not declared Enzymatic hydrolysis (Enzyme not declared)	Asp (0.99); Glu (0.74); Ala (0.41); Gly (0.36); Val (0.33) Glu (5.4); Asp (3.3.); Leu (2.4); Lys (1.9); Ser (1.7)	Organic matter: 23% Free amino acids: 1.5% Total amino acids: 5.1% Organic nitrogen: 5% Organic carbon: 19% Free amino acids and soluble peptides: 31%	Soppelsa et al. (2018) Paul et al. (2019a)
Chickpea; liquid formulation	Enzymatic hydrolysis (proteases and cellulases)	Arg (0.22); Glu (0.18); Pro (0.17); Leu (0.14); Met (0.12)	Total carbon: 23. 6% Total nitrogen: 2.7% Organic nitrogen: 5.1%	Ertani et al. (2019)
Animal				
Gelatin capsules	Not reported	Gly (27.2); Pro (15.5); Hyp (13.3); Glu (11.6); Ala (11.3)	MW: 40% of peptides around 50–150 kDa	Wilson et al. (2018)
Chicken feathers; liquid formulation of PH (Amino-Hort)	Acid hydrolysis (H_2SO_4/H_3PO_4)	Glu (23); Pro (20); Gly (19); Asp and Leu (17)	Not reported	Popko et al. (2018)
Porcine by-products; micro granular form (Pepton 85/16®)	Enzymatic hydrolysis (Enzyme not declared)	Leu (10.9); Asp (9.9); Glu (7.2); Lys (7.2); Ala (6.9)	Average MW distribution around 2–3 kDa; 66% of peptides are considered short-chain (with less than 50 amino acids per chain) and 16% are considered long-chain peptides (>50 amino acids)	Casadesús et al. (2020)
Chicken feathers; sprayed PH	Alkaline hydrolysis (KOH)	Pro (13.1); Glu (8.63); Leu (6.86); Gly (6.65); Val (5.40)	Total protein 72.8% Total N: 11.7%	Ebru and Atici (2019)

(continued)

Table 6.2 (continued)

Source	Hydrolysis principle	General composition Amino acid content (five major concentrations, %)		References
Fish by-products (heads and tails); sprayed PH	Enzymatic hydrolysis (Alcalase)	Glu (22.72); Gly (15.79); Ser (14.45); Val (7.42); Leu (7.02)	Total organic matter: 87.2%	Al-Malieky and Jerry (2019)
Chicken feathers; sprayed PH	CO_2-assisted pressure hydrolysis	Ile + Leu (25.9); Gly (24); Val (18.1); Phe (10.1); Lys (8.0)	2.81 g/L of peptides and 0.039 g/L of free amino acids	Schmidt et al. (2020)

option for farmers and informed consumers (Bradshaw et al. 2012; Colla et al. 2015; Caruso et al. 2019; Madende and Hayes 2020).

Several methods exist to determine DH during protein hydrolysis, but there is no standard technique to accomplish reliable results for samples that have been produced by chemical or enzymatic hydrolysis (Spellman et al. 2003). For instance, DH can be measured by determining the amount of nitrogen released during hydrolysis, which becomes soluble in the presence of a precipitating agent such as trichloroacetic acid (Hung et al. 1984). Another approach to determine DH is by quantification of the free amino groups released during hydrolysis using compounds that react specifically with amino groups such as trinitrobenzene-sulphonic acid (TNBS) and *o*-phthaldialdehyde (OPA); several modifications of these techniques exist (Polychroniadou 1988; Caer and Colas 1993; Nielsen et al. 2001). Another technique used to measure DH is the pH-stat method, having the advantage of monitoring the hydrolytic process in real-time, taking advantage of the dissociation of protons from the free amino groups that occurs when hydrolysis is carried out at neutral or alkaline conditions (Adler-Nissen 1986).

Since hydrolysis on protein structure causes a decrease of molecular weight (MW) and also increases the number of ionizable groups and the accessibility of hydrophobic regions in the protein structure (Panyam and Kilara 1996) the biostimulant effect of PH can be affected. For example, Lucini et al. (2020) analyzed the effect of peptide fractions on the performance of a legume-derived PH biostimulant in tomato; interestingly, the smallest (MW <1 kDa) peptides showed the most active stimulatory activity.

6.4 Protein Hydrolysate Production

The production of protein hydrolysates has been increased in the last three decades (CAGR of 6.5% and a market size value of $844.2 m by 2019), and are specially used as additives in food products and feed for animals. This process converts raw agricultural materials or pure proteins into value-added products for use in several agro-industries However, recently, its uses as plant biostimulants (PB) in agricultural practices has gained relevance for improving nutrition, quality, yield, and abiotic tolerance in different crops (Colla et al. 2015).

PH-based biostimulants can be manufactured from agro-industrial by-products or by using pure proteins. The use of isolated proteins results in better quality products; however, it increases the costs of production. Therefore, the use of protein-rich by-products is more attractive. Protein sources are pre-treated by either heating them with acid, fermented with specific microorganisms, applying separation procedures (e.g., pressing, defatting, sedimentation, centrifugation, filtration, etc.), or adding enzymes to remove undesirable material, as was discussed in previous sections. PH is a complex mixture of polypeptides of different sizes and free amino acids, and their composition and properties are highly variable depending on the protein source, type of hydrolytic method, degree of hydrolysis, fractionation, etc. (Moreno-Hernández et al. 2020). Protein hydrolysate production by enzymatic methods has been the preferred process (around 70% of the PH is produced by this procedure) due to its higher efficiency than acid and alkaline treatments since these last can destroy essential amino acids such as lysine, serine, arginine, and threonine (Fiormarket 2020). On the contrary, enzymatic methods are eco-friendly and hydrolytic-conditions controllable, which yields products with characteristics and quality reproducibles.

6.4.1 Protein Substrates Treatment

Most commercial protein hydrolysate-based biostimulants are plant protein-derived (P-PH); however, animal protein-derived (A-PH) have also gained acceptance due to their satisfactory results and their lower cost (Lucini et al. 2020). Several plants (e.g., legume seeds, alfalfa hay, corn wet-milling, and vegetable by-products) and animal sources (e.g., leather by-products, collagen, blood meal, fish by-products, chicken feathers, and milk proteins), have been used for this purpose. Protein-rich plant material for PH production can be used either minimal processed (raw) or pre-treated to concentrate the protein before its proteolytic enzymatic processing. For example, sunflower defatted seed meal (SDSM) (a by-product from oil production), is concentrated by a sedimentation/flotation fractionation procedure. If required, a further alkaline extraction followed by precipitation at the isoelectric point (pH 4.3) is used to obtain a protein isolate (PI) (Ugolini et al. 2015).

A legume-derived PH-PB, known as Trainer®, is a commercial product manufactured by Italpollina (Rivoli Veronese, Italy) and has become the focus of several studies due to its high activity as a plant biostimulant. It contains 27–31% of amino acids and soluble peptides and has been obtained through a process of enzymatic hydrolysis of proteins derived from legume seed flour, followed by separation of insoluble residual compounds by centrifugation and concentration to obtain a product with a final acid pH (Colla et al. 2015; Lucini et al. 2020).

Proteins from animal sources (e.g., leather by-products, collagen, blood meal, fish by-products, chicken feathers, and milk proteins) have been also used for PH production. Collagen, elastin, and keratins are the prevalent fibrous proteins found in animal by-products generated from meat production. It is estimated that approx. five million tons chicken feather are generated worldwide by the poultry industry, representing an attractive source of protein (approx. 90% of keratin) to convert into PH-PB. However, due to the insolubility and hydrolysis resistance efficient hydrothermal, chemical, biological, or enzymatic processes are required (Callegaro et al. 2019). The use of microorganisms with high keratinolytic activity has been one of the preferred processes for keratin feather hydrolysis. *Bacillus licheniformis, B. subtilis, B. pumilus,* and *B. cereus* are among the most effective feather-degrading microorganisms. However, other bacteria genera such as *Chryseobacterium, Serratia,* and *Stenotrophomonas,* and the fungi *Chrysosporium spp.* and *Aspergillus spp*. have been also considered (Callegaro et al. 2019; Gurav et al. 2020).

Feather microbial fermentation (whole or milled) is usually produced through submerged cultivations with mesophilic (5–20 g feathers/L, 30–40 °C, 24–96 h) or thermophilic bacteria (30–50 g milled feathers /L, 45–50 °C, pH 10.0, 48 h). The hydrothermal and enzymatic process, alone or combined, has been also used for this purpose (Callegaro et al. 2019).

Bryndina et al. (2019) describe a procedure to hydrolyze non-ground pen keratin by applying a pre-treatment with sodium sulfide, urea, sodium thioglycolate, or sodium tetraborate (0.3% by weight) using a ratio of 1:20 (solid: liquid) and pressure of 0.15 MPa for 2 h. Then a protease preparation from *Str. chromogenes* s.g. 0832 at a concentration of 3 U/g of protein was used and the enzymatic hydrolysis was carried out for 6 h with continuous stirring, at 40 °C, pH 8. The degree of hydrolysis (DH) after enzymatic treatment was higher in pen keratin pre-treated with sodium tetraborate (DH 80%), followed by sodium thioglycolate (DH 50%) (Bryndina et al. 2019).

A recent strategy for whey valorization has been the production of PH-based biostimulant. A fermentation process using *Lactobacillus ramnosus* (considered as a plant growth-promoting bacterium, PGPB) under controlled conditions (pH 5.5, 37 °C and agitation at 300 rpm with 0.1% of protease added as inductor), was developed. Lactic acid, peptides, and free amino acids and the biomass of *Lactobacillus rhamnosus* were fractionated with a triple system membrane device (MMS AG membrane System) using a 0.2-μm PVDF membrane to separate *L. rhamnosus* biomass (microfiltration) and a 200-Da MW cut-off TFM membrane to separate the protein hydrolysate (nanofiltration), and the lactic acid recovered by distillation. *L. rhamonsus* presented biocontrol activity against some phytopathogenic microorganisms and the PH and the lactic acid were used as soil biostimulant which induced microbial activity and had a modifying effect on microbial biodiversity, favoring the growth of plant growth-promoting bacterial (Caballero et al. 2020).

6.4.2 Enzymatic Proteolysis Performance

To maximize bioactive peptide/oligopeptide proportion and yield, proteolytic enzymes (proteases) must be added at specific ratios and under controlled conditions. Enzymatic hydrolysates have been developed by utilization of pure proteases, enzymatic mixtures, and raw aqueous preparations extracted from food or by-products themselves (Salazar-Leyva et al. 2017). However, proteolytic enzymes such as Alcalase, Flavourzye, Neutrase, Pepsin, and Protamex are used frequently in both commercial and experimental PH manufacture (Mazorra-Manzano et al. 2018). The selection of proteolytic enzyme is based on operative conditions and specificity. Generally, fermentation-produced enzymes by specific microbial strains (i.e., *Bacillus subtilis*, *Bacillus lichenifromis, Aspergillus flavus Asperguillus niger*) show thermostability in comparison with animal proteases (pepsin, chymotrypsin, trypsin) improving hydrolysis rates (dos Santos Aguilar and Sato 2018). The chemical nature of active sites in protease structure provides remarkable insights into its proteolysis mechanism (enzyme-substrate interaction), pH conditions, and specificity. Specificity parameter determines the positions of clave sites in which the enzyme catalyzes peptide bond break, as well as reveals the nature of amino and carboxylic terminal groups of the released peptides. For instance, flavourzyme ad alcalase preferentially hydrolyses peptide bonds between aromatic residues (Phe, Trp, and Tyr), while papain preferentially cleavages peptide bonds containing large hydrophobic side chains (Tavano 2013). It has been observed that acceptable DH (20–50%) percentage is obtained by using 1–5% w/v of E/S ratio respect protein basis. Higher doses of proteases, especially in their purified forms, induce excessive proteolysis, increasing the content of free amino acids, and might reduce bioactivity by overhydrolysis of oligopeptides. Actually, there are a lot of proteases from animal, microbial, and plant sources employed in the production of a protein hydrolysate, and extensive literature have been generated around protein hydrolysates manufacture and characterizations, as well as a hydrolysis optimization parameter, protease selection, enzyme/substrate ratio, pH, temperature, DH, additives, etc. (dos Santos Aguilar and Sato 2018; Mazorra-Manzano et al. 2018; Etemadian et al. 2021). Most of these enzymes are actually explored in the production of PH-based biostimulant at experimental and commercial levels (Moreno-Hernández et al. 2020). The releases of peptides and amino acids will depend on the extent of hydrolysis and appropriated protease selection, since the specificity of the protease, protein substrate digestibility, DH, among other factors, determining the final characteristics of PH.

6.5 Effects of PH-Based Biostimulant on Crops' Traits

Agro-industry and farmers search for products that promote plant growth, productivity, and quality crop. Biostimulant are other than fertilizer, induce growth and resistance of biotic and abiotic stress; many biostimulants are made from diverse agro and seafood sources, these have macromolecules or chemicals substances, that can modify physiological processes of plants that enhance nutrient uptake, resistance to biotic or abiotic stresses, and remarkable improvement on crop yield and quality (Xu and Geelen 2018; Ricci et al. 2019; Shukla et al. 2019). Additionally, the fertilizers supplemented with biostimulants (protein hydrolysate, chitosan, algal extract, humic acid, or microorganism) might reduce the drawbacks of agrochemicals fertilizers or pesticides (Drobek et al. 2019; Aktsoglou et al. 2021).

6.5.1 Foliar and Radicular Administration of Hydrolysates

Crops exhibit different physiological responses to the application of PH, which seems to be affected by the protein source and amino acid composition of hydrolysates, application mode, and dosage used (Aktsoglou et al. 2021). PH as liquid, soluble powder, and granular forms promotes macro and micronutrient assimilation when these compounds are applied in a foliar spray and radicular manner (Fig. 6.1), stimulating plant metabolism with a potential effect on the quality and yield, in many crops (Calvo et al. 2014; Nardi et al. 2016; Colla et al. 2017). Amino acid composition is a key feature in the stability of PH employed in crop nutritional programs. It has been proposed that PH incorporated into fertigation solutions requires a high balance of hydrophilic/hydrophobic oligopeptides (with high solubility) to prevent insoluble aggregates or undesirable interactions with other nutrients, especially mineral, due to chelating properties of some amino acids (Schiavon et al. 2008; Ertani et al. 2013b). Moreover, radicular plant systems, root secretions (hydrolytic enzymes), soil biochemistry, and root absorption coefficient are some critical aspects for optimal utilization of peptides through the root system (Moreno-Hernández et al. 2020). While leaves' porosity (stomata activity) is critical for proper peptide or amino acid acquisitions during the foliar application, other factors involved are peptide size/sequence, relative humidity, temperature, evaporation parameters, and leaf area to improve diffusion (Koukounararas et al. 2013). For instance, Sestili et al. (2018) showed that the application of PH is more effective in improving plant growth and total N uptake than foliar sprays. This is because free amino acids in PHs have been reported to activate nitrate transporters.

Amino acids in PH represent an important source of nitrogen that could be equally effective such as inorganic nitrogen fertilizers when they are used as a nutrient hydroponic solution (Aktsoglou et al. 2021). The hydroponic cultivation of peppermint and spearmint has not affected plant growth either positively or adversely by the addition of PH Amino16® (Evyp LLP, Greece) in the nutrient solution and was attributed to either the increased root growth on or to the low rate of PHs applied (lower than 0.5%) (Aktsoglou et al. 2021). Pieces of evidence suggest that amino acids and small peptides derivated from PH, are uptake and translocated by amino acid transport proteins involved in phloem loading and unloading, xylem-phloem transfer, import into seed, and intracellular transport in plants from leaves or root tissues (Yang et al. 2020). Foliar application of PH can increase amino acid and peptide availability for plant uptake by reducing the competition with a microorganism (Colla et al. 2015). Glu is rapidly absorbed by creeping bentgrass foliage and

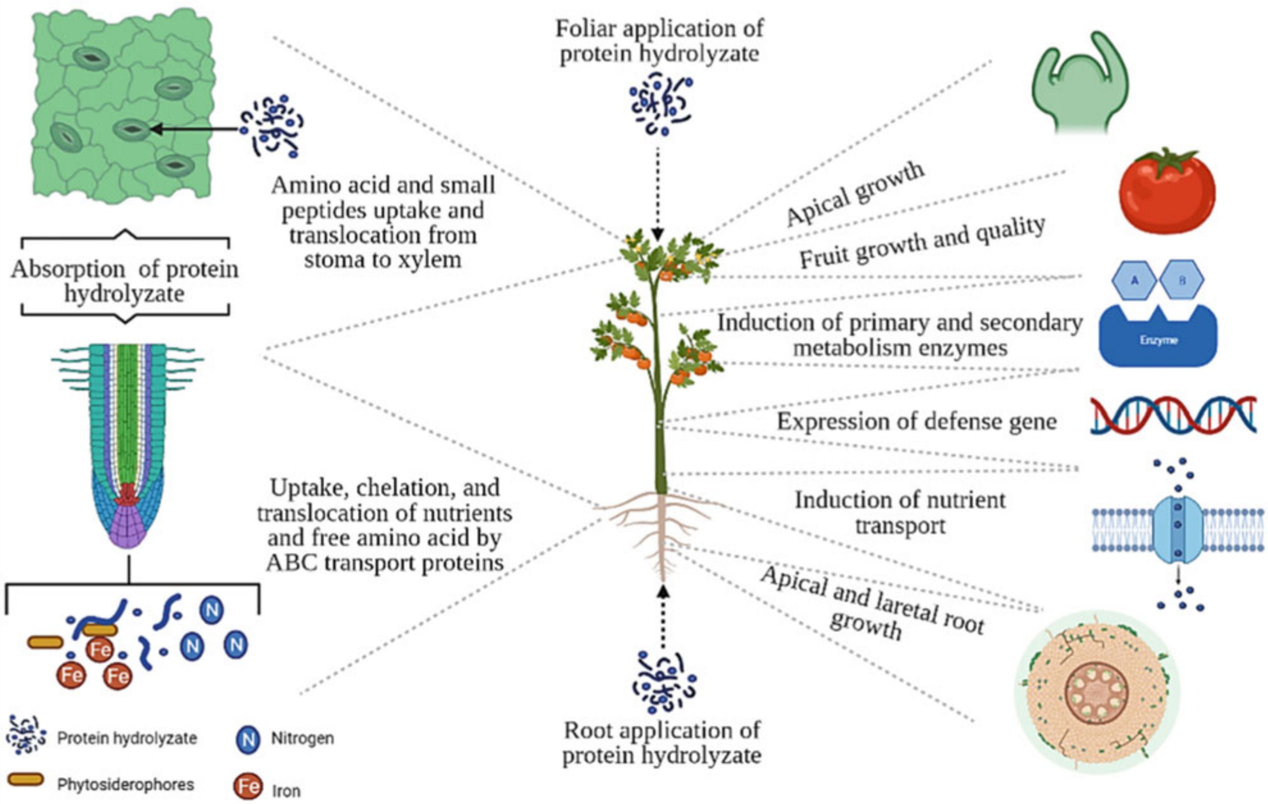

Fig. 6.1 Schematic view of the effect of foliar and root application of protein hydrolysate effects on tomato plants. (Created with BioRender.com)

directly utilized as a precursor to synthesize gamma-aminobutyric acid and proline, two important metabolites with well-known roles in plant stress adaptation (Rouphael and Colla 2020). Tryptophan is considered a fundamental amino acid for the synthesis of indoleacetic acid (IAA), a hormone with important functions on plant growth. However, its activity can be affected when it is applied separately. In a study, L-methionine stimulated lettuce growth parameters; however, distinct effects have been observed when L-Gly and L-Trp were applied radicular on butterhead lettuce hydroponically grown (Rouphael and Colla 2020). Paul et al. (2019a) suggest that foliar application of PH reach mesophyll cells by absorption trough cuticle, epidermal cells, and stomata, while in drench or hydroponically application, the absorption occurs through root epidermal cells via ABC membrane transport and gets redistributed through the xylem. Most PH-based biostimulant induces positive physiological effects as growth and development; moreover, it enhances uptake nutrient from soil or microorganism of the rhizosphere; however, to perform the desired effect, PH must be able to penetrate the plant tissue at low dosages, depending species, cultivars, and vegetative stage, but also depend on environmental condition, stomata, and cuticle that act as a barrier (Pecha et al. 2012).

6.5.2 Effects in Crop Growth and Quality

Biostimulants have significant effects on many crop traits related to productivity and quality, including root architecture, change in endogen phytohormone levels, photosynthetic rate, increased pigment content, protein, phenolic contents, stimulate the growth, antioxidant activity, and enhance macro and microelements in vegetal tissues (Yakhin et al. 2017; Drobek et al. 2019; Ertani et al. 2019; Ambrosini et al. 2021). Table 6.3 includes a description of the most applied PH-based biostimulants, their intended use, and primary functions.

PH-based biostimulants probably contain molecules that display phytohormone-like activities as has been proposed in a recent revision (Moreno-Hernández et al. 2020). PH-containing peptides might act as auxin-like and gibberellin-like elicitors, triggering signaling as naturally occurring peptides in plants and promoting vegetative plant growth, and early maturation of fruits (Drobek et al. 2019), effects triggered by some endogenous regulatory signaling peptides and protein-like hormones (e.g., phytosulfokine) influencing productive traits such as fruit maturation, root length, and thickness of stem, or inducing primary and secondary metabolism biosynthesis through the activation of multiple signaling pathways that involve second messengers that stimulate enzymes of the nitrate assimilation pathway, like nitrate reductase and glutamine synthetase which catalyze a rate-limiting step in nitrogen assimilation (Ertani et al. 2013a). In this context, Ertani et al. (2019) reported indole-3-acetic acid (IAA)-like and gibberellin (GA)-like activities of PH, obtained from *Cicer arietinum* L. and *Spirulina platensis*, and that they induced plant growth and accumulation of N-compounds (proteins, chlorophylls, and phenols) on hydroponically *Zea mays* L. culture; furthermore, PH from *C. arietinum* and *S. platensis* increased the activity of two enzymes (peroxidase and esterase)

Table 6.3 Commercial PH-based biostimulants' functionality and target crops

Trademark	Purpose	Intended use	Source
AminoHort®	Micronutrient deficiency corrector Nutrient uptake enhancer	Fruit trees, grapevine, greenhouse vegetables, industrial crops	USAGRO (2021)
AminoPrim®	Stress modulator Metabolic regulator Increase stress tolerance and plant recovery Improve yield quality and quantity	Flax, fruit trees, olive trees, fruit bushes, berries, grapevine, citrus, coffee, vegetables, ornamentals, lawns, plant nurseries	INTERMAG (2021)
Brown's Fish Hydrolysate®	Nutrient uptake regulator	Broccoli, ornamentals, grasses, oats	BrownsFish Genesis (2021)
Hydrostim®	Growth promoter Anti-stress regulator	Citrus, greenhouse vegetables, kiwifruit, olive, strawberry, grape	Hydro Fert (2021)
Ilsadrip Forte N9®	Growth promoter Radicular stimulant	Banana, fruit trees, horticultural crops, mango, vid, wheat	Ilsagroup (2021)
Pepton 85/16®	Growth promoter Phytohormonal-like action Photosynthetic activity enhancer Increase nutrient uptake, yield, and quality	Carrot, chili, citrus, industrial crops, lettuce, onion, ornamentals, potato, rice, tomato, vid, watermelon	FEMSSA (2021)
PROTIFERT LMW®	Nutrient uptake enhancer metabolic stimulant	Citrus, fruit trees, pineapple, melon, onion, ornamentals, peanut, rice, tomato, carrot, broccoli, sugar cane	SICIT (2021)
Siapton®	Growth promoter Anti-stress regulator (salinity, drought, low temperatures, transplant shock) Increase micronutrient intake and pollen germination	All crops	Isagro (2021)
Sinergon 3000®	Promotes vegetative and productive growth Plant recovery from environmental and physiological stress Increases fruit size and development of new vegetal tissues (buds, sprouts) and on fruit swelling	Non declared by the manufacturer	Cifo Srl (2021)

(continued)

Table 6.3 (continued)

Trademark	Purpose	Intended use	Source
StresSal®	Osmotic regulator for saline stress	Citrus, fruit trees, greenhouse vegetables, olive, ornamentals, strawberry	Bioiberica (2021a)
Terra-Sorb® Radicular	Growth promoter Improves nutrient absorption	Leafy vegetables, citrus, fruit trees, tomato, olive, spinach, strawberries, vid, industrial crops	Bioiberica (2021b)
Trainer SP®	Anti-stress regulator Growth and yield stimulator Fruit color and sugar content, fruit caliber, homogeneity enhancer	Non declared by the manufacturer	ITALPOLLINA (2021)

related with plant growth and differentiation of organogenesis; in the same way, Casadesús et al. 2020 reported hormonal signaling for improving root growth in tomato (*Solanum lycopersicum*, var. Ailsa Craig) plants, mediated by chorismate-derived hormones, in particular by salicylic acid.

Recently, Ceccarelli et al. (2021) reported foliar application of vegetal-derived PH on tomato cutting-promoted rooting and biomass density, length, and the number of lateral root branching, with promoting plant growth and development owing to stimulation of auxins (particularly precursors as 4-(indol-3-yl) butanoate and tryptamine), cytokinin, and gibberellin biosynthesis or IAA precursors. The foliar application (spraying) of animal-derived PH (from fish by-products) on lettuce, showed significant effects on the total number and area of leaves, carbohydrate, proline, shoot-fresh weight of plants, dry matter, total soluble solids, and total yield (Al-Malieky and Jerry 2019). In celery, Plant-PH Tyson® obtained from soy protein extract, Trainer® (legume-derived PH), and animal-PH Aswell® (bovine epithelium hydrolysate) improved plant growth and nutritional balance in both foliar and radicular applications (Consentino et al. 2020). Recently, the biostimulant activity of five plant-derived PH on tomatoes was evaluated for their ability to promote rooting in tomato cuttings following quick dipping. All PHs increased root length (45–93%) and root number (37–56%) (Ceccarelli et al. 2021).

Since, in addition to its biostimulant activity, PH also mediates plant adaptation to several stress conditions, including mineral depletions, cold, thermal, drought, and saline. StresSal® and Trainer® have been employed as osmoregulator in both fruit trees (persimmon) and horticultural crops (lettuce), to avoid the negative impact of saline stress (Visconti et al. 2015; Luziatelli et al. 2019). Experimental blood-derived PH and commercial Amino 16®, displayed protective effect in lettuce cultivated under extreme climate conditions, hydrolysates showed thermo-protective functionality toward warm and chilling temperatures, respectively (Polo et al. 2006; Tsouvaltzis et al. 2014). Some studies suggest that the accumulation of glycine betaine and proline is associated with increased stress tolerance, and the exogenous application of protein-based compounds in maize, barley, soybean, alfalfa, and rice has been highly correlated (Ahmad et al. 2013). Ambrosini et al. (2021) evaluated on hydroponic culture the capacity of a commercial PH derived from bovine collagen to mitigate Fe deficiency stress in roots of *Zea mays*, and observed that PH exhibited an increased growth and absorption by chelation of Fe area of the roots compared with control treatment; these studies show how PH have a positive effect by foliar or hydroponic culture; in another way, PH drench application on *Solanum lycopersicum* L. plants enhance transpiration rate and transpiration use efficiency with a positive impact on the biomass and metabolic profile (Paul et al. 2019a). Protective effects in some PH-based biostimulants have been attributed to proline or proline-precursors compounds (glutamate an/or ornithine) in hydrolysates, due their osmolyte and chemical chaperone roles under various stressful sceneries during plant development.

A clear mechanism for peptides found in PH-PB remains uncertain, but most pieces of evidence suggest that PH does not only provide nutrients directly to plants but these compounds also stimulate plant nutrient acquisition processes and is an alternative to diminishing chemical fertilizers. Protein hydrolysates induce a positive effect on plant crops, containing signaling peptides and free amino acid that enhance germination, seedling growth, fruits, and vegetable quality as well as crop productivity (Rouphael and Colla 2020). Table 6.4 shows some examples of PH whose potential as plant biostimulants has been tested for many plant traits such as morphophysiological parameters (stem, leaves number, foliar area), flowering time, fruit set-filling, crop productivity, and nutrient use efficiency. Until now, clear identification and characterization of the peptides (or amino acids) in PH related to the PB activity and associated mechanism of action has not been determined. Therefore, an important characteristic to be included in PH-PB characterization could be the peptidic size fraction, in addition to the common parameter considered such as DH, chemical composition, and amino acids content (Moreno-Hernández et al. 2020).

6.6 Approaches to Elucidate PH Mode of Action

High crop productivity is the ultimate goal of agricultural systems that employ PH-based biostimulants in their production practices. Farmer decision about the application of a particular biostimulant must be supported by accurate information about biostimulant quality and safety, effectivity, and also by the mode of action of the active ingredients or any other parameter about primary-secondary function (EBIC 2021). A collection of evidence about PH function as plant biostimulant has been recovered experimentally under different production systems and are based on the agronomic parameter; however, the trend around these biostimulants is turned into a more multidisciplinary approach, to validate a new generation of PH-based biostimulants employing precision testing technologies and genomic-based tools.

Table 6.4 Effect of PH-based biostimulant on crop traits under different growth systems

Crop	Biostimulant	Application mode	Enhanced traits	Reference
Growth chamber				
Lettuce	Alfalfa hydrolysate	Radicular	Primary-secondary growth/ development Root architecture, branching, and root tip density Root physiology by increase in nutrient uptakes Root system length, mass, surface area	Ertani et al. (2009)
	Hemoglobin hydrolysate	Radicular	Induce root stress responses to thermal stress Root dynamic/phenology maintaining primary specific growth rate, mass and surface area Leaves biomass and yield	Polo et al. (2006)
Maize	Connective-tissue hydrolysate	Radicular	Stimulated secondary root growth/development Stem length, size, and mass Root physiology by increase in nutrient uptakes	Ertani et al. (2009)
	Cow connective tissue	Radicular	Enhance root physiology stress response reactive species Stem length and biomass	Ambrosini et al. (2021)
Tomato	Skin fish hydrolysate	Radicular	Seedling vigor, germination rate Leaves dynamic, chlorophyll synthesis Photosynthetic rate Stimulated secondary root growth/development	Horii et al. (2007)
	Legume-derived hydrolysate	Foliar/ Radicular	Root length, mass, surface area, hair density	Ceccarelli et al. (2021)
Greenhouse				
Chickpea	Chicken feathers hydrolysate	Radicular	Germination rate, seedling vigor, transplant adaptation Stimulated secondary root growth/development Root dynamic/phenology maintaining primary specific growth rate, mass, and surface area	Paul et al. (2013)

(continued)

Table 6.4 (continued)

Crop	Biostimulant	Application mode	Enhanced traits	Reference
Cucumber Broccoli	Gelatin hydrolysate	Radicular	Early hypocotyl emergency (seed priming) Seedling length, mass, stem length and diameter Shoot mass, dry weight Root dynamic uptake nutrient	Wilson et al. (2018)
Lettuce	Legume seeds hydrolysate	Foliar and radicular	Increase stem growth rate protein and chlorophyll production Root physiology, stress response to ROS indicators Osmoregulation un saline conditions	Lucini et al. (2015)
Lettuce	Trainer®	Foliar	Enhanced plant growth, productivity Uptake nutrients Osmoregulation to salt stress	Luziatelli et al. (2019)
	Amino 16®	Foliar and radicular	Nutrient uptake Leaves morphology uniformity, yield Leaves chemistry quality, secondary compounds (antioxidant)	Tsouvaltzis et al. (2014)
Maize	Alfalfa hydrolysate	Radicular	Leaves length, foliar area, mass Grain yield Root dynamic, macronutrients content	Schiavon et al. (2008)
	Sicit2000®	Radicular	Root growth, length, and surface area Uptake nutrients, micronutrients content Stress response indicators	Santi et al. (2017)
Pepper	Alfalfa hydrolysate	Foliar	Foliar fresh weight Fruit set, filling Number of fruit per plant Root chemistry secondary compounds Fruit nutraceutical quality, yield	Ertani et al. (2014)
Snapdragon	Hydrostim®	Foliar/ radicular	Leaves photosynthetic rate Transpiration rate, conductance Root chemistry nutrient uptake and root nitrogen content, photosynthetic rate, transpiration rate, and stomatal conductance	Cristiano et al. (2018)

(continued)

Table 6.4 (continued)

Crop	Biostimulant	Application mode	Enhanced traits	Reference
			Primary/secondary growth/ development Branching, root tip density, branching intensity	
Strawberry	Hemoglobin hydrolysate	Radicular	Root length, mass, surface area Increases biomass production and yield Flowering time Fruit set and filling	Marfa et al. (2008)
Tomato	Trainer®	Radicular	Root dynamic, phenology, chemistry Root anatomy and architecture, branching density Root dry weight	Sestili et al. (2018)
Field trial				
Apple	Alfalfa protein hydrolysate	Foliar	Fruit quality trait (color index, sugar content) Fruit nutraceuticals (anthocyanin content) Biotic post-harvest resistance	Soppelsa et al. (2018)
Banana	Chicken feathers hydrolysate	Foliar/ radicular	Leaves photosynthetic rate and chlorophyll content Flowering time Fruit set, filling, and yield Fruit quality (antioxidant and nutraceutical)	Gurav and Jadhav (2013)
Celery	PHs from soy extract and bovine animal epithelium	Foliar	Whole plant length, weight Nutrient uptake Yield	Consentino et al. (2020)
Grapevine	Carob germ hydrolysate	Radicular	Fruit quality traits, nutraceutical value Plant growth/development	Parrado et al. (2007)
Lettuce	Fish hydrolysate	Radicular	Leaves number Stem diameter, shoot fresh and dry weight Quality trait succulence and nutraceutical	Xu and Mou (2017)
Maize	Chicken feathers hydrolysate	Foliar	Root surface area, nutrient uptake Grain yield	Tejada et al. (2018)
Persimmon	StresSal®	Radicular	Plant adaptation Stress response indicator ROS Osmoregulation	Visconti et al. (2015)

(continued)

Table 6.4 (continued)

Crop	Biostimulant	Application mode	Enhanced traits	Reference
Soybean	Terra-sorb® complex	Foliar	Pods numbers Seed yield Seed quality (phenolic, flavonoid) Oil content	Kocira (2019)
Tomato	Pepton 85/16®	Foliar/ radicular	Plant height Stem diameter Flowering time Fruit set and yield	Polo and Mata (2018)
Wheat	Terra-sorb® complex	Foliar	Leaves area, mass, photosynthetic rate Seed sugar content, yield	Martinez-Esteso et al. (2016)

6.6.1 High-Throughput Phenotyping Characterization

Biostimulants induce significant changes in crop development at different physiological levels. Most results on biostimulant efficiency have been based on agronomical traits (germinations, adaptation, flowering time, fruit set, and yield), monitoring phenotypical changes to identify biostimulant candidates, and also provide clues about its modes of action. However, conventional phenotyping protocols for reporting these traits might prove time-consuming, laborious, with low reproducibility, and strong subjectivity. Additionally, some of these methods are destructive and unsuitable for large-scale probes. This has driven the development of new tools for the automatic management of crops, and the continuous monitoring of plants treated with biostimulants. The concept of high-throughput phenotyping (HTP) in agriculture emerged with the necessity of high-precision systems for data recovery in agronomy; these powerful robotic tools can analyze broad scenarios and their influences on plant traits, also known as plant phenomics. In the field of biostimulants, HTP has been used to evaluate the influence of active components in biostimulants on physiological plant traits quantitatively and enables to compare dynamic plant-environment responses in a real-time manner (Dalal et al. 2019).

During the development of new biostimulants, HTP platforms have provided an accurate assessment of different active products contained in protein hydrolysates. An interesting feature of this platform is that a set of biostimulants can be assayed in a wide range of conditions, including water stress, nutrient deficiency, temperature stress conditions (heat/cold), and light intensity, in a continuous lab-field-lab cycle (Rouphael et al. 2018). In a drought model, this technology was used to analyze the morphophysiological traits of Trainer®-treated (spray or substrate drench) tomato plants, under semi-controlled greenhouse conditions. By employing imaging sensors, visible red, green, blue images for digital biomass increase, and fluorometers to report photosynthetic performance, HTP identified the best-performing plants as an effect of biostimulant applications. Moreover, analysis of spectral data revealed the most active photosynthetic tissues and their correlation with biomass accumulation, and the metabolomics profiling of stimulated plants, annotated over 1900 compounds associated with ROS signaling, sterols, carotenoids, membrane lipids, phytohormones, polyamines, and chlorophyll-related molecules (Paul et al. 2019a, b). Similar approaches have been applied to understand the role of biostimulants on peppers' productivity and survival under drought conditions (Dalal et al. 2019). Recent advances in imaging acquisition technology as spectrograph (hyperspectral analysis) offers new opportunities in the field of biostimulant research by analysis of whole plants; till date, around 16 crops, including barley, maize, potato, grapevines, wheat, oak, and peppers have been analyzed with this technology (Mishra et al. 2020). Although these studies do not contemplate the use of biostimulants, the tests evidence the efficacy of these systems for the high-throughput phenotyping in a variety of conditions.

6.6.2 Metabolomic Analysis

Plant metabolite profiling is an emerging field to describe cellular plant response to a wide range of biotic or abiotic conditions. The metabolomic approach seeks to establish a relationship between cellular metabolites and a specific variation factor, as well as its influence on particular traits shown by plants (Schauer and Fernie 2006). In the field of plant sciences, metabolomics is a powerful tool to integrate new metabolic pathways to omic-data, for high-throughput analysis, combining analytical techniques like Liquid/Gas Chromatography-MS/MS systems to explore primary/secondary plant metabolites in many economically important crops like maize, rice, tomato (Sharma et al. 2021). Metabolomics analyses have been applied to profile the metabolite change of plant to HP-based biostimulants.

Plant crops treated with protein hydrolysates derivatives from animal, plant, or algae sources induce growth and development of fruits, leaf, roots, and phytochemicals metabolites—in tomato, the application of PH-enhanced root and quality of fruits with increased diameter, weight, and volume—these effects were reported in diverse crops such as, kiwifruit, papaya, banana, passionfruit, and vegetables such as lettuce and pepper (Rodrigues et al. 2020; Ceccarelli et al. 2021). Furthermore, several studies show that application of PHs stimulate secondary metabolites with antioxidant activity such as carotenoids, polyphenols, and flavonoids, as well as defense metabolites like alkaloids, salicylic acid, jasmonates, and ethylene, as well as phytoalexins as indole-3-carboxyl and psoralen (Casadesús et al. 2020; Lucini et al. 2020; Ambrosini et al. 2021), thus promoting crop productivity. The benefit of PH is not only over crops but also has beneficial effects on the microbiome of the rhizosphere, improving physiological and development processes in plants, favoring greater nutrient and water uptake as well as enhanced resistance against biotic and abiotic stress. PH also promotes nitrogen assimilation via coordinate regulation of carbon and nitrogen metabolism, by inducing activity enzymes as nitrate reductase, nitrite reductase, glutamine synthase, glutamate synthase, and aspartate aminotransferase (Ertani et al. 2009, 2019; Sestili et al.

2018; Paul et al. 2019a), and carbon metabolism as malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase (Ertani et al. 2013a), or esterase and peroxidase enzymes that have a role in meristematic growth that induces vegetative development (Ertani et al. 2019), as well HP drench, foliar, or hydroponic application induce high-affinity nitrate transporters belonging to NRT2 family: NRT2.1 and NRT2.3, that play a key role in the coordination of root development, acting on lateral root initiation and nitrate uptake and long-distance transport system from root to shoot (Sestili et al. 2018; Paul et al. 2019a), as well as iron transporters (ZmTOM1 and ZmIRT1) involved in phytosiderophores and FeII assimilation (Ambrosini et al. 2021), the PH application favoring minerals absorption and transport, known as nutrient acquisition response (Rouphael et al. 2021).

Metabolomic data of four plant-derived PH indicate a reprogramming of phytohormones profile by modulating gibberellin and cytokinin biosynthesis with a lower effect on auxins and brassinosteroids biosynthesis. The hierarchical cluster analysis (HCA) revealed that metabolomic profiles in roots were significantly influenced by PHs foliar application, in a PH-dependent specific manner, providing evidence that such hormone-like activity of PHs depends on the protein source (Ceccarelli et al. 2021).

6.6.3 Differential Gene Expression Analysis

In the last decade, improvements in Next-Generation Sequencing (NGS) technology have led to a substantial increase of data about plant genetics, providing relevant knowledge about genome functionality. This information is fundamental in the construction of transcriptomes, employing RNA-seq analysis. Due to the transcription process is the very first genome response to biotic and abiotic stimulus; this provides a complete scenario about changes in expression patterns, genomic information flux, and gene network enrichment in plant cells for a particular time-lapse. Fundamentally, RNA-seq reflects the total RNA identities produced by a single cell or tissue that has been successfully sequenced and mapped to an annotated genome or curated transcriptome dataset. Coupled with bioinformatics exploration, RNA-seq tells us about a group of genes and gene networks significantly upregulated or downregulated as a response to certain factors (Differential Gene Expression Analysis; DGEA) like variations in nutritional status, phenological stage, light/dark cycle, environmental conditions, and also biostimulant application.

Advances in NGS have reduced the cost of sequencing services and allowed access to this technology by biostimulant developers. This approach has gained relevance in the field of biostimulant research, providing not only accurate evidence about the effects of these compounds in crop development but also recovering robust data to elucidate the mode of action of biostimulant at the molecular level. Although NGS is relatively time-consuming and costly in comparison with non-omic approaches (limiting its application to some horticultural crops), the number of available genomes in databases is growing faster than ever, and this is impacting the number of scientific reports documenting biostimulant uses. Transcriptomic-wide identification of DEG has been used to explore the mechanistic process of plant growth-promoting bacteria (González-Morales et al. 2021), humic substances (Galambos et al. 2020), and PH-based biostimulants (Trevisan et al. 2017). For instance, DGEA has been used to understand the influence of PH-based biostimulants at the transcriptional level in crops like cucumber, maize, soybean, and tomato (Table 6.5). Wilson et al. (2015) evidenced the complexity of transcriptional regulation on two-week-old cucumber seedling by hydrolyzed collagen (gelatin) capsules, employing the RNA-seq data to coexpress gene network construction. In this spot trial controlled conditions, gelatin biostimulant induced 620 differentially expressed genes (DEGs), grouped in five modules by Weighted Gene Coexpression Network Analysis (WGCNA) with interconnected hub networks. An upregulation of phloem amino acid, N-transporter genes (amino acid transporter, amino acid permease, ammonium transporter), and nitrogen metabolism, as well as detoxifying mechanism (Glutathione S-transferase), was evidenced, indicating its relevance at first-level of plant response during the early stage of biostimulation process (Wilson et al. 2015). Other reports have evidenced new target molecules in tomato seedlings exposed to alfalfa hydrolysates, inducing overexpression of stress-related genes as phytohormone modulation, antioxidant-related genes, phenylpropanoid pathways, detoxification process, and MAPK signaling pathway in crosstalk between biotic and abiotic stress responses (Ertani et al. 2017). A recent study reveals insights on the mechanistic action of PH-stimulant by combining transcriptomic and quantitative proteomics approaches. By examining maize seedlings exposed to commercial PH-stimulant under chamber controlled conditions, Ebinezer et al. (2020) identified 608 DEGs and 242 differentially abundant proteins (DAP) at a high dosage of biostimulant. The bioinformatics to construct Gene Ontology Enrichment (GO terms), clustered DEG and DAP into 20 categories associated with stimulus responses, including osmotic, salt, hormone regulation, brassinosteroid metabolic, and biosynthesis, phenylpropanoid, lignin, siderophore, and homeostasis maintenance. Pathway Enrichment Analysis discriminates significant metabolic pathways impacted by PH, mainly metabolic pathways, biosynthesis of secondary metabolites, glutathione metabolism, and amino acid metabolism, all with up/down-regulated genes under essay conditions (Ebinezer et al. 2020).

These studies evidence all possible target molecules in broad metabolic pathways for biostimulant design; however, this scenario evokes a major challenge in the development of active molecules as plant biostimulants. Information about the size, sequences, and functionality of peptides are characteristics that need to be included in PH characterization to associate their putative role as plant biostimulants (Lucini et al. 2020; Moreno-Hernández et al. 2020). Until now, a clear identification of peptides found in PH (with diverse composition, characteristics, and properties) responsible for the plant biostimulation activity has not been completed and established. Figure 6.2 proposes an interdisciplinary approach to biostimulant rational design to provide accurately integrated evidence on the effectivity of plant stimulation phenom. To accelerate the re-investigation on the first and new generation of plant biostimulant based on protein hydrolysates.

Table 6.5 Transcriptional changes in crops exposed to PH-based biostimulant

Crop tissue	Biostimulant	Experimental conditions	Change in expression pattern	References
Cucumber leaves	Collagen hydrolysate	Seeds planted with gelatin capsules (radicular) in spot trial under controlled greenhouse conditions. Temperature 24/21 °C, 14/10 h photoperiod	620 DEGs respect control conditions KEGG: Cell wall degradation, photosynthesis, hormone metabolism, abiotic stress response, signaling, plant development, transcription factors, amino acid transporter, and metabolism	Wilson et al. (2015)
Maize seedling lateral roots	APR®	Seedlings treated by irrigation with two increased concentrations of APR under pot trial essay, grown in a climatic chamber to maintain standard conditions, 70/90% relative humidity, 14 h day/10 h night cycle, and 280 µmol m^{-2} s^{-1} photon flux density	608 DEGs in response to the high concentration of APR KEGG: Biosynthesis of secondary metabolites, metabolic pathways, phenylpropanoid biosynthesis, monoterpenoid biosynthesis, glutathione metabolism, cysteine, and methionine metabolism	Ebinezer et al. (2020)
Soybean cotyledons	KIEM®	Seed soaked with biostimulant solution for a complete distribution on the seed surface. Primed seeds incubated under heat stress (35 °C), 24–48 h until germination	Biostimulant-treated seeds showed 879 DEGs after 24 h of incubation at 35 °C, 93% of genes were downregulated GO: stimuli and chemical response, hormone stimuli response, programmed cell death, oxidative stress response, carbon metabolism, transferase activity, cell wall organization or biogenesis	Campobenedetto et al. (2020)

(continued)

Table 6.5 (continued)

Crop tissue	Biostimulant	Experimental conditions	Change in expression pattern	References
Tomato seedling leaves and roots	Alfalfa hydrolysate	Tomato seed germinated on agar medium under grow chamber conditions, 70/85% relative humidity, 2621 °C air temperature, 14 h day/10 h night cycle, and 280 μmol m^{-2} s^{-1} photon flux density. Seedling in spot trial treated by fertigation with hydrolysate	2988 DEGs (1938 leaves, 1054 roots) GO: organic substances metabolic process, primary metabolic process, cellular metabolic process, nitrogen compounds metabolic process, response to stress, catabolic process, cellular component biogenesis, response to abiotic stimulus	Ertani et al. (2017)

Differentially expressed genes (DEGs), Kyoto Encyclopedia of Gene and Genomes (KEGG) Pathway, Gene Ontology (GO) terms

6.7 Conclusions

Emerging agriculture is necessary to meet the millennium goal of food security. Biostimulants are a strategic issue to break with limitations of traditional crop production practices and overcome the challenges of the climate crisis. PH-based biostimulants offer broad opportunities for the development of efficient and eco-friendly systems for food production. These compounds can be produced from a variety of protein substrates from food wastes or by-products discarded by industries, promoting revalorization of these residues and mitigating their environmental impacts. The collective efforts on biostimulant development and research have to lead to the identification of functional PH, experimentally and commercially probed, with agricultural applications. Many of these bioactive PH with attractive improvements on crop vegetative growth, plant nutrition, stress adaptation, yield, and quality.

Advances in high-throughput phenotyping and genomics have increased our understanding of the mode of action of some PH-based biostimulants at the physiological and molecular levels. Nonetheless, only a few studies report the evaluation of biostimulants under these approaches, partially due to the availability of reference genomes and transcriptomes, as well as the inherent limitations on the technology transferences process. To face the challenges of PH-based biostimulant production, the structure-function relationship of peptides released during the hydrolytic process must be conducted, to link specific peptide sequences to particular bioactivity detected in plants. Coupled to phenomic-metabolomic-transcriptomic studies, structural data will lead us to a rational design for “ad-hock” production of biostimulants

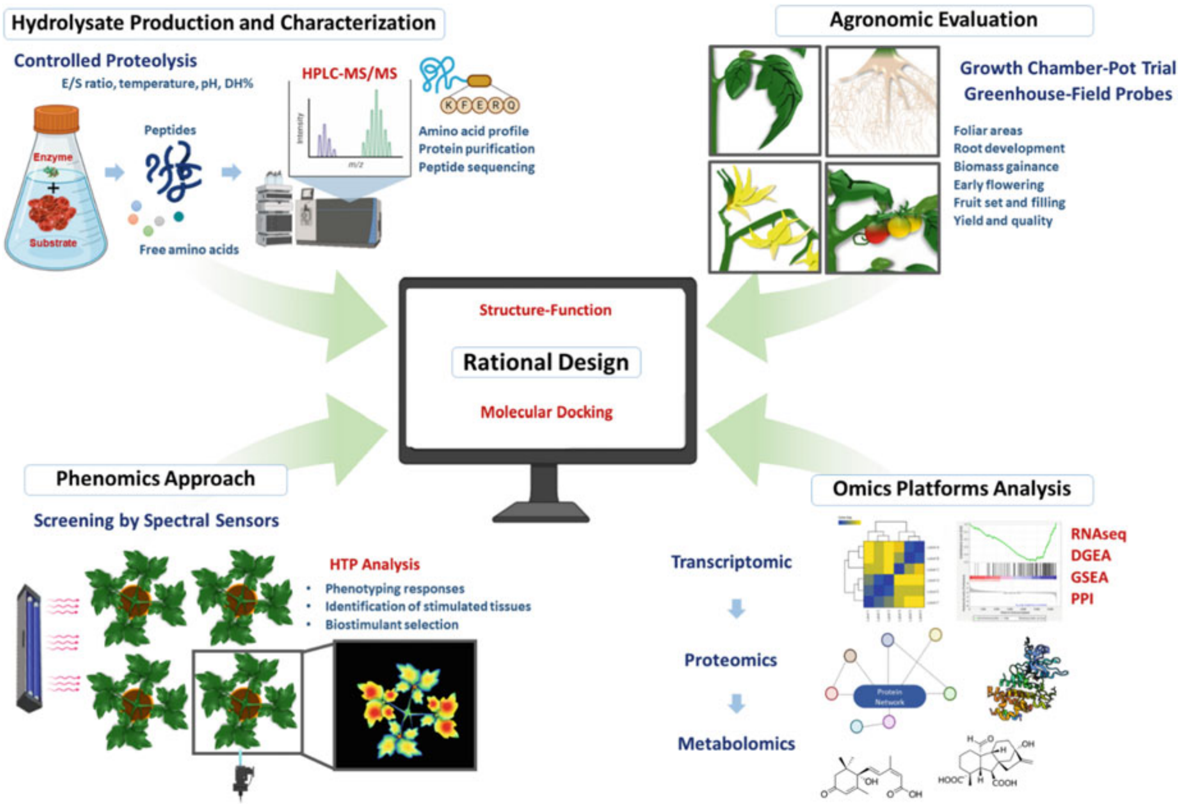

Fig. 6.2 Rational design for PH-based biostimulant production. Schematic representation of multidisciplinary approach for "ad-hoc" design bioactive peptide for plants. *DGEA* Differential Gene Expression Analysis, *DH%* hydrolysis degree, *E/S* enzyme-substrate ratio, *GSEA* Gene Set Enrichment Analysis, *HPLC-MS/MS* High-Performance Liquid Chromatography-Mass Spectrometry, *HTP* High-Throughput Phenotyping, *PPI* Protein-Protein Interaction, *RNAseq* RNA-sequencing, (RNAseq)

based on proteins, by developing specific peptide mixtures, biologically or synthetically produced, to improve target traits for a particular crop, and finally migrate toward more sustainable agriculture.

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7

Biostimulants and Their Extraction from Food and Agro-Based Industries

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Abstract

The bioactive compounds present in the waste and by-products of the food and agro-based industries, can be utilized for the valorization of the unutilized biomass generated by the food and agriculture industries. Several classes of organic wastes generated by food and agro-based industries consist of various active ingredients which have an utmost potential to be exploited as biostimulants. Biostimulants can be of natural or synthetic origin, and are used to treat the seeds, plants, and/or soil. Biostimulants are known to improve the abiotic stresses tolerance of the plants and thereby improve the quality and yield of the produce. The availability of huge quantity and diverse types of agricultural and food processing waste provide us with an opportunity to test a vast range of biostimulant substances in order to expand the possible ways for their valorization. Number of conventional techniques are being utilized and researched for the extraction and development of biostimulants from the waste biomass generated by the food and agro-based industries. This utilization, if scaled up efficiently, will not only provide a strategy of utilization of agro-industrial waste but will also help in reduction of agro-chemical usage in an eco-friendly way.

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_7

Keywords

Biostimulants · Agro-industrial waste · Organic waste · Sustainable farming · Plant extract

7.1 Introduction

Biostimulants can be of natural or synthetic origin that can be used to treat seeds, plants, or soil, influence the plant growth by improving the abiotic stresses tolerance, and simultaneously improve the quality and yield of the produce (Ana and Lucia 2019).Various researchers have defined Biostimulants differently. Biostimulants are the preparations that are composed of substances or some beneficial microbes, which increase the abiotic stress tolerance, enhance the nutrient uptake and nutrient efficiency, thereby improving the crop yield and quality (Przybysz 2014). Yakhin et al. (2017) defined biostimulants as biological-formulated products that result in the improved plant productivity due to their multiple constituent composition. These biostimulants comprise plant growth regulators, plant protective compounds, essential plant nutrients, amino acids, phenolic compounds, sugars, sterols, etc. (Puglia et al. 2021).

Initially, the categorization of biostimulants was attempted by Filatov (1951), who had suggested four groupings of biogenic stimulants. Followed by that, biostimulants have been vastly classified by various researchers and sources. A list of 15 biostimulants and their 59 components was compiled by Karnok (2000). Ikrina and Kolbin (2004) systematized the patent literature and categorized the raw materials used to derive biostimulants into nine categories. In 2008, another researcher, Basak suggested a biostimulants classification based on origin and mode of action of the active ingredient. He also suggested that they can be grouped on the basis of number of components in formulations—single or multiple. The scientific rationale of classification was pioneered by Du Jardin (2012a), in which he considered eight classes of biostimulants, but subsequently he reduced them to seven classes, wherein he explicitly excluded microorganisms from his categorization, as microorganisms are already categorized as biopesticides and plant hormones sources. Another classification based on the action of the biostimulants on the physiological responses of the plant has been proposed by Bulgari et al. (2015). One additional categorization is based on different origins and characteristics of the primary sources from which the biostimulants are derived such as plant and animal protein hydrolysates, sea weed and vegetal extracts, humic and fulvic substances, and a few microorganisms.

Biostimulants hold a high potential to stimulate plant growth, increase the stress-tolerance, and positively influence the crop yield, through regulating or modifying the plant's physiological processes. When applied appropriately, the biostimulants provide potential benefits to the growth and development of the crops and also act on the physiological processes of the plants to cope with the salinity, water stress, and toxicity of elements (Du Jardin 2015; Couto et al. 2012). Biostimulants have a direct or indirect response on the plant productivity. In a direct response, the plants or soils respond directly to the biostimulant application, whereas, in an indirect response, the biostimulant impacts the soil and plant microbiome with consequent effects on the plant productivity (Yakhin et al. 2017). The biomass with potential biostimulant activity has active ingredients that can be categorized into a range of molecules, including amino acids (Colla et al. 2017), phytohormones (Letham and Palni 1983; Werner and Schmülling 2009; Pacifici et al. 2015), polyamine (Fuell et al. 2010), etc. Biostimulants are efficient even in small concentrations in enhancing the nutrition efficiency, crop quality traits, abiotic stress tolerance, regardless of its nutrients content. According to Yaronskaya et al. (2006), these biostimulating substances have effects similar to those of the plant growth hormones such as auxins, gibberellins, and cytokinins.

The present statistics predict that global production and consumption of all types of food commodities is likely to continue to escalate (FAO and OECD 2018). The industrial waste streams and by-products of food and agro based industries consist of various active ingredients that are of utmost potential for the extraction of biostimulants. The extraction of biostimulants from the waste and by-products of food and agro-based industries provides a good opportunity for the valorization of the waste produced in hefty amounts. Food and agro-based industries generate various classes of source material that are composed of protein hydrolysates, chitin and chitosan, and other biostimulants. Protein hydrolysates manufactured from hydrolyzed protein-rich waste generated in food industry are commonly used biostimulants (Dieterich et al. 2014). Thus, the extraction of biostimulants not only provides a means of valorization of the organic wastes and by-products generated from Food and Agro-based industries but is also helpful in waste management. not only provides a means of extracting Organic wastes are the promising sources for production of biostimulants.

7.2 Biostimulants from Food and Agro-Based Industries

While developing a biostimulant, the selection of the biomass resource is imperative. Plants, animals, microorganisms, seaweeds are the prime sources of the biostimulants (Table 7.1). Apart from the several primary sources of biostimulants, the waste generated from food and agro-based industries are undoubtedly the potential significant sources for biostimulant development. With the escalating demand for healthy food, the food and agro-based industries are flourishing to meet that demand, thereby generating huge amount of waste and by-products during food production and processing. The scale of biomass production substantially varies with the type of crop, source of food, and it’s processing. Among various challenges that have to be overcome in agriculture and food production systems, waste management is one of the major on the list. The extraction of biostimulants from the industrial waste and by-products of food and agro-based industries renders a good opportunity for valorization as well as for waste management. However, when these raw materials are being used, a basic knowledge of their biochemical

Table 7.1 Examples of biostimulants extracted from different wastes and by-products of agro- and food-based industries

Biostimulant	Source	Reference
Protein hydrolysates (PH)	By-product extracts (rapeseeds, apple seeds, rice husks)	Donno et al. (2013)
Protein hydrolysates (PH)	Waste biomass from tomato plants	Baglieri et al. (2014)
Protein hydrolysates (PH)	By-products of carob (Carob germs)	Parrado et al. (2008)
Chitin	By-products of the mushroom industry	Tao et al. (2004)
Biostimulant extract	Viticulture waste (wine-shoot aqueous extracts)	Sánchez-Gómez et al. (2017a, b)
Protein hydrolate	Pea and tomato residues	Colla et al. (2014)
Fish PHs	Fish skin	Chalamaiah et al. (2012)
Chitin and chitosan	Shrimp waste	Khanafari et al. (2008)
Biostimulant extract	By-products extracts (lemon, fennel, barley grains)	Chehade et al. (2017)
Biostimulant extract	Seaweed extract	Billard et al. (2014)
Biostimulant extract	Plant waste such as leaves, seeds, and exudates of *Amaryllidaceae*, *Brassicacae*, *Ericaceae*, *Fabaceae*, *Fagaceae*, *Moringaceae*, *Plantaginaceae*, *Poaceae*, *Rosaceae*, *Solanaceae*, *Theaceae*, *Vitaceae.*	Yakhin et al. (2014); Parrado et al. (2008); Apone et al. (2010); Ertani et al. (2014); Colla et al. (2014); Yasmeen et al. (2014); Ugolini et al. (2015)

characteristics such as preservation of the specific bioactive ingredient is a prerequisite (Povero et al. 2016). Further in this section, various source materials from plant and animal origins that are considered to be wastes and by-products in the food and agro-based industries such as protein hydrolysates, chitin and chitosan, and others will be discussed.

The biostimulants of plant origin can be extracted from the plant waste such as seeds, leaves, roots, exudates from families such as *Brassicacae, Amaryllidaceae*, *Ericaceae*, *Fabaceae*, *Fagaceae*, *Rosaceae*, *Poaceae*, to name a few (Naumov et al. 1993; Yakhin et al. 2014; Parrado et al. 2008; Apone et al. 2010; Ertani et al. 2014; Colla et al. 2014; Yasmeen et al. 2014; Ugolini et al. 2015), generated in food and agro-based industries. According to Food and Agriculture organization (2019), the inedible plant residues generated from agro- and food-based industries are annually recorded as being approximately 250 million tons. These huge wastes are rich in lipids, carbohydrates, and aromatic molecules that can be used to extract biostimulants.

Protein hydrolysates (PH) are an important class of biostimulants that are produced from the protein hydrolysis found in by-products generated from the food and agriculture industries based on the food from plant and animal origins (Colla et al. 2014). Protein hydrolysates (PH) of plant origin are considered to be more eco-friendly and are generally developed or extracted through enzymatic hydrolysis. This results in the mixture of amino acids and peptides of different lengths (Colla et al. 2015a, b). Recently, the protein hydrolysates developed from organic wastes have gained popularity as plant biostimulants. Different sources such as the extracts from the by-products of rapeseeds, apple seeds, and rice husks (Donno et al. 2013), waste biomass from tomato plants (Baglieri et al. 2014) have been used for the production of biostimulants. The carob germs, which are the high-protein by-product obtained during treatment of carob fruit, have also been explored for the production of amino acids and proteins using enzymatic process (Parrado et al. 2008). Parrado et al. (2007) also studied the effect of enzymatic extract of PHs derived from vegetable by-products, along with the phytohormone, on the anthocyanin levels in grapes.

Another class of biostimulants that have potential applications in a number of industries such as food, cosmetics, and industrial processes is Chitin, which is a biopolymer derived from crustaceans' shells. Chitosan, which is the deacetylated form of chitin, also holds a great potential as a biostimulant. These can be derived from the food and agro industry wastes (Olsen et al. 2014). The physico-chemical and biological properties of chitin and chitosan are determined by the ratio of each monomer in the polymer chain (Pichyangkura and Chadchawan 2015). Chitosan is a bio-waste product that is much cheaper as compared to other biopolymers. The sources of chitin are majorly marine animals' and crustacean residues, but can also be obtained from insects and microorganisms. The chitosan has antibacterial and antifungal properties, along with mucoadhesivity, atoxicity, and biocompatibility (Santos et al. 2020).These biostimulants can be derived from the industries generating mycelial waste and mushroom industry. White et al. (1979) were pioneer in isolating chitosan from fungal mycelium, which was followed by many other researches (Elsoud and El Kady 2019). Crude fungal chitin on fresh basis (0.65–1.15%) was yielded by Tao et al. (2004); this anticipated the potential of the by-products of mushroom industry for the development of biostimulants.

One other attractive sector of agriculture that produces an enormous quantity of waste is viticulture. The waste generated by viticulture is responsible for environment problems related to its disposal (Sánchez-Gómez et al. 2017a, b). Approximately, 1.4–2.0 tons per hectare of vine-shoot waste is generated annually (Peralbo-Molina and Luque de Castro 2013) that can be utilized for the production of biostimulants owing to their high content of phenolic compounds (Garcia Martinez et al. 2021). Sánchez-Gómez et al. (2017a, b) used some extraction methods to obtain several vine-shoot aqueous extracts which then they tested on *Leptinotarsa decemlineata*, *Lactuca sativa*, and *Lolium perenne*. A few other studies also have addressed the utilization of residues of vine-shoot. Water extract from the shoots of vine, when applied to the grapevine itself, has been reported to improve the wine quality and aroma, volatile and phenolic compound content (Pardo-García et al.

2014a, b). Garcia Martinez et al. (2021) have also reported an increase in the alcohol content and grape yield.

These biostimulants extracted from agro food-based industries have proven effects on the various parameters of plant growth. The hydrolyzed extract developed from the residues of rice husks, apple seeds, and rapeseed was tested for its effect on the growth and quality parameters of Kiwi. An increased fruit weight, ascorbic acid, and antioxidant activity in kiwi was recorded resulting due to the presence of auxins, gibberellins, and cytokinins in the extract (Donno et al. 2013). Colla et al. (2014) tested a protein hydrolate derived from residues of pea and tomato on maize crop and recorded an increase in the shoot length, SPAD index, total biomass, nitrogen content, and induced rooting. Chehade et al. (2017) created a biostimulant extract from fennel, lemon, and brewer's spent grain residues and recorded the effect of the product on the metabolic and agronomic performance of *Solanum lycopersicum*. This biostimulant product was reported to increase the nutrient content, vitamin C content, shoot growth, dry matter, and in *Solanum lycopersicum*. The maize waste was tested for Trans-zetain and other phytohormones by Choez et al. (2014) in order to develop a biostimulant. The residues of blueberry fruit, red grape skin material and hawthorn leaf extracts were studied for their biostimulative effects on maize crop by Ertani et al. (2016) and it was reported that they increased the protein content, chlorophyll, and nitrogen uptake by the crop.

Biostimulants of animal origin are also gaining pace and can comprise protein hydrolysates and amino acids derived from wastes and by-products from animal-based food industries (Mladenova et al. 1998; Rodríguez-Morgado et al. 2014), and chitin and chitosan derived from insects (Sharp 2013). Another animal-based industry that generates vast amounts of waste is the fishing industry. The fishing industry produces enormous amounts of exoskeletons of crustaceans (crab, shrimp, and lobster) that account for 5.9 Mt. global production, out of which 35–45% is discarded (Sharp 2013). Head, skin, fins, frames, etc., consists of over 60% of the biomass of the fish by-product (Chalamaiah et al. 2012). Fish skin is a source of collagen and gelatine and also can be used for extracting protein hydrolysates (Chalamaiah et al. 2012). By-products such as fish head, viscera, muscle, bone, frame, liver, and egg (Chalamaiah et al. 2012) are rich in fats, proteins, amino acids, antioxidants, and have already been valorized as food or feed and are now being explored as plant biostimulants (Halim et al. 2016). Another source of protein hydrolysates is the keratin-rich feather waste (Korniłłowicz-Kowalska and Bohacz 2011). High amounts of feathers of chicken are discarded in poultry slaughterhouses that are the most common keratin waste and have the potential for development of protein hydrolysates (Korniłłowicz-Kowalska and Bohacz 2011). Owing to the biostimulating properties of protein hydrolysates to increase crop quality and quantity, they are now widely accepted in agriculture (Colla et al. 2017). The protein hydrolysates generated by the enzymatic hydrolysis of plant proteins are more preferred over those produced from animal by-products for sustainable farming. Preferring plant-based PHs also at the same time avoids the possible risk of pathogens contamination.

In addition to protein hydrolysates, another class of biostimulants generated by agro-food industries of animal origin is chitin and chitosan. In many countries, seafood is a popular choice of animal protein. Seafood industry generates about 10^6 tons of waste per annum, which is either composted or is converted into animal feeds or fertilizers that are generally low-value commodities (Schmitz et al. 2019). According to Muñoz et al. (2018), annually 2000 tons of chitosan is extracted from the residues of crab shell and shrimp, which can be utilized efficiently for the chitin extraction. The conversion of chitin to chitosan is done by a simple process of deacylation (Nessa et al. 2010). The crustaceans (crabs, shrimps, lobsters, and krill) are the major sources of chitin, out of which shrimp waste is the most significant source of chitin for commercial use (Yadav et al. 2019). Khanafari et al. (2008) used both chemical and microbiological methods of extraction to extract chitin and chitosan from waste of *Penaeus semisulcatus*.

Apart from this, in recent years, several other types of by-products have been reported to enhance plant production and food quality. The pruning process of vineyards generated vine shoots that are rich in volatile, phenolic, and mineral compounds, which, when applied to grapevine, acted as a biostimulant as well as a foliar fertilizer (Sánchez-Gómez et al. 2017a, b). Chehade et al. (2017) reported that the by-products extract from fennel, lemon, and barley imparted biostimulating effect on the fruit quality and yield in tomato. Another study by Ertani et al. (2013b) reported that the by-products extract of castor oil, rapeseed, and flaxseed show biostimulating activity in plantlet growth of maize. The nutrient by-products generated by aquaculture are also reported to have biostimulatory effects. There is a lack of firm evidence to determine whether the effects are due to the added primary nutrients (Nicoletto et al. 2018) or due to the role of microorganisms and dissolved organic matter in water (Delaide et al. 2016).

Biostimulants have also been derived from the microorganisms such as bacteria, yeasts, and fungi. The biostimulant preparations of microorganism origin may be composed of microorganisms and their metabolites. The establishment of the concept of biostimulant preparations based on microorganisms and their metabolites was first reported by Xavier and Boyetchko (2002). Biostimulants are also commonly extracted from various species of algae (mostly seaweeds) (Crouch and van Staden 1993; Aremu et al. 2015). In the process of producing food, fine chemicals, alginate, agar, etc. from the seaweeds, significant amounts of waste are produced, which is generally discarded in the landfills. The waste generated from seaweed is rich in the compounds responsible for promoting the growth of the plant and yield, root development, seed germination, and abiotic stress resistance (Katuzewicz et al. 2018).

7.3 Modes of Action of Biostimulants from Organic Waste

The diversity of source materials and various technologies of extraction makes it difficult to determine a shared mode of action for all classes of biostimulants. The varied characteristics and properties of raw materials used for the biostimulant development and the diverse composition of biostimulant products renders it difficult to identify the exact component(s) accountable for the biostimulating activity and what is the mode of action (Parađiković et al. 2011).

Biostimulants do not substitute, but rather supplement the products used for plant protection and nutrition such as pesticides and fertilizers. Their mode of action is different to that of pesticides and fertilizers, as they do not directly affect the pests, and their mode of operation is also different from that of fertilizers (http://www.biostimulants.eu). According to Brown and Saa (2015), the biostimulants that originate from food and agro-industrial wastes, macro and micro-algae and living microbial cultures are extremely complex, attributing to the multiple components involved resulting into biostimulant products that are poorly characterized and has a limited understanding of its mode of action.

Different categories of biostimulants are known to positively impact the plant productivity through improvement in the physiological processes of the plants (photosynthesis, phytohormone modulation, senescence, nutrients and water uptake) (Khan et al. 2009). Biostimulants are also responsible for activation of genes responsible for abiotic stress resistance, altered plant phenology and architecture (Sharma et al. 2012). In a study reported by Wozniak et al. (2020), under drought conditions, the damage measured by electrolyte leakage of *Arabidopsis thaliana* leaf tissue was reduced by fermentation metabolites present in the biostimulants. Some reports suggest that many biostimulants increase the assimilation of carbon, nitrogen, and sulfur and improve photosynthesis, stress responses, and ion transport, thereby improving the plant productivity (Jannin et al. 2012; Khan et al. 2009; Parađiković et al. 2011). Most of the biostimulants are known to impart the protective effect against biotic as well as abiotic stresses. This protective property has been linked to a reduction of stress-induced reactive oxygen species followed by activation of the plants' antioxidant defense system or elevated levels of phenolic compounds (Ertani et al. 2011, 2013a).

To understand the mode of action of biostimulants, a multidisciplinary and systematic approach is required, where the technologies from the different fields such as biology, genomics, and chemistry need to be considered together (Povero et al. 2016). After the application to the plants, the stages of the plants need to be considered such as tissue penetration, reaction with the plant metabolites, binding to metabolic enzymes, and impact of the compound on the plant's physiology and gene expression modulation (Yakhin et al. 2016).

For a better insight into modes and mechanisms of biostimulant action, Yakhin et al. (2017) have categorized the biostimulant's action on plants into the stages such as (a) penetration of biostimulants into the plant tissues followed by its translocation and transformation; (b) gene expression, plant signaling, and the regulation of hormonal status; and (c) metabolic processes and integrated whole plant effects. The PHs penetrate into the tissues through the diffusion via membrane pores, which is an energy-dependent process (Kolomazník et al. 2012; Parrado et al. 2008). Solubility of the biostimulant is an important property for its efficiency. It must be readily soluble in water and other solvents. Sometimes, to overcome the solubility and lipophilicity, surfactants and other additives may be required (Pecha et al. 2012).

The observations that the biostimulants induce the genes and benefit the plant productivity only under abiotic and biotic stress complicates the pursuit for mode of action even more.

The role of signaling molecules in how a plant responds to the environment has been an active research area. The signal transmission process involves (a) synthesis of signaling molecules (ligands); (b) their translocation, binding to receptors; (c) the resulting cellular responses; and (d) degradation of the signaling molecules (Zhao et al. 2005; Wang and Irving 2011). When the signaling molecule binds to its receptor, it activates the secondary messengers that further leads to consecutive cellular responses.

Biostimulants that have shown to affect the hormonal status of the plants have been developed from complex organic materials, seaweeds, humic substances, antitranspirants, etc. (Du Jardin 2012a). The crude extracts of lower and higher plants have also been demonstrated to have the same effect (Kurepin et al. 2014). It has been reported that biostimulants might induce the de novo synthesis of plant hormones in the treated plants (Jannin et al. 2012). On the other hand, in many biostimulants, compounds such as polysaccharides, glycosides, amino acids, etc. are present that might act as activators/precursors of endogenous plant hormones (Parađiković et al. 2011). Thus, the biostimulants derived from microorganisms, algae, higher plants, animal and humate-based raw material could therefore be responsible for the hormone-like effects or hormonal actions.

Chitin and chitosan are used for the improvement of crop resistance to abiotic stress and pathogen attack, attributing to its potential to induce plant defense mechanisms and initiate the stress response pathways. The chitin molecule activates a key plant defense enzyme, Phenylalanine ammonia-lyase (PAL), that is responsible for accumulation of phenolic compounds in the plants, including papaya (*Carica papaya* L.), sweet basil (*Ocimum basilicum* L.), and sunflower (*Helianthus annuus* L.) (Pichyangkura and Chadchawan 2015). Chitosan is responsible for abiotic stress gene regulation through phytohormone regulators such as abscisic acid (ABA), jasmonic acid (JA), and phosphatidic acid (PA) (Pichyangkura and Chadchawan 2015). A proteomic approach study by Lucini et al. (2018) revealed that chitosan was responsible for phenylpropanoids' biosynthesis in grapes.

7.4 Extraction of Biostimulants from Food and Agro Waste

A number of techniques are being used for using the by-products and wastes generated from agro and food industries in order to extract and prepare biostimulants. These techniques include extraction, cultivation, fermentation, processing and purification, hydrolysis, and high-pressure cell rupture treatment (Halim et al. 2016). The final biological properties, the final composition, and commercial use of the biostimulant greatly depends upon the techniques that are used for extracting or developing the biostimulants and the raw material used (Traon et al. 2014). A number of studies have focussed on valorizing the food and agro-industrial waste considering their high content of high value molecules into the plant biostimulants. They have used various techniques for the extraction and development of the biostimulant extract that is rich in phytohormones, amino acids, protein, and phenolic compounds (Puglia et al. 2021). Sometimes, if different production methods and different sources are used, the result can be a mixture of components.

For producing a commercial product that is uniform, frequently the extracts are utilized rather than the raw biomass through a standardized manufacturing process (Michalak and Chojnacka 2014). The processes being opted for the production of many commercial products are decided and driven by the marketing demands, rather than the target to optimize the efficacy of the commercial biostimulant. According to Lötze and Hoffman (2016), the biostimulants that are derived from same raw materials are usually marketed as similar products, but can significantly vary in terms of their composition as well as efficiency. Craigie (2011) mentioned that the biostimulants produced from the same species of the seaweed extract, are rarely equivalent. Most of the manufacturers do not disclose the process/technique of production of biostimulant, as it is a trade secret (Traon et al. 2014).

For the production of the protein hydrolysates from the by-products and wastes of food and agriculture industries, chemical and enzymatic protein hydrolysis are the most preferred methods (Colla et al. 2014). The technique/process to be used for the protein hydrolysis depends on their source of origin. For example, proteins from animal origin such as horns, feathers, beaks, wool are keratin rich and thus are usually hydrolyzed through acidic or alkaline treatment, often keratinase enzymes of bacterial origin are also used (Pasupuleki and Braun 2010). On the other hand, the animal waste and by-products from whey, intestines, casein and meat, rice, pea, cottonseed, etc. are hydrolyzed by general enzymatic or microbial processes (Dieterich et al. 2014; Pasupuleki et al. 2010). The protein hydrolysates originated from the plants are generally extracted by enzymatic hydrolysis that produce a blend of peptides and amino acids of varying lengths and low salinity (Colla et al. 2015a, b).

The protein hydrolysates derived from organic wastes through acid and alkaline hydrolysis have been widely endorsed as efficient biostimulants for plant. Acid hydrolysis has an advantage of being cost efficient as compared to the alkaline hydrolysis. The complete hydrolysis of proteins through alkaline hydrolysis method is done at a high temperature with the help of chemical agents, viz., sodium, calcium, or potassium hydroxide (e.g., 4 mol/L for 20 h). Alkaline hydrolysis obtains both soluble and insoluble fractions (Dai et al. 2014). The chemical properties of protein hydrolysates produced majorly depend on the source of protein and the production process (Colla et al. 2015a, b).

A novel technique, i.e. Cavitation-assisted extraction (CAE) is also an alternative to the conventional methods such as reflux, percolation, maceration using organic solvents. CAE process adds to the “(a) increase in temperature and pressure resulting into high mass transfer rate; (b) improved diffusion and implosion of agitating bubbles; (c) enlargement of pores; and (d) production of exceedingly reactive free radicals aiding cell disruption” (Panda and Manickam 2019). Xu et al. (2015) have described the effects of pulse electric field on dairy protein extraction. His study reported the cell disruption and extraction of β-LG band. In another recent study by Ghosh et al. (2019), 78 ± 8 mg/mL protein content was generated by the waste meat using high voltage. Fermentation has also been mentioned as an effective method of obtaining hydrolysates from underutilized fish protein by-products (Ruichang et al. 2021).

During the chitin extraction, the extraction conditions determine the various characteristics of purified chitin like degree of deacetylation, molecular weight, purity, and polydispersity index (Yadav et al. 2019). The chemical extraction method has three basic steps (a) an alkaline solution deproteinization; (b) an acid solution demineralization; and (c) discoloration (Garcia et al. 2019). All these steps have an impact on the final chitin product and its physicochemical properties (Ali et al. 2018; Küçükgülmez 2018).

Although the chemical extraction methods are considered uneconomical and detrimental to the environment, known to unfavorably affect the chitin properties, still it is the most common method on the commercial scale. To overcome these disadvantages, biological extraction method is seeking interest due to its economic viability and safer treatment. The biological extraction methods are still limited to laboratory scale only. Tao et al. (2004) used alkali treatment and acid reflux to extract chitinous material from alkali insoluble material and examined it's purity, crystallinity, degree of acetylation (DA), and yield.

The yield was recorded as 0.65–1.15% on a fresh basis. Chitin and chitosan were also extracted through alkali-acid treatment by Khanafari et al. (2008) from *Penaeus semisulcatus* waste collected from a shrimp processing landing center, and the yields recorded were 510 and 410 mg/g, respectively.

7.5 Conclusion

The global agriculture and food production industry is under pressure for meeting the escalating demand of human food, animal feed, and energy production. This leads to the generation of huge amounts of waste and by-products that ultimately find their way into the environment. Finding alternative ways to make use of such material is imperative. In this context, the valorization of the waste produced in food and agro-waste industry into the development of a high value product such a biostimulants. Biostimulants (PB) have their applications in agriculture and are a heterogeneous, complex, and varied group of substances. Biostimulants regulate and modify the plant's physiological processes in order to stimulate their growth, to impart stress resistance and to increase plant yield. Biostimulants can be categorized into those from plant and animal origins, protein hydrolysates, chitin and chitosan, etc. Various techniques are being opted for extraction and development of the biostimulants such as extraction, cultivation, fermentation, processing, purification, hydrolysis, and high-pressure cell rupture treatment. The utilization of the waste generated by the agro and food industry will not only help in valorization and waste management but also provide a substitute for the reduction of agrochemicals.

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Foliar Application of Microbial and Plant-Based Biostimulants on Plant Nutrition

8

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Abstract

Natural or commercial substances or their consortium employed for enhancing the plant growth and quality are referred to as Biostimulants. They boost the physiology of the plant and its entire vital characteristic owing to their activity in enhancing the nutrient availability, membrane stability, root zone stability, osmoprotection, etc. Biostimulant's mode of action has been comprehended by several modern molecular approaches which indicate what changes they bring about in the plant and its physiology at the cellular level. Various amalgamations and formulations of biostimulants are available, but it is their source and composition, based on which they are classified into three main groups, viz. humic substances, hormonal products, and amino acid products. The use of biostimulants, as opposed to fertilizers and chemical treatments, can promote plant growth, increase nutrient use efficiency, and enhance the nutritional content of the products when applied in small quantities in addition to being an ecofriendly and sustainable plant enhancement methodology.

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_8

Keywords

Biostimulants · Foliar applications · Humic acids · Hormonal products · Amino acid · Microbial

8.1 Introduction

There is a continuous effort to increase food production under limited land resources to feed the growing population. This has led to the excessive use of fertilizers and pesticides that has led to environmental pollution and human health hazards. Seeing its consequences on land productivity and human health, several technologies have been put forward which may supplement chemicals and fertilizers and create a conducive environment for nutrient uptake and crop growth. Such environmentally sound nonpolluting substances are the natural biostimulants derived from plant materials, which increase plant growth, production of crops, and efficient utilization of nutrients and also gives the plant the capacity to endure hardship (Youssef and Giuseppe 2020). Thus it can be said that biostimulants are materials that can not only promote plant growth but also increase nutrient use efficiency when applied in small quantities.

Nowadays' use of biostimulants in practices involving agriculture have increased widely in many crops of high value like fruits and vegetables, but still knowledge on its efficiency and mode of action is very limited. Agricultural biostimulants may be derived organically or synthetically, which helps in restoring natural processes within a plant and assist the plant in all growth stages from germination to maturity of a crop. Although biostimulants have been used by fruit growers since a long time, it has only been recently included into crop production system to increase the yield and quality of certain important crops like cereals, corn, oilseeds, soybeans, and more (Naik et al. 2020).

Any substance or microbes that enhance the nutritional efficiency, tolerance to stress, or quality of plant, together with the increase in nutrient content, is called a plant biostimulant. It includes commercially available products having mixtures of such substances and/or microbes. (Calvo et al. 2014; Du Jardin 2015). According to Du Jardin (2012), plant biostimulants are materials that are diverse in character or composition, including microbial and complex organic materials, humic substances and materials obtained from industries, sewage sludge, urban wastes, etc., with elements beneficial to plants like Co, Al, Si, and Se and certain inorganic materials. The organic plant biomass or biostimulants that include seaweed extracts (SWE), mainly the green, brown, and red macroalgae, are the most common seaweed generally used in agriculture as well as horticulture crops (Youssef and Giuseppe 2020). Microalgae as a renewable source of plant biostimulant were proposed by Pasquale et al. 2018.

Biostimulants are found in different formulations and with different ingredients, but are generally classified into three main groups based on their source and composition. This group includes humic substances, hormonal products, and amino acid products. Hormonal products, such as marine weed extracts contain large amounts of active plant growth regulators such as auxins, cytokines, or their derivatives and other microbes that can strongly influence plants' vigor (Du Jardin 2015). The performance of these plant biostimulants varies depending upon the species/cultivars of the same species, rate, or dose (Assal et al. 2019) and their time of application (Kunicki et al. 2010). Assal et al. (2019) reported that foliar application of 300 mL felloton/1000 mL of water produced higher amount of plant growth parameters as well as yield of wheat as compared to control as well as other concentration. Besides this, the performance of bioinoculant also depends upon the timing of application or plant physiological stage (Canellas et al. 2015).

8.2 Mode of Action of Biostimulants

The mode-of-action of biostimulants can be explored at diverse echelons covering comprehensive analysis of the morpho-physiological, biochemical, and molecular characteristics. A biostimulant may be produced from multiple biologically active compounds due to its source materials' miscellany and chemical complexity. Therefore, a combinatorial effort is made to study the mode of action rather than a single approach (Brown and Saa 2015). With the advent of high-throughput omics approaches, it has become feasible to explore the action mechanism of complex combinations of biostimulants (Fig. 8.1). A prominent feature of biostimulants is that they contribute to the stress alleviation system of the plant by maintaining normal growth and development. Consequently, the most effective pathway portraying biostimulatory effects have been investigated and found to belong to photosynthesis, nutrient absorption, and localization, accrual of antioxidant secondary metabolites. The contemporary research attempts have aimed to investigate the biostimulant mode of action through varied molecular tools vis-à-vis genomic, proteomic, transcriptomic, phenomic, and metabolomics analysis (Yakhin et al. 2017a, b).

Gene expression is intensely predisposed to the environment, permitting plants to produce such molecules and compounds, which permit their adaptation and protection from the varying environment when such cues are perceived (Ren and Gray 2015). Over a span of several years, scientists have endeavored to decrypt the gene expression patterns by studying the phenotypic changes induced by biostimulants.

To explore the transcriptional regulations of biostimulants, their involvement in impacting other phytohormones can also be taken into account. *Trichoderma spp* has been used over the years for its biocontrol ability. An expression study was conducted to understand its mode of action as to how it can manage nutrient efficiency, abiotic stress, organ growth, and morphogenesis. This study employed an auxin- and ethylene-related mutant gene expression procedure, which revealed that Trichoderma spp. could modulate the auxin and ethylene-dependent MPK6 activity by regulating root growth and root hair formation (Contreras-Cornejo et al. 2015; Du Jardin 2015; Colla et al. 2015a, b).

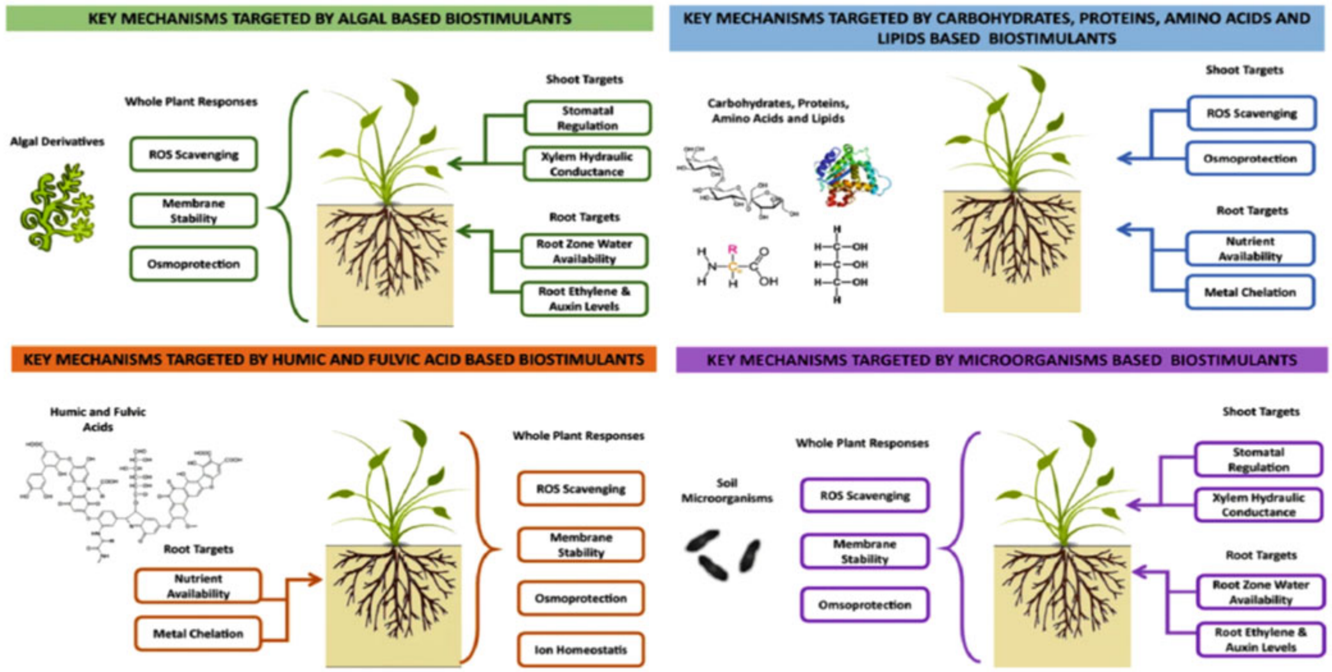

Fig. 8.1 Role of plant and microbial biostimulants in alleviating stress induced by various environment factors (Van Oosten et al. 2017)

To comprehend the action mechanisms of biostimulants at molecular level, effect of biostimulants on plant at the transcriptomics level was assayed. The RNA samples from the biostimulant treated plants' tissues were extracted, followed by cDNA library construction. These cDNA library were further subjected to experimental procedure through multiple approaches. One approach in the absence of prior sequence information is the cDNA-amplified fragment length polymorphism (cDNA-AFLP) enabled genome-wide expression analysis, (Vuylsteke et al. 2007). A cDNA-AFLP based study to identify the candidate genes of *Arabidopsis* treated with plant-humic substance uncovered differential expression of 133 transcript fragments functionally associated with stress response and auxin signaling (Trevisan et al. 2011).

Semi-quantitative RT-PCR-based approach was utilized in maize (*Zea mays*) seedlings treated with animal-derived protein hydrolysate (PH) to evaluate the relative expression of transcript of enzymes associated with C-metabolism and N-assimilation and (Ertani et al. 2013; Schiavon et al. 2008). Transcriptomic approaches like quantitative real-time PCR (qRT-PCR) and microarray data analysis permit the investigation of patterns of gene expression in a massive quantity of genes simultaneously. These methods have been used to unveil the mode-of-action in *Brassica napus* employing global transcriptional changes. They have been successful in obtaining above 300 differentially expressed genes (DEGs) by treatment for 3 days with a biostimulant prepared from humic acid (HA7) (Jannin et al. 2012). In the same species, a three-day treatment of seaweed *Ascophyllum nodosum* extract (AZAL5) induced 724 DEGs in the shoot and 298 DEGs in root (Jannin et al. 2013). In *Arabidopsis*, ANE A, a commercially used seaweed extract biostimulant, was found to regulate 1011 DEGs (Goni et al. 2016). In tomato (*Solanum lycopersicon* L.), treatment with alfalfa-based biostimulant exhibited 1938 DEGs in leaves and 1054 DEGs in roots (Ertani et al. 2017). In a similar study conducted on maize, microarray analysis of PH- treated plants identified 587 DEGs when compared with N-treated plants and 431 DEGs when compared with AA-treated plants (Santi et al. 2017). Functional annotation of these DEGs to ascertain the effects conferred on the physiological level by the biostimulants revealed common functional groups, including cell wall components, stress responses, transcription factors involved in root development and N availability, transport processes, and hormone metabolism (cytokinin[CK], auxin and gibberellin [GA]) (Jannin et al. 2012; Jannin et al. 2013; Goni et al. 2016; Ertani et al. 2017; Santi et al. 2017). These findings through transcriptomic approaches on the different tissue types established that a range of biostimulants can improve plant growth and yield by functioning as a trigger molecule to escalate gene expression levels in stress-related genes by upregulating genes associated with nutrient mobilization and metabolism.

Next generation sequencing is another high-throughput technology which is currently in very high demand for transcriptome profiling of a particular cell type, tissues, or developmental stage, under a range of stress situations (Villar et al. 2011; Guo et al. 2014; Senthil et al. 2015; Xu et al. 2012). With respect to detection of any biostimulant's mode of action, NGS derived transcriptome data can give us an idea as to which gene/allele may be responsible for expression of a particular response in the plant. In maize, sequencing the plant tissue after application of a plant biostimulant demonstrated that its application could activate such genes which can, in turn, stimulate stress responses in the maize plant (Trevisan et al. 2017).

The total protein expressed by the genome represents its proteome (Wilkins et al. 1996) and proteomics-based approaches have been used to estimate the response of plants to the application of biostimulants. The effects of ROS on plant defense response proteins was unraveled using proteomics approach (Tanou et al. 2009). Using proteomics study, the effect of biostimulant under salt and zinc stress was elucidated in lettuce plants (Lucini and Bernardo 2015). In citrus, pretreatment with biostimulant, followed by exposure to salt stress, exhibited reduction in the effects of oxidative stress, which is evident from the decrease in the accumulation levels of S-nitrosylated and NaCl-response proteins (Tanou et al. 2009). Proteomics approach using 2D PAGE and Chromatography on a biostimulant produced from an auxin-like compound, melatonin, revealed cold stress tolerance in maize (Kołodziejczyk et al. 2016). Hydropriming of seeds with melatonin was able to induce cold stress resistance in maize by improving the overall metabolism. This was revealed by analyzing the defense related proteins which changed the overall proteome of maize under cold stress, which, in turn, improved the crop growth and survival (Kołodziejczyk et al. 2016). Trichoderma spp., which is known for its biocontrol ability and growth-promoting activity, have also demonstrated to increase the abiotic stress tolerance in vegetables by regulating nutrient uptake, thus exhibiting its biostimulatory property which have been confirmed by proteomics studies (Colla et al. 2015a, b; Rouphael et al. 2017a, b). In another proteomics study, 28 proteins involved in protein synthesis, protein folding, ethylene biosynthesis, isoprenoid biosynthesis and ROS scavenging activity were identified from *Trichoderma asperellum* strain T34 inoculated in cucumber plants (Segarra et al. 2007). Trichoderma biostimulant also exhibited to upregulate the proteins involved in photosynthesis, metabolism, and defense mechanism (Shoresh and Harman 2008). Similar studies for the proteomic profiling of host pathogen interaction was conducted in common bean inoculated with *Trichoderma harzianum* (ALL-42) as biostimulant (Pereira et al. 2014).

Metabolites are produced as a response of an organism to environmental stimuli generally at the end of a cellular process (Fiehn 2002). The metabolome comprises a cumulative of all metabolites which maintain the normal function of the plant by participating in the metabolic processes that arise as a response to environmental stress (Dunn and Ellis 2005). Metabolites can be classified into the primary metabolites, which are essential for maintaining processes like photosynthesis and respiration, and the secondary metabolites, which are produced as a response to stress environment and become essential for the survival under such conditions (Aharoni and Galili 2011; Wolfender et al. 2013). The impact of biostimulants on the plants' metabolism can be assessed through metabolic profiling, which can deliver better data as compared to transcriptomics analysis. This may be due to the fact that metabolic profiling gives us the necessary idea about the physiological and morphological response that a plant would exhibit in response to a biostimulant treatment, whereas DEGs obtained through transcription profiling may not produce an actual change in plant behavior or responses (Fiehn et al. 2000; Urbanczyk-Wochniak et al. 2003). Biostimulants can modulate the N and C biochemical pathway by regulating the activity of enzymes involved in N assimilation, Krebs cycle, and glycolysis (Ertani et al. 2014). On the other hand, biostimulants are also capable of regulating secondary metabolites and enable the plants to adapt and combat stress conditions. Metabolic profiling in biostimulant-treated lettuce reported the expression of carbohydrates, phenolic compounds, phytohormones, glucosinolates, and lipids under zinc or salt stress conditions (Rouphael et al. 2016). While, the metabolic profiling in bell pepper treated with biostimulants exhibited the generation of phenolic acids, ascorbic acid, and β-carotene (Ertani et al. 2014). Over the years, chitosan has proved to be an effective biostimulant owing to the fact that its application mimics a pathogen attack and thus acts as an elicitor to activate the plant defense mechanism (Hadwiger and Beckman 1980). Another metabolomics analysis revealed that biostimulants improved the inhibitory effects on phytochemical contents under nutrient deficiency conditions suggesting an improvement of secondary metabolite contents (Aremu et al. 2014; Lucini et al. 2019).

8.3 Plant Biostimulants on Crop Growth and Yield

As stated earlier, plant biostimulants mainly categorized into humic and falvic acid, protein hydrolysates and other N-containing compounds, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds like Al, Co, Na, Se, and Si, beneficial fungi like Trichoderma spp. and AMF, bacteria like Rhizobium and ‘plant growth-promoting rhizobacteria’, etc. (Du Jardin 2015). All these plants’ biostimulants are known to have a great impact on crop growth and yield. The spraying of plant growth-promoting bacteria, viz. *Herbaspirillum seropedicae* in combination with humic acids only in later stage of crop growth increased the root dry weight as well as maize grain production (Canellas et al. 2015). The production of more root growth was due to the presence of auxin and auxin-like compounds in *H. seropedicae* (Monteiro et al. 2012). The Plant growth promoting rhizobacteria (PGPR) also enhanced growth as well as yield of pea under organic cultivation, (Ahmet et al. 2010).

In maize, irrespective of method of application, the PGPB, especially *Azotobacter chroococcum*, significantly enhanced the chlorophyll content as well as photosynthetic rate of the crop. However, the effect was more pronounced under soil application than the foliar application (Efthimiadou et al. 2020). The beneficial effect of PGPB might be due to its ability to produce more amount of plant hormones (Egamberdiyeva 2005, Garcia de Salamone et al. 2001, Gutierrez-Manero et al. 2001, Sahin et al. 2004) and solubilization of nutrients (Jeon et al. 2003).

The foliar spray of plant biostimulants such as Bio-1 was more effective under the adequate soil potassium supply that resulted in enhancement in growth parameters like total shoot leaf area, shoot length, and biomass of almond tree (Saa et al. 2015). Among the plant biostimulants, the chitosan is a natural biopolymer derived from chitin known for its ability to enhance plant growth and development (Mondal et al. 2012); however, very limited research was done in this aspect. The okra crop when fertilized with chitosan at 100 or 125 ppm at early growth stage of crop growth helped in achieving the maximum fruit yield as well as increasing the fruit quality parameters like number of fruits per plant, fruit length, individual fruit weight, etc. (Mondal et al. 2012). In an experiment, when different concentration of chitosan were sprayed on crops like tomato, green gram, and maize, it greatly enhanced biometric parameters as well as yield and yield-attributing characters as compared to control (Islam et al. 2016). The augmentation in seed yield in rice and soybean was also reported by Chibu et al. (2002). They opined that chitosan influenced the early stage of crop growth and ultimately increased the crop yield. The spraying of seaweed extracts, viz. *Duvillaea potatorum* and *A. nodosum* increased the plant density as well as root length in strawberry crop (Mattner et al. 2018). The plant biostimulants enhanced the growth parameters even under nutrient stress condition (Soppelsa et al. 2019). The spraying of different groups of plants biostimulants such as vitamins, chitosan, and silicon and alfalfa hydrolysate promoted almost four- to seven-fold increase in root biomass as well as 20% increase in strawberry yield. While, the B-group vitamins showed its efficiency in enhancing the leaf area besides increasing the root growth of strawberry (Soppelsa et al. 2019). The spraying of seaweed extract *A. nodosum* also increased the root growth and tolerance capacity of strawberry plants against iron deficiency (Spinelli et al. 2010). The foliar spray of plant-based biostimulants, i.e. legume-derived protein hydrolysate (LDPH) and tropical plant extract (TPE) expanded the leaf surface of baby rocket plant (*Diplotaxis erucoides* L.) by 66% and 80%, respectively (Mola et al. 2019). The LDPH was also found to increase the green pigment in crops like baby lettuce, brinjal, and maize (Tadros et al. 2019; Carillo et al. 2019), perhaps because of the presence of abundance of amino acid in LDPH-treated plants that helped in enhancement of chlorophyll pigment as well as net photosynthesis rate of crop. The foliar application of sole or combined application of tropical plant extracts (TPE) or protein hydrolysates significantly increased the total yield as well as dry biomass yield of perennial wall rocket during harvest (Giordano et al. 2020). However, no significant difference was observed in the soil plant analysis development (SPAD) throughout the growing period of perennial wall rocket.

The plant-derived biostimulants such as root extracts of Licorice (*Glycyrrhiza glabra*) also played a significant role in enhancing the vegetative growth of almond seedlings (Giordano et al. 2020; Babilie et al. 2015).

Nowadays, some synthetic biostimulants are also applied in crops for boosting the crop growth and yield. The synthetic biostimulants mainly include growth regulators, inorganic salts, essential elements, phenolic compounds, *etc* (Przybysz et al. 2014). For example, the foliar spray of 0.3% Atonik, a synthetic biostimulant, improved the yield without any negative effect on their qualities of bean (Kocira et al. 2017). Smidova (1960) stated that potassium humate was a good plant growth biostimulant that greatly increased the physiological parameters, including photosynthesis and plant root respiration, resulting in greater plant growth and yield.

Microbial inoculants were also known for increasing nutrient availability to the crop and for enhancing nutrient use efficiency of applied chemicals because of production of higher yield further deterioration in the quality of the produce (Adesemoye and Kloepper 2009). Numerous studies have reported crop yield increase due to the use of different biostimulants (García Martínez et al. 2010). The yield and quality of the produce varies depending upon the kind, dose, time, and method of application of biostimulants (Panfili et al. 2018). Tarantino et al. (2018) reported effective results of biostimulants such as carboxylic acid, humic acid, and fulvic acid on yield of apricot. However, polysaccharides-based biostimulants could not show promising effect on the same variety of apricot trees. Parađiković et al. (2011) reported yield increase of vegetables and two varieties of yellow pepper with a biostimulant produced by a mixture of polysaccharides, amino acids, humic acids, vitamins, organic carbon, and enzymatic proteins. The mixture also brought about a favorable condition in the soil plant system which helped in achieving the desired effect.

A good size and healthy looking fruits and vegetables are always preferred by customers and sellers. Fawzy (2012) reported that the use of combinations of amino acids, nitrogen, and auxins increased the average weight of cucumbers. Concentration of humic acid at 3 g/L increased the average fruit size by 9.9 cm and 12.2 cm in the first and second season, respectively. Similarly, the diameter of the cucumbers was also increased by 1.23 and 1.55 cm during the first and second season crop, respectively. A biostimulant containing auxin, amino acid, and nitrogen helped in the elongation of cucumber by 3.85 cm and 3.49 cm in the first and the second season, respectively. An increase in the diameter of the cucumbers by 1.12 cm and 1.56 cm in the first and the second season, respectively, was also reported. Tarantino et al. (2018) reported a ten-fold increase in the size of apricot fruit by using biostimulants humic, fulvic, and carboxylic acids. The greatest effect was contributed by carboxylic acid, which led to the increase in breadth of the fruit by 2.6 mm. Consortia of microorganisms have proved to be efficient biostimulants on growth and quality of fruits and vegetables. Bona et al. (2018) reported that species Rhizophagus, Claroideoglomus and various other species of arbuscular mycorrhizal fungi, have shown very good result in increasing the average weight of tomatoes.

8.4 Implications of Biostimulants for Abiotic Stress Tolerances

Abiotic stresses, such as low or high temperature, deficient or excessive water, high salinity, heavy metals, and ultraviolet radiation, are detrimental to plant growth and development, leading to reduction in crop yield and to strive under such unfavorable conditions, plants can modify their cellular, physiological, and molecular conditions (Huang et al. 2013).

Hostile environmental conditions like drought, acidity, salinity, extreme temperature, etc. are responsible for 70% of yield reduction influenced by the climate change scenario of the globe (Wang et al. 2003). In these contexts, these abiotic pressures are supposed to exert an increasingly deleterious impression, causing serious misfortunes on crop productivity, and consequently, food-security (Rouphael et al. 2018b). The effectiveness of biostimulants to fight stress conditions depends on various conditions, for instance, the time of application and mode of action of the stimulants. The biostimulants may be applied as foliar applications within various time frames, viz. before the effect of stress is visible on the crop (as a prophylactic measure), during the stress, or even after removal of the stress. Depending upon the objectives and desired results, application of biostimulants may be done in different stages of the crop right from the seedling stage to the matured stage (Kunicki et al. 2010). As a thumb rule, the biostimulant compounds or products that have anti-stress properties, viz. glutamic acid or proline, must be applied during the stress period. Conversely, those that are entangled in the stimulation and activation of bioactive compounds' biosynthesis have to be used or applied before the occurrence of stress condition (Franzoni 2021). Timing of foliar spray depends upon critical growth stages of crop and that varies from plant to plant and species to species. Therefore, to find out the correct time of foliar application of biostimulant is equally important as the fixing of the precise dose of application to enhance their performance, to prevent wastage of chemicals and thereby reducing application costs and preventing unexpected results. However, there is no fixed formula that operates for a particular plant species in various stressful situations.

The application of microbial- and plant-based biostimulants to enhance plant stress tolerance mechanism has been suggested as one of the most promising techniques to get rid of this problem (Rouphael et al. 2018a). Foliar application chemicals containing amino acids and soluble peptides were found to provide drought tolerance in tomato grown in controlled condition (Paul et al. 2019a, b; Rouphael and Colla 2020). The mechanism tolerance may be due to (1) improved tolerance to increase of Reactive Oxygen Species (ROS) facilitated and (2) intonation lipids profiles and phyto-hormones. Casadesús et al. (2019) also reported that the foliar application of pepton on water-stressed tomato plants benefited protection of antioxidants and provided a major phyto-hormonal effect in leaves of tomatoes grown in water-stressed conditions by enhancing the content of endogenous cytokinin, auxin, and jasmonic acid. Lee et al. (2019) also studied the effects of foliar spray of biostimulants like methyl jasmonate, salicylic acid, brassinosteroid, sodium-nitroprusside on some physiological as well as photosynthetic characteristics and antioxidant enzyme activity of cabbage subjected to temperature stress. These biostimulants decreased physiological injury and boosted the action of antioxidant enzymes, thus improved stress-tolerance in the crop. Tommaso et al. (2021) reported that *Ascophyllum nodosum* extracts as foliar spray in soil moisture limiting conditions might be a better tool to enhance physiological activities of grapevine while conserving integrity of photosystems and vineyard resilience even during severe water stress conditions. Several other workers throughout the globe have also extensively studied the role of foliar spray of biostimulants on plant stress tolerance mechanisms. Some of their findings are listed in Table 8.1.

Apart from the conventional ways of mitigating abiotic stresses through application of plant growth regulators like gibberellins, cytokinins, etc., biostimulants nowadays play a great role not only in increasing productivity but also helps in

Table 8.1 Foliar application of different biostimulants to cope with various kinds of stress

Sl No	Type of stress	Crop	Type of biostimulant	Mode of application	References
1	Salt stress	Maize	Humic acid	Foliar spray	Kaya et al. (2018)
2	Drought stress	Lima bean	-do-	Foliar spray	Beheshti and Tadayyon (2017)
3	Drought stress	Repressed	Fulvic acid	Foliar spray	Lotfi et al. (2015)
4	Drought stress	Common bean	Chitosan	Foliar spray	Abu-Muriefah (2013)
5	Growth room condition	Potato	Phosphite	Foliar spray	Burra et al. (2014)
6	Drought stress	Soybean	Seaweed extract	Foliar spray	Shukla et al. (2016)
7	Drought and heat stress	Wheat	Plant extract of *Brassica* family crops (Exp. mustard) sorghum & sunflower crops	Foliar spray	Farooq et al. (2017)
8	Salt stress	Wheat	Leaf extract Moringa plant	Foliar spray	Yasmean et al. (2013)

regulating and modifying the functional processes in plants to enhance growth and escape unfavorable conditions (Yakhin et al. 2017a, b). Different commercial products of biostimulants can offset environmental stress such as moisture stress, osmotic stress, exposure to sub-optimal growth temperatures and heat stress, etc. in several ways (Du Jardin 2015; Pokluda et al. 2016; Le Mire et al. 2016; Van Oosten et al. 2017). Biostimulants interact with several processes involved in plants' responses to stress, and enhance the accumulation of antioxidant compounds and thus improves stress tolerance (Franzoni 2021).

In present days, protein hydrolysates (PH), that contained free amino acids and signaling peptides are extensively used in agricultural crops largely for improving plant nutrient uptake, growth, yield, and fruit quality and also for their ameliorating effect on crop tolerance to abiotic stresses (Colla et al. 2015a, b). A legume-based protein hydrolysates which contained soluble peptides and amino acids applied as foliar and drench helped to reduce the adverse effect of drought in tomato by improving the use efficiency of transpiration (Paul et al. 2019a, b). Casadesús et al. (2019) evaluated the effect of hormones in animal-based protein hydrolysate containing L-a amino acids, free amino acids, iron, potassium, and organic nitrogen in tomato grown under water stress condition. Volpe et al. (2018) reported that tomato plants could tolerate drought stress through application of microbial biostimulants derived from arbuscular mycorrhizal fungi (AMF), and the effect of two strains of AMF *Rhizophagus intraradices* and *Funneliformis mosseae* were also assessed. Several PGPR (Bacillus spp.) isolated from durum wheat rhizosphere were cultivated in an extreme saline condition and showed a number of growth assisting characteristics (Verma et al. 2018).

A number of strains of these PGPR were also able to improve plant growth-attributing characteristics of mung bean. Tolerance to salinity can be enhanced by use of microbial and non microbial plant biostimulants. Polysaccharides from brown seaweed or acidic polysaccharides increased the tolerance of wheat seedlings against salinity (Zou et al. 2019). This was attributed to the increase in chlorophyll content, decreased membrane lipid peroxidation, and improved antioxidant activities. They also reported that polysaccharides derived from both brown algae and red algae (Pyropia yezoensis) were proved to be efficient in eradicating the negative effects of salinity on wheat seedlings grown under saline conditions.

8.5 Enhancing Nutrient Uptake and Translocation

Biostimulants can be applied as soil application either as granules or powders or it may also be applied in the leaves as foliar spray in the form of solutions (Kocira et al. 2018a, b). Humic substances and nitrogen containing biostimulants can be best used as soil application, but biostimulants containing different types of plant extracts and sea weeds are used as foliar spray. Incorporation of biostimulants in irrigation water enables the plant to draw up such nutrients along with water. Kocira et al. (2018a, b) reported a biostimulant Kelpak SL (Ecklonia maxima extract) which showed prominent result when sprayed on Phaseolus vulgaris as an aqueous solution. According to Tejada et al. (2016), application of biostimulants as foliar application is more effective compared to soil application. Maize leaves showed an increasing level of macro and micro nutrients when a biostimulant obtained from sewage sludge was applied as foliar spray in maize plant. There was also an increase in nitrogen content in maize leaves by 26%. Foliar spray of biostimulants is done in the morning when the stomata are open and the rate of assimilation of nutrients is highest (Goñi et al. 2018).

Many workers have reported that the biostimulants prepared from marine weeds and microbial cultures and extracts might stimulate nutrient uptake and translocation (Parađiković et al. 2011; Dodd and Perez-Alfocea 2012; Jannin et al. 2013; Calvo et al. 2014; Sabir et al. 2014; Yakhin et al. 2017a, b; Rouphael et al. 2018). The mechanisms which may describe the penetration of foliar-applied biostimulants through the leaf surface is rather complex and is influenced by the size and polar nature of the applied molecules (Fernandez and Brown 2013). The initial barrier to leaf penetration is the negatively charged, hydrophobic cuticular layer which hinders the entry of cations and hydrophilic molecules (Fernandez and Brown 2013). It is supposed that the stomata and trichomes available in the cuticle might also act as an entry-route of small molecular weight compounds inside the plant cell (Eichert et al. 2008). The apoplastic room present in plant leaves is covered by a negatively charged exchange complex which, besides, may interact with cationic molecules, limiting their movement in cell. The metabolism of leave-absorbed compounds in the apoplast is not well understood, even so, depending upon the chemical composition and concentration of the applied biostimulants may influence plant metabolism unswervingly through the supply of nutrients, metabolites, or molecules that corrects mineral deficiencies or alter metabolic pathways or indirectly providing temporary effects on cellular pH of electrochemical balance (Saa et al. 2015). Even after discussing all these, it is worthy to mention that though, in recent years, biostimulants are highly promoted and even used in many agricultural operations, more particularly in advanced countries, for growing high-value fruits and vegetable crops, their efficacy or mode of action is not yet clearly understood. Predicting plant response to the application of biostimulants is complex due both to the uncertainty surrounding the foliar absorption and the lack of knowledge of the mode of action of these products. Drobek et al. (2019) reported that during the last few years, different strains of bacteria have been used in the preparation of biostimulants out of which species such as Arthrobacter, Enterobacter, Pseudomonas, Rhodococcus, and Bacillus proved to be very promising. Plant growth-promoting bacteria are commonly applied as foliar spray over the leaves of the crop. Foliar application brings about an interaction between the microorganism and the plant leaf surface (Preininger et al. 2018). Foliar spray can be done throughout the season at critical growth stages to improve the growth of the plant or under certain conditions to combat plant diseases. The mechanism of absorption of foliar-applied nutrients is complex and is greatly affected by the polar nature and size of the applied nutrient molecule (Fernandez and Brown 2013). The stomata and trichomes are present in the cuticle which favor the entry of small molecular weight compounds (Eichert et al. 2008). According to Calvo et al. (2014), efficiency of foliar application of Zinc to crops can be increased by addition of plant biostimulants. Tian et al. (2015) reported that foliar applied microbial inoculants along with Zn greatly increased the efficiency of foliar Zn application in sunflower. Leaf Zn content was also found to increase when amino acid chelate was applied in pear (Koksal et al. 1999). Foliar application of Zn with various biostimulants, like amino acids, fulvic acid, seaweed extract, and microbial incubates significantly increased Zn concentration in wheat grain confirming the compatibility of Zn with various biostimulants. Besides, it was also concluded that Zn + AA and Zn + FA, when applied as foliar spray, increased Zn concentration above 45 mg kg^{-1} (Wang et al. 2020).

Zodape et al. (2011) found significant increase in the concentration of macro (13.24–67.50%) and micro (23.84–42.61%) nutrients over control by foliar application of a seaweed sap (*K.alverezii*) in tomato. Nutrient uptake by fruit and shoot was also found to increase along with the resistance to diseases brought about by leaf curl bacteria and fruit borer. Foliar application of biostimulants like protein hydrolysates and seaweed extract (brown microalgae: Eklonia maxima) showed a promising effect in increasing productivity and NUE in baby lettuce grown under greenhouse condition and low input cropping systems (Di Mola et al. 2019). The same research group also worked on two other leafy vegetables, viz. baby spinach and lamb's lettuce, and treated with a legume-based protein hydrolysate under optimum N condition and found that foliar application of this biostimulant significantly increased N uptake and N use efficiency for both the leafy vegetables (Di Mola et al. 2020). Foliar spray of humic acid was found to be very effective in calcareous soil in maize crop and significant increase in the uptake of primary, secondary, as well as micro nutrients was also observed with its application (Celik et al. 2011). However, promising results are dependent on concentration, source, frequency of application, and species of the plant (Nardi et al. 2002).The efficiency varies from plant to plant. It may be beneficial to one crop, but may not be so effective in another (Vaughan and McDonald 1976).

8.6 Improving Nutrient Use Efficiency

Nutrient Use Efficiency (NUE) of a plant not only depends on its ability to uptake nutrients efficiently and effectively but also depends upon their internal transport in plant tissues, storage, and remobilization (Prieto et al. 2017). Foliar application of biostimulants can provide essential nutrients to plants, thereby improving the ability of plants in ingesting nutrients through the leaves and is thereby supposed to improve the NUE of Plants (Rouphael and Colla 2020).

There are great evidences that there is increase in use efficiency of nutrients and productivity of crop by biostimulants by making the best use of added fertilizers and proper utilization of residual nutrients. According to Halpern et al. (2015), there are four major groups of biostimulants which greatly affect growth of roots and uptake of nutrients. They are (1) protein hydrolysate (2) humic substances (HS) (3) different types of amino acid (AA), (4) plant growth promoting microorganisms and (5) seaweed extract (SE).

Humic substances (HS) possess characteristics that can favor nutrient uptake in plants and increase nutrient use efficiency. Nitrate uptake has been found to increase by application of HS (Nardi et al. 2000). Sources of HS from different parent materials proved to be efficient in improving the uptake of Nitrogen, Phosphorus and micronutrients like Manganese, Copper, and Zinc throughout the growing season in barley (Ayuso et al. 1996). Lee and Bartlett (1976) reported that P and Fe were efficiently taken up by maize when HS were applied in soils with low organic matter. Many researchers reported very good response of micronutrient uptake, especially in micronutrient deficient soils (Sanchez-Sanchez et al. 2005). Fe and Zn solubility can be increased by adding HS as a result of metal humic complexe formation (Chen et al. 2004). HS also increases P availability by inhibiting ca-phosphate formation (Delgado et al. 2002).

The use of N fertilizers may be decreased by the use plant biostimulants containing amino Acids (AA) that restored NO_3 assimilation enzyme and work as chelators most effectively. They can also be effectively used for eradication of micronutrient deficiencies by forming micronutrient chelate (Halpern et al. 2015). Amino acids enhance the efficiency of foliar application of micronutrients like $FeSO_4$ against chlorosis in grapevine (Maini 2006). Some AA can act as a reductant and increase the availability of micronutrients. The uptake of Cu by maize crop was increased by application of cystein due to which Cu II was converted to Cu I, thus making Cu available to the plant (Zhou et al. 2007).

Seaweed extract (SE) is considered as an important biostimulant that is known for its stimulating effect for increasing nutrient use efficiency in many crops. For many years, seaweeds have been used as fertilizers in areas near the sea (Craigie 2011). Booth (1969) first patented the methodology for liquefying seaweed to be used for agricultural purposes. A brown seaweed, Ascophyllum nodosum, is commonly used for manufacturing liquid seaweed extract, although species like Durvillaea potatorum, Macrocystis pyrifera, Durvillaea Antarctica, etc. are also used (Khan et al. 2009). Plant growth was found to increase by application of SE (Van Staden et al. 1994). Besides**,** positive effect was also seen in flowering and yield (Eris et al. 1995), germination of seeds (Kumar and Sahoo 2011), and plant protection against pests and pathogens (Jayaraman et al. 2011) by application of SE. In soybean grown under rainfed condition, SE derived from red algae increased the Nitrogen, Phosphorus, Potassium, and Sulfur concentration in grains by 36%, 61%, 49% and 93%, respectively.

The plant growth-promoting bacteria are found near the rhizosphere which helps in promoting plant growth under different conditions, and control of pathogens (Bashan and Bashande 2005), increase tolerance to salts (Alavi et al. 2013), and resistance to heavy metals (Lucy et al. 2004)**.** PGPB favors N fixation in legumes and solubilization of P (Vessey 2003). Gamalero and Glick (2011) also reported that Phosphorus availability can be increased by mineralization of organic P by bacteria. Siderophores, which are produced by certain bacteria, chelates Fe and renders it more soluble (Masalha et al. 2000). Sharma et al. (2003) reported that Fe uptake in mung can be increased by Pseudomonas sp. by producing siderophores.

However, it is reported that nutrition efficiency, tolerance to stress, crop yield, and product quality can be improved by application of certain products derived from fungal origin (Calvo et al. 2014). The main problem in using such products is the difficulty in mass scale production of AMF due to their biotrophic character (Dalpé and Monreal 2004). There is convincing evidence that many plant responses are also induced, including increased tolerance to abiotic stress, nutrient use efficiency, and organ growth and morphogenesis (Colla et al. 2015a, b; Shoresh et al. 2010). Based on these effects, these fungal endophytes may be regarded as biostimulants, though their agricultural uses are currently supported by claims as biopesticides only. Similarly, interaction of plants and bacteria also influence plant life cover in the biogeochemical cycles through supply of nutrients, increasing nutrient use efficiency, induction of disease resistance, enhancement of abiotic stress tolerance, modulation of morphogenesis by plant growth regulators, etc. (Ahmad et al. 2008). When biostimulants aim at increasing NUE, combinations between fertilizers and biostimulants must be optimized for their compatibility. Nevertheless, practical challenges comprise the formulation and mixing of biostimulants with other fertilizers and/or plant protection chemicals. In this concern, positive interaction between microbial compositions of the biostimulant mixtures on the one hand, and between the biostimulant inoculants and the resident rhizosphere and phyllosphere microflora, on the other, must be studied in detail (Rouphael and Colla 2020). Proper observation of the crops and for deciding ‘4R’ nutrient stewardship provides a framework to achieve the optimum effect of biostimulants that may include the right combination at the right time, at the right rate and in the right place. However, the situation is complex when the foliar application of biostimulants must be applied with a crop protection chemical, for which the incidence of pests and diseases is comparatively easier to detect and compute, and for which calculated models are available to optimize the application of the pesticides. NUE is also complicated to appraise to judge the application of a biostimulant that would aim for this trait, because it is barely supported by measurable plant attributes in the field.

8.7 Improving Nutritional and Nutraceutical Quality of Plant Products

The expediency of biostimulants for a wide range of crops has already been discussed by several notable studies and pronouncements (Beckett et al. 1994). A number of authors have explored the application of biostimulants on enhancing the nutraceutical and nutritional aspect of the plant and its products (D'Amato et al. 2018; Koyama et al. 2018; Soppelsa et al. 2018; Vergara et al. 2018). A major portion of the primary and secondary metabolism in plants can be regulated by the utilization of both microbial and non-microbial biostimulants (Colla et al. 2015a, b; Rouphael et al. 2015). This encourages the accrual of several important compounds in the plant like vitamins, antioxidants, acidity, micronutrients, minerals, secondary metabolites, etc. which in turn upgrade overall nutritional quality of the human diet (Singh et al. 2018). Biostimulants derived from yeast extract, chitosan, arachidonic acid, jasmonic acid, and aminobutyric acid, in addition to Nano-gro and Kelpak, have revealed promising results on the nutraceutical and nutritional quality of the food produced from those plants (Kocira et al. 2015; Złotek et al. 2016).

Biostimulant's application is widely recognized to increase the content of dissolved solids in the plants which, in turn, lead to better taste (Grajkowski and Ochmian 2007). The application of a biopolymer containing humic, fulvic, and carboxylic acids with polysaccharides as biostimulants in apricots demonstrated an increase in the dissolved solid content reflected by an increase in the brix content from 10.7 to 14.1 brix, improving its overall taste (Tarantino et al. 2018). The concentration of dissolved solids in the fruits of raspberry was found to be reduced through the application of phenolic complexes or chitosan-derived biostimulants. On the contrary, an increase in the dissolved solids content was observed when the biostimulants used were derived from titanium compounds (Grajkowski and Ochmian 2007). The application of plant-based biostimulants increases the dissolved solids by 10–15 times the titratable acidity and this rise is directly proportional to the nutritional quality of the fruits (Parađiković et al. 2011; Kulkarni et al. 2019).

Acidity is an important modulator of fruit taste and its preference mostly varies from consumer to consumer. Therefore, no conclusive statements can be made if the changes in the acidity of fruits brought about by the application of biostimulants can be interpreted in positive or negative terms. Use of biostimulants from phenolic and titanium compounds leads to an increase in the acidity in raspberry fruits

(Grajkowski and Ochmian 2007). Whereas, the application of polysaccharides, fulvic and humic acids, and carboxylic acid-enriched biostimulants reduced the fruit acidity in apricot (Tarantino et al. 2018).

Nitrogen is an important growth modulator in plants, while being an indispensable component of nucleic acids and building blocks of amino acids and proteins (Jamtgard et al. 2010). The protein content of a plant is mainly significant in the case of grain plants. Application of biostimulants from chitosan and phenolic and titanium compounds leads to an increase in the level of nitrates in raspberries (Grajkowski and Ochmian 2007). Biostimulants formed by the hydrolysis of a combination of chicken feathers and nitrogenous fertilizers increase the protein and nitrogen content of maize (Lichtfouse et al. 2009; Tejada et al. 2018; Nasir et al. 2016). Another biostimulant prepared from sewage sludge causes increase in the protein content in maize (Tejada et al. 2016). *Moringa oleifera*, in combination with K_2SO_4 and $ZnSO_4$, proved to be an efficient biostimulant for mandarin trees, leading to an increase in the nitrogen content (Nasir et al. 2016). In another study, spraying of 0.1% Atonik, a synthetic biostimulant, on beans leads to a reduction in the overall albumin and globulin contents without any further decrease in nutritional quality (Candela et al. 1997; Nestares et al. 2001). This decrease in the protein content may be a result of stress response in the plant. This finding conforms to that of similar observation in potato and celery (Czeczko and Mikos-Bielak 2004). However, several studies have reported that the application of biostimulants increases the overall starch content and protein content in fenugreek and green gram (Pise and Sabale 2010).

An investigation was carried out to understand how the polyphenolic content is affected by a combination of biostimulants and herbicide. Polyphenols are known to possess anti-cancer properties and are therefore, of actual prominence to the human health (Zarzecka et al. 2019). The application of this combination resulted in a 4 mg/kg rise in the polyphenols in the tubers of potato. In potato leaves, the use of this combination, leads to a 2–3 mg/kg increase in the polyphenolic compounds. When A. nodosum seaweed was used as a biostimulant, it significantly increased the phenol content of “Sangiovese” grapes than in the control (Frioni et al. 2018). A combination of seaweed and silicon biostimulant led to a decrease in the phenolic compounds in strawberries throwing light onto the fact that such compounds are produced during stress and hence the biostimulant can be said to have lowered the stress levels in strawberry (Weber et al. 2018).

A plant is said to have nutraceutical prospect if it possess some amount of antioxidant property due to the composition of its compounds (Scalbert and Williamson 2000). In the high stress-laden and toxic lifestyles of the people nowadays, antioxidant property of the consumed foods come to the rescue and reduce the occurrence of many deadly diseases and disorders (Han et al. 2019). Biostimulants have demonstrated to change the enzyme activity of superoxide dismutase, catalase, peroxidase, etc. and cleansed several reactive oxygen species like O_2, H_2O_2, etc. through its antioxidant properties (Abdalla 2014). Atonik, Nano-Gro, and Kelpak biostimulants have been used to increase the antioxidant and phenolics content of beans (Kocira et al. 2015; Pise and Sabale 2010). These biostimulants have also been reported to increase the ascorbic acid and phenolic content in carrot, potato, celery, and tomato (Czeczko and Mikos-Bielak 2004; Tigist et al. 2013). Biostimulants prepared from protein hydrolysate lead to a 34.9% and 27.3% increase in the lycopene and ascorbic acid content of tomato, respectively (Rouphael et al. 2017a, b). The antioxidative abilities of apricot fruits was significantly increased from 1 year to the other following the use of biostimulants (Tarantino et al. 2018). The total phenols and ascorbic acid content and thereby the overall antioxidant activity in spinach can be increased by the application of a consortium of biostimulants like protein hydrolysate, vegetal oils, *Ecklonia maxima* extract, seaweed (*A. nodosum)* extracts (Fan et al. 2011). Foliar application of plant hormone derived from alfalfa plants lead to an increase in the chlorogenic acid concentration and antioxidant activity in the fruits of green pepper (Ertani et al. 2014)

Application of *M. oleifera* extract-based biostimulant resulted in two contrasting effects. Its increasing concentration was seen to decrease the activity of catalase, peroxidase, and superoxide dismutase, whereas, the phenol and ascorbic acid contents were seen to rise with the increase in concentrations of the biostimulants (Abdalla 2014). A higher concentration (200 μg/L) of a biostimulant based on an extract of aqueous garlic amplified the peroxidase and superoxide dismutase activity in tomato indicating its positive effects on improving the tomato oxidation properties. A lower concentration of this extract, however, had no effect on the activity of these enzymes (Hayat et al. 2018). Biostimulant prepared from nanoparticles of salicylic acid and chitosan (SA-CS) increased the activity of the enzyme superoxide dismutase, phenylalanine ammonia lyase, polyphenol oxidase, peroxidase activity, and catalase activity, as opposed to salicylic acid treatment alone (Kumaraswamy et al. 2019; Singleton and Rossi 1965). The antioxidant system of sunflower (*Helianthus annuus* L.) was activated by soaking its seeds in a biostimulant containing a combination of magnesium and corn seed extract. This biostimulant increased the intensity of the photosynthesis, activity of peroxidase, catalase, and superoxide dismutase in sunflower (Rehman et al. 2018).

The chemical composition of a plant is determined by its macro- and micro-elements and glucose, fructose, sucrose, ascorbate, and protein contents. A biostimulant consortium of arbuscular mycorrhizal fungi (AMF) + Pseudomonas sp. 19 Fv1T (PFv1T) and P. fluorescens C7 (PC7) demonstrated an upsurge in the fructose and glucose content as opposed to their respective controls. Seaweed extract and a silicon extract-enriched biostimulant increased the levels of glucose, fructose, and sucrose (Rouphael et al. 2015; Weber et al. 2018). In pumpkin, an extract of the leaves of M. oleifera increased the total soluble sugar by about 80.6% (Abd El-Mageed et al. 2017).

Vitamin C is another essential health-related nutrient dependent mainly on the variety of the crop. The application of phenolic compound-rich biostimulants amplified the vitamin C content in raspberries (Grajkowski and Ochmian 2007). In another study, the application of natural biostimulants exhibited the same influence on the Vitamin C content (Drobek et al. 2020). In another interesting finding, it was observed that the biostimulant containing a consortium of AMF, PFv1T and PC7, directed an increase in the ascorbate content in tomato, while a consortium of AMF and PC7 decreased the same. The latter consortium, furthermore, increased the β-carotene content, which in turn gets converted to Vitamin A and protects against free radicals (Bona et al. 2018). Spent mushroom substrate (SMS) biofortified with *T. harzanium* and grazed earthworm brought about better fruit quality in tomato with respect to lycopene, lutein, and b-carotene contents (Singh et al. 2018).

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The Role of Biostimulants in Plant Growth, Development, and Abiotic Stress Management: Recent Insights

9

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Abstract

Modern agriculture is facing a crucial time due to extreme environmental climate change, where trailblazing strategies need to grow for sustainable agriculture and food security. Non-nutrient substances or microorganisms, also known as biostimulants, are the ones that enhance plant growth and productivity, reduces the need for fertilizers, and also provides sustainable and beneficial solutions to enhance crop productivity. Based on visual observation, it is suggested that the basic potential function of biostimulants is to modulate and modify the physiological processes in plants by increasing nutrition efficiency, reducing stresses, and simultaneously improving quality and yield. Moreover, various raw materials such as algae/brown algae (Phaeophyceae), humic acids, protein hydrolysates, plant hormones, plant growth-promoting bacteria, and other compounded mixtures have been used in biostimulant composition. With respect to this, the main purpose of this section is to define the use of biostimulants for the plant growth and development as well as their effects on them with respect to abiotic stress.

Keywords

Biostimulants · Abiotic stress management · Algae · Humic acids · Protein hydrolysates

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_9

9.1 Introduction

Global climate changes usually apply to the strongest possible environmental conditions and which are now being experienced more frequently. So, in the current scenario, the agriculture is facing consequent hurdles and showing sensitivity to extreme changes in environment. Several technological elevations have come into existence from the last three decades to increase the productivity of agricultural production systems by cutting out use of hazardous chemical fertilizers and pesticides (Du Jardin 2015; Rouphael and Colla 2018) along with reducing the ill-effects on earth ecosystem and human health (Paul et al. 2019; Bulgari et al. 2017). Plant development and productivity increases by the use of biostimulants and plant-based biostimulants alone or in combination (Dalal et al. 2019). They cannot protect plant from a pest attack but can act as a regulator.

Biostimulants, either natural or synthetic, have been considered as an innovative agronomic tool which can be applied directly to seeds, soil, and whole plant, which usually cause some structural changes in plant processes that ultimately improve plant growth and productivity by alleviating negative effects of abiotic stresses, and thus cause increase in the yield and quality of crop (Calvo et al. 2014; Bulgari et al. 2015). In addition to this, the biostimulants also lower the need for fertilizers (Bulgari et al. 2015).

Out of all the reported definitions, the most widely accepted and complete definition of biostimulants is by Du Jardin's knowledge article, in which biostimulant is defined as "any substance or microorganism that applied to plants, regardless of its nutrients content, is able to enhance nutrition efficiency, abiotic stress tolerance and quality traits of crop".

According to the Du Jardin theory, the biostimulants were classified into eight classes: humic acid, seaweed extracts, protein building blocks, complex organic materials, beneficial chemical elements, inorganic salts, chitin and chitosan derivatives, and anti-transpiring elements. Biostimulants do not fall in the category of pesticides as they don't have any direct effect against pests (Van Oosten et al. 2017a). Some of the major known components consist of mineral elements, vitamins, amino acids poly/oligosaccharides and trace of natural plant hormones. However mechanisms involved in the activity of biostimulant is still under study (Povero et al. 2016).

The mode of action of biostimulant activity can be understood by high capacity physical composition and omic technologies. Soil conditions can be improved by their direct action on plant physiology and metabolism (Bulgari et al. 2019a). They also enhance primary and secondary metabolism as well as modify molecular process that improves water use efficiency, plant growth, and counteract abiotic stresses. The activity of which component is most active is difficult to understand because of the complexity of the products used and the complicated molecules involved. The action of biostimulant involves activity of many different molecules together, so it's difficult to understand the action of a single molecule and its isolation (Yakhin et al. 2017a). Also, time of application is variable. One of the most important aspects is its use in stress conditions, not their role as nutrients but instead as remedies (De Pascale et al. 2017).

Many writers have proposed various types of biostimulant substances depending on their components and mechanism of action over the years (Du Jardin 2015; Rouphael and Colla 2020). The present classification is based on the raw material source, though it doesn't provide the information on the biological action involved (De Pascale et al. 2018). As a result, biostimulants are divided into the following categories:

1. Humic and Fulvic acids (HFs): HFs are a type of naturally produced soil organic matter that results from plant, animal, and microbial decomposition (Nardi et al. 2016a). The exact mechanism through which HFs alter plant physiology is unknown because of the complex nature of the molecules as well as the amount and diversity of plant responses that are influenced by their use (Trevisan et al. 2010; Canellas et al. 2015). HFs help intolerance to environmental stresses and also improve plant growth by increasing root, shoot elongation, and leaf nutrient accumulation, which is same effect as auxin and are able to stimulate plasma membrane H+ ATPase activity and enhance plant development (Dobbss et al. 2010).
2. Seaweed extracts (SWE): Seaweeds are a red, green, and brown group of microscopic sea algae. Seaweed extracts (SWE) are becoming more commonly available as biostimulants which are used as plant growth promoters that improve abiotic stress tolerance in commercial formulations (Norrie and Keathley 2006). In agriculture, seaweed extracts have been used since ancient times and are an important source of organic matter and other nutrients (Sharma et al. 2014). They can be administered directly to the soil or used as a foliar spray.

   It helps in enhancing crop growth, productivity, photosynthetic activity, abiotic and biotic stress tolerance (Battacharyya et al. 2015; Chiaiese et al. 2018; Oancea et al. 2013; Mogor et al. 2018).
3. Protein hydrolysates and products containing amino acids: Protein hydrolysates are a mixture of amino acids, peptides, polypeptides, oligopeptides, and denatured proteins that results from breakdown of plant and animal proteins (Nardi et al. 2016a, b). According to study, animal-derived commercial protein hydrolysate products are more hazardous to plants than plant-derived commercial protein hydrolysate (Lucini et al. 2015a; Rouphael et al. 2017). But in an experiment by Botta (2012) on lettuce plants, contrary results were seen that the plants treated with an animal-based protein hydrolysate showed higher fresh and dry weight than those in the control group. They improve plant tolerance to abiotic stress by strengthening plant defense mechanisms (Colla et al. 2017).
4. Microorganisms: This category includes all microorganisms such as bacteria, yeast, filamentous fungi, and microalgae that have been isolated from manures, organic materials, soil, plants, and water sources (Ruzzi and Aroca 2015). When applied to soil, crop productivity rises due to metabolic activities, while nutrient uptake rises due to nitrogen fixation and nutrient solubilization. They boost plants' abiotic stress tolerance mechanism by promoting the manufacture of

plant hormones such as auxins, cytokinins, and other hormones. It also emits volatile organic compounds (VOCs), which have the ability to cause direct harm to plants. Plant growth-promoting rhizobacteria (PGPR) improve physical, chemical, and biological processes to help plants respond better to abiotic stresses (Turan et al. 2017). Microorganisms that form a protective biofilm on the root surface improve nutrient and water uptake.

Some biostimulants like vermicompost, composts, and compost manures, residues from water body, waste streams, and waste from food and industries and sewage treatments are included in another category of biostimulants. The activity of the enzyme phenylalanine ammonia lyase is raised, and secondary chemical synthesis is implicated in many plant physiological responses, among other things by biostimulants produced from agro-industrial by-products.

Juarez-Maldonado et al. (Juarez-Maldonado et al. 2019) have proposed a novel biostimulant product category that includes nanomaterials and nanoparticles. They are particles with size ranging from 1 to 100 nm that demonstrate features which are not found in their bulk form. They improve the production's quality and resilience to abiotic problems if used in tiny amounts as a foliar spray or in nutrient solutions (Fig. 9.1).

9.2 Biostimulants as Plant Growth and Development Regulators: An Efficient Agronomic Tool

Plant biostimulants have received a lot of attention in the recent decade as a possible answer for reducing the negative effects of climate change on agriculture, and it can act as one of the key tools for long-term food production, a new agricultural revolution is needed. Despite the fact that biostimulants were initially proposed in 1933, it has only been in the last few years that attention and studies from various domains have focused on defining, describing, and understanding plant biostimulants and their modes of action (Bulgari et al. 2015; Van Oosten et al. 2017a; Rouphael and Colla 2020). In their paper, Yakhin et al. (2017b) present a comprehensive analysis and timeline of the term "biostimulant". Presently, definition of biostimulant can be said as any substance/molecule or microbe that is not used as a nutrient, pesticide, or soil improver but has the ability to improve overall plant growth by altering its natural biological processes.

Plant biostimulants can assist to overcome nutrient shortages in organic farming by increasing nutrient availability and their absorption. However, when biostimulants are used in conjunction with chemical fertilizers in well-managed soils, the best output is obtained. While there are several commercial plant biostimulants in the market, their performance on a crop is unpredictable because it depends on the soil, climate, dose and time of application, species, and cultivars. This underlines the knowledge gap in plant biostimulant mechanisms at the cellular and molecular levels. In this section, however, it is necessary to highlight some of the

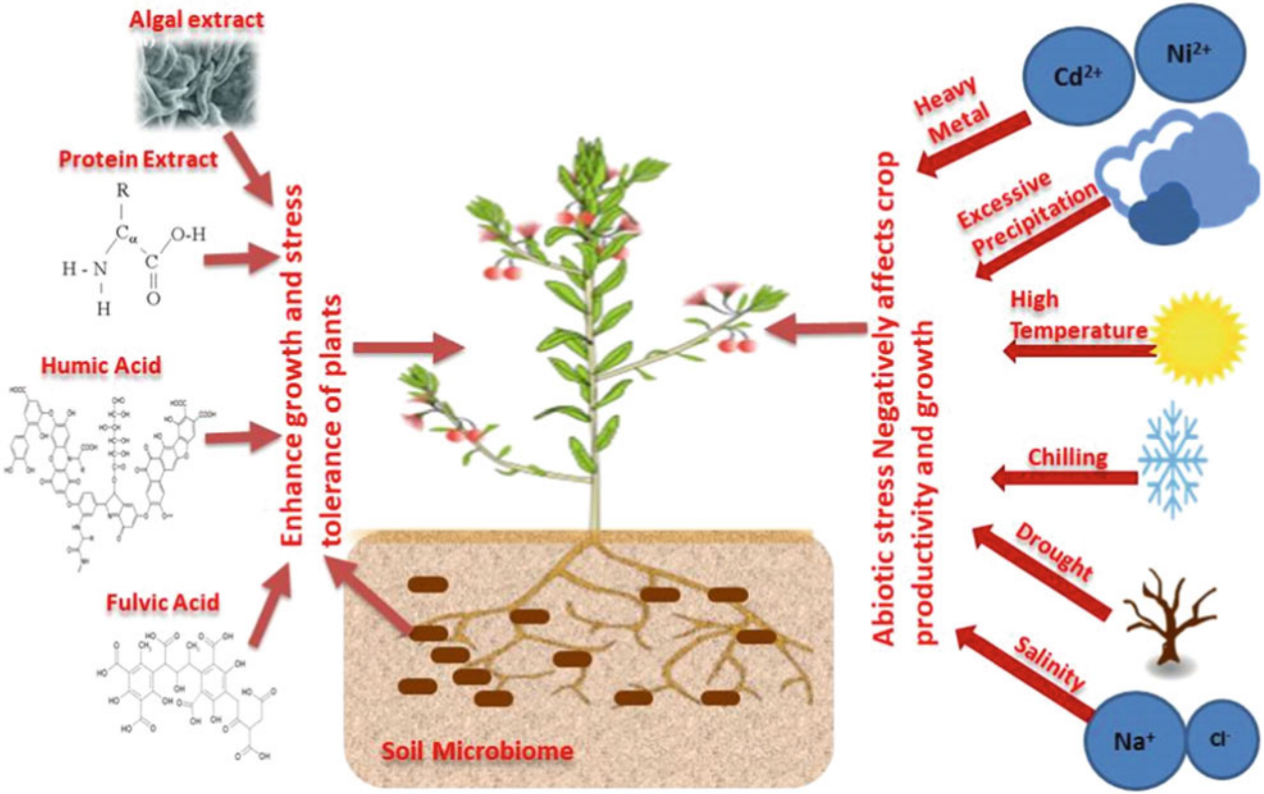

Fig. 9.1 Physiological effects and abiotic stress tolerance induced by biostimulants

basic mechanisms used by different types of biostimulants in plant-biostimulant interactions (Van Oosten et al. 2017b).

9.2.1 Abiotic Stress

Plants are constantly exposed to various stressful environments, beginning from seed germination until the end of a plant's life cycle. The stresses are split into two categories depending on the sort of trigger factor: Stresses caused by biotic and abiotic factors. Biotic stress is a result obtained by other living organisms consisting of fungi, insects, weeds, and bacteria which have an impact on plant growth and development. Abiotic stresses are usually associated with the environment's climatic, edaphic, and physiographic components. Drought, salt, temperature, and inadequate soil fertility are among the most important abiotic stresses affecting plant growth and survival nearly everywhere on the planet, which is why abiotic stress management is one of the most important challenges in agriculture. Due to the ongoing climate change scenario and the environmental degradation as a result of human activity, abiotic stress has emerged as a significant threat to food security. Abiotic stress causes plants to undergo a variety of molecular, cellular, and physiological changes to be able to act and adjust (Huang et al. 2013). Therefore, much research has been concentrated on understanding the molecular biology of abiotic stress response and the development of better, stress-tolerant plants. A plant's growth conditions, as well as availability of water, nutrients, and plant growth regulators (PGRs—auxins, cytokinins, gibberellins, strigolactones, and brassinosteroids), can all help to mitigate abiotic stress. Biostimulants have become an important tool for the alteration and/or regulation of plant physiological mechanisms to promote growth, reduce the influence of abiotic stress-induced limits, and boost production and productivity. Acclimatization and mitigation are necessary to strengthen the adaptability of agricultural systems while also assuring crop output and quality in the near future. As environment is beyond control and cannot be regulated, a variety of measures at multiple levels will be necessary to use various agronomical approaches or create more tolerant types.

9.3 Abiotic Stress Management Through Biostimulants Application

9.3.1 Salinity

One of the most critical hindering factors for crop development and productivity is salt stress. Plant growth may be hampered by salts in the soil water by lowering the plant's ability to absorb water, resulting in a decrease in growth rate (Kumar et al. 2021; Saddiq et al. 2021). Furthermore, the cells in the transpiring leaves may be harmed if too much salt enters in to the plant through the transpiration stream, resulting in further growth restrictions (Mudgal et al. 2010; Parihar et al. 2015; Hao et al. 2021). These salinity impacts result in ion imbalance, toxicity, and disruptions of ion homeostasis; this altered water status results in early growth restriction and plant production is limited (Parihar et al. 2015).

Salt stress affects various metabolic processes in plants, including photosynthesis, respiration, phytohormone control, protein biosynthesis, and nitrogen absorption, as well as causing secondary oxidative stress (Parihar et al. 2015; Sayyad-Amin et al. 2016; Hao et al. 2021). It usually results in a decline in yield, retards root and leaf growth, along with change in texture and color of leaf (Mudgal et al. 2010; Parihar et al. 2015; Sayyad-Amin et al. 2016; Hao et al. 2021). Salinity stress prevention has been examined using biostimulants based on the humic compounds (Tahir et al. 2011; Khedr et al. 2021). The physical and chemical properties of soil subjected to salt stress are improved by humic compounds and extract of *Moringa oleifera* leaf as a biostimulant (Desoky et al. 2018; Bulgari et al. 2019b). Even though the precise action mechanism is uncertain, humic acids are known to have a variety of positive effects, including expansion of the shoots and roots, and increasing resistance to environmental stress. These actions were verified in a variety of vegetable crops cultivated under various salt stress circumstances, including lettuce (Fasciglione et al. 2015; Bulgari et al. 2019b) and sweet pepper (Del Amor and Cuadra-Crespo 2012).

Plant tolerance to abiotic stressors can also be improved by bioactive chemicals found in seaweed extracts. Despite the saltwater environment, Super Fifty® and Acadian, two plant biostimulants made from seaweed *Ascophyllum nodosum*, were used, resulting in a considerable increased root dry weight and yield (Guinan et al. 2013). After treatment with sulfated exopolysaccharides produced from the microalgae Dunaliella salina., plant growth was boosted, antioxidant enzymatic activity, and various metabolic process such as jasmonic acid pathway, which reduces salt stress damage (El Arroussi et al. 2018). The use of *Sargassum muticum* and *Jania rubens* seaweed extracts dramatically reduced the deleterious effects of salt by regulating amino acid metabolism, balancing ionic content, and improving antioxidant defense (Abdel Latef et al. 2017). A study on biostimulant based on honeybee improves onion plant resistance to salt stress. The treated plants produced more biomass, photosynthetic pigments, and increased bulb yield. In addition, osmoprotectants such as proline, soluble sugars, and total free amino acids, as well as the membrane stability index and enzymatic and non-enzymatic antioxidant activity, all improved (Semida et al. 2019).

Therefore, application of biostimulants in salt stress causes the deposition of osmolytes, which increases the cell's osmotic potential and the quantity of antioxidant molecules.

9.3.2 Drought

Drought is a prevalent abiotic stress, particularly in arid and semiarid areas. It's a multi-faceted stress that can change a plant's morphophysiological, molecular, biochemical, and ecological features (Punia et al. 2020). It has a negative impact on plant growth and development. The plants which have been adapted to drought circumstances are determined by the period and intensity of the drought, plant's species, age, and stage of growth. Root biomass growth increases by use of biostimulants in soils which are less fertile and have limited water availability. The biostimulant can be directly applied to seed or at the start of development, helps seedlings recover and grow faster in drought conditions (Bulgari et al. 2019a). Drought stress has a significant impact on plant gas exchange, which affects photosynthetic and transpiration rates, all of which are directly related to yield (Bahrami-Rad and Hajiboland 2017; Wang et al. 2018). *Ascophyllum nodosum* has been used to treat broccoli (Kałuzewicz et al. 2017a) and spinach (Xu and Leskovar 2015a), which increase plant resilience to water stress due to increased gas exchange via stomatal closure reduction. During leaf senescence, chlorophyll breakdown, leaf yellowing occurs, which is a dependable evidence of metabolic and energy imbalance in stressed plants and is another prominent indicator of drought stress. Biostimulants from *A. nodosum* boosted overall chlorophyll content in tomato leaves and reduced water loss, withering damage, and 3-carbon dialdehyde MDA levels *(*Goni et al. 2018*)*. In response to Megafol treatments, Petrozza et al. (Petrozza et al. 2014) achieved similar outcomes in tomato plants. In terms of biomass and chlorophyll fluorescence, the treated plants outperformed the non-treated ones. Moreover, when provided source of water, the biostimulant-treated plants recovered swiftly.

9.3.3 Cold/Chilling Stress

The metabolic and physiological reactions of plants are slowed and delayed in low temperatures, which lead to a reduction in activity of photo system II, called photoinhibition. The cell membranes are damaged and destabilization of the phospholipid layer occurs due to cold. Tomato plants treated with psychrotolerant soil bacteria had increased level of tolerance to cold and showed higher rate of germination, less damage to membrane, and antioxidant systems activation on their exposure to cold temperatures. During winter, many strains are isolated from the soil and used as a cold protectant (Subramanian et al. 2015; Subramanian et al. 2016). These soil bacteria are thought to operate as biostimulants, protecting plants against cold stress. Plants are stressed by low temperatures, especially during transplantation. The similar impact of enzymatic hydrolysates was tested on lettuce plants that had been exposed to cold stress, and the biomass, relative growth rate and specific leaf area, all increased (Polo et al. 2006).

The use of an amino acid-based biostimulant (Terra-Sorb® Foliar) on lettuce plants increased fresh weight and stomatal conductance (Botta 2012). 5-aminolevulinic acid treatment of pepper (Capsicum annuum) seedlings improved cold tolerance using three distinct methods: sprinkling the leaves, soaking the seeds, and watering the soil. In terms of stress tolerance, all of the applications were beneficial. Membrane permeability was reduced, but water content, proline, sucrose, and fresh biomass increased (Korkmaz et al. 2010a). There was favorable effect on metabolic pathways in Coriander plants grown under cold conditions on biostimulant application. Stress markers such as antioxidant activity, photosynthetic pigment concentration and activity, hydrogen peroxide, and malondialdehyde concentration all revealed beneficial ways for stressed plants to acclimatize to low temperatures (Pokluda et al. 2016). Biostimulant activity against cold stress mainly enhances osmotic molecule accumulation by activating cold-protective chemical biosynthetic pathways. Additionally, these biostimulants improve membrane thermal stability, reducing the danger of chilling injury.

9.3.4 Heat Stress

The effects of global warming have resulted in a rise in temperature, which has significantly impeded plant development and growth. High temperatures can cause significant damage to plant cells, resulting in poor germination, slowed growth, reduced photosynthetic efficiency, and, in some cases, death (Challinor et al. 2014; Fahad et al. 2017). Protein synthesis and activity are also disrupted, rendering enzymes inactive and causing membranes damage. Biostimulants provide a long-term solution for stress mitigation by boosting the plant's defense system through defensive characteristics (Salehi-Lisar and Bakhshayeshan-Agdam 2016). In plants, biostimulants reduce heat stress (Quintero-Calderon et al. 2021). Temperatures above 60 °C cause irreversible denaturement in plants, hence the temperature range of 30–45 °C is considered the ideal temperature range for physiological and enzymal activity. Seed germination is inhibited, and plant growth is slowed as the temperature rises above the optimum. Heat stress can disrupt the reproductive phase, reducing pollen viability and germination, limiting flower development, and fruit set reduction, all of which reduce crop growth and production (Hasanuzzaman et al. 2013). When used to combat heat stress, biostimulants increase cell membrane integrity and minimize or prevent the buildup of reactive oxygen species (ROS). When various crops' seeds are exposed to high temperatures, the negative effects on germination can be mitigated by using biostimulants. Biostimulants are thus possibilities for reducing such impacts and, by presenting a defensive mechanism against extreme temperature variations; they can help plants cope with such stressors. *A. nodosum* extract improved lettuce growth, chlorophyll content, leaf senescence, membrane thermostability, seed size, biomass, and heat stress tolerance (Yildirim et al. 2002).

Brassinosteroids increased biomass accumulation and net photosynthetic rate in tomato (Ogweno et al. 2008a) and snap bean (El-Bassiony et al. 2012), as well as production and quality of snap bean pods when it comes to NPK content and total free amino acids in leaves. This could be because brassinosteroids protect the photosynthetic machinery from oxidative stress by enhancing RuBP regeneration and carboxylation efficiency. The impact of exogenous glutathione administration against heat stress was examined by Nahar et al. (Nahar et al. 2015) for mung bean seedlings that were given a treatment before being exposed to high temperatures showed lower levels of methylglyoxal, a reactive chemical that destroys cells and oxidative stress. As a result, the antioxidant defense system becomes more effective. Plant physiological adaptability was improved by glutathione-based pre-treatment, which increased heat stress (short-term) tolerance. The relative water content and turgidity of the leaves rose, which is unusual at high temperatures.

9.3.5 Heavy Metals

Microbial inoculants have been demonstrated to have an indirect effect in soil restoration and fertility in recent years, according to a study. Bioremediation is widely recognized as a valuable tool for reforesting degraded terrain and ecosystems. It is the plant that is the most important part of the phytoremediation (process of removing pollutants) process, and using microbial inoculants in polluted soil bioremediation and reforestation is a workable study topic (De-Bashan et al. 2012; Grandlic et al. 2008). Tomato, canola (*Brassica napus*), and Indian mustard (*Brassica juncea*) cultivated in soils with strong zinc and nickel percentages benefited from the application of Kluyveraascorbata, which raised various plant characteristics and stimulated plant development (Ma et al. 2011). Another investigation also refers to the growth of *Pseudomonas* spp. boosted canola and common reed (*Phragmites australis*) when copper or polycyclicaromatic hydrocarbons are present (Reed and Glick 2005). Microbial inoculants could improve the qualities of plants used for phytoremediation, but choosing microorganisms that can live and thrive in phytoremediation is crucial (Kohler et al. 2008). Other macro and micronutrients have been reported to be enhanced by microbial inoculants, although the techniques included are yet unknown. Indirect ways for boosting nitrogen uptake include increasing root biomass, root surface area, or root hairs. *Pseudomonas* spp. and *Acinetobacter* spp., *Azospirillum* ssp., *Bacillus* spp., and AMF have all been found to increase Zn, Cu, Mn, Ca, and Mg absorption. According to one study, inoculation with a mixture of two bacteria, *Pseudomonas mendocina* and the AMF Glomus intraradices, dramatically improved Fe, Ca, and manganese (Mn) uptake in lettuce (Lactuca sativa).

Plants have long relied on bacteria that make ACC deaminase to help them cope with a wide range of stresses. These bacteria defend plants from ET-induced growth inhibition in a variety of stressful situations, including flooding, toxic chemicals (both organic and heavy metals), excessive salt concentrations, drought, and pathogenic assault, by lowering the stress hormone ET (Glick et al. 2007; Saleem et al. 2007).

Plants' resistance to a variety of heavy metals is influenced by amino acids and peptides. Many plants under significant metal stress accumulate proline, even when no extra metal ions are present; some plants that are resistant to metals have a high constitutive proline level (Sharma and Dietz 2006). Proline may play a role in osmoregulation by compensating for the water deficit caused by exposure to heavy metal; metal ions could be chelated inside plant cells and in the xylem sap; it may operate as an antioxidant as a result of scavenging free radicals produced by heavy metal uptake; and it may operate as a regulator. Histidine levels are higher in nickelhyperaccumulating plants, and it indicates that histidine has a role in Ni transport from the root to the shoot (Kerkeb and Kramer 2003). According to research, additional amino acids, including asparagine, glutamine, and cysteine, as well as peptides like glutathione and phytochelatins, may have a role in Zn, Ni, Cu, As, and Cd chelation (Sytar et al. 2013).

9.4 Future Thrust and Conclusion

Agriculture can become more resilient and sustainable by the use of Plant Biostimulant. It could be a potential new source of agricultural income, complementing agrochemicals like pesticides and fertilizers, while also improving abiotic stress resilience and increasing agricultural commodity productivity. Crops are frequently subjected to abiotic stresses throughout their life cycle, which can substantially impair production and product quality when acting separately or in combination. Biostimulants may show to be a useful and long-lasting strategy promoting plant development and productivity by enhancing abiotic stress tolerance. In fact, biostimulants have been successfully applied:

- To increase crop nutrient and water efficiency.
- To increase resistance to abiotic stressors.
- To boost agricultural crop yields and quality.

It's vital to keep in mind that the complicated and changeable nature of the raw resources that were used, as well as the complexity of bio-preparations and the mixture of components that is heterogeneous as the end product, can make it impossible to assign a specific activity mode to any biostimulant. The high number of plants, microorganisms, and other compounds that fall under the category of plant biostimulants further complicates the situation. Furthermore, the kind and intensity of abiotic stress may influence the biostimulant activity of a substance. A variety of factors influence the impact of biostimulants, including the raw products and procedures used to produce crop species, processing conditions, and climate. However, because the substance of its therapeutic benefits is unknown, their modes of action in some situations remain a challenge that must be identified. Given the large spectrum of compounds that might be utilized as source materials for biostimulants, including humic compounds, seaweed extracts, plant hormones, and plant growth-promoting rhizobacteria, it's also important to look at the mechanisms that cause these effects. The use of a biostimulant can boost root and shoot vigor; however, choosing the right biostimulant is crucial because its effects might differ significantly between species. As a result, biostimulants are still one of the newest agricultural innovations that require further investigation. The availability of cutting-edge research methods will undoubtedly improve our understanding of biostimulant composition, yet this knowledge will be incomplete. The physiological, biochemical, and molecular reactions of plants can thus be used to deduce the biostimulant's mode of action (Table 9.1).

Table 9.1 Effect of biostimulants in alleviating abiotic stress in crops

Sr. No.	Plant species	Biostimulants	Stress	Responses on growth and development and stress tolerance	References
1	*Phaseolus vulgaris*	Humic acid	Salinity	Nitrogen, phosphorus, and nitrate levels in plants all increased Reduced dry weight of plant roots and shoots, as did proline and electrolyte leakage	Aydin et al. (2012)
2	*Capsicum annuum*	*Azospirillumbrasilense* and *Pantoeadispersa*	Salinity	Dry weight, K+: Na+ ratio, GS relative growth rate, net assimilation rate, and NO3 and CO2 assimilation all improved in the plant Cl-accumulation concentration decreased	Del Amor and Cuadra-Crespo (2012)
3	*Cicer arietinum*	*Azospirillumbrasilense*	Salinity	More nodule formation and increase in shoot dry weight	Hamaoui et al. (2003)
4	*Cucurbita pepo*	*Bacillus species, Trichoderma harzannum*	Salinity	Weight gain has improved, as has potassium uptake and the K+: Na+ ratio Decrease in sodium uptake	Yildirim et al. (2006)
5	*Lactuca sativa*	*Protein hydrolysates*	Salinity	Plant nitrogen metabolism, fresh yield, biomass, root dry weight, osmolytes, and glucosynolates all improved Decrease in oxidative stress	Lucini et al. (2015b)
6	*Solanum lycopersicum*	*Achromobacterpiechaudii*	Salinity	Increase in weight, both fresh and dried, uptake of phosphorous and potassium Decrease in ethylene	Mayak et al. (2004)
7	*Phaseolus vulgaris*	Propolis and maize grain extract	Salinity	Seedling development, relative water content, free proline, total free amino acids, total soluble sugars, cell membrane stability index, IAA, gibberellic acid, antioxidant system activity increased Reduce in lipid peroxidation, electrolyte loss, and ABA levels	Semida and Rady (2014)
8	*Cicer arietinum*	*Sargassum muticum* and *Jania rubens*	Salinity	Plant growth, chlorophyll content, carotenoids, soluble sugars, phenols, and potassium levels all improved Decrease in Na+, declined H_2O_2 level CAT, SOD, POD, APX activity increased	Abdel Latef et al. (2017)

9	*Solanum lycopersicum*	*Azospirillum brasilense* (BNM65)	Drought	Increased plant height Enhanced dry weight and xylem vessel area	Romero et al. (2014)
10	*Spinacia oleracea*	*Ascophyllum nodosum*	Drought	The amounts of chlorophyll, carotenoids, soluble sugars, phenols, and potassium in the plants all improved	Xu and Leskovar (2015b)
11	*Cucurbita pepo*	Moringa leaf extract	Drought	Enhanced growth, soluble sugars, free proline Less electrolyte leakage Increased membrane stability	Abd El-Mageed et al. (2017)
12	*Brassica oleracea* var. italica	*Ascophyllum nodosum* + amino acids	Drought	Improved photosynthetic rate, stomatal conductance and chlorophyll	Kałuzewicz et al. (2017b)
13	*Pisum sativum*	*Pseudomonas* spp.	Drought	Improved yield and plant growth Chlorophyll content and the quantity of pods per plant increased	Arshad et al. (2008)
14	*Ocimum basilicum*	Titanium dioxide with gibberellic acid	Drought	Enhanced CAT activity Decrease in lipid peroxidation	Kiapour et al. (2015)
15	*Solanum lycopersicum*	Brassinosteroids	Heat	Increased antioxidant enzyme Activities and shoot weight Decreased H_2O_2MDA	Ogweno et al. (2008b)
16	*Cicer arietinum*	Proline	Heat	Improved % germination, shoot and root length Decrease in electrolyte leakage Increased chlorophyll RLWC Decreased lipid peroxidation and H_2O_2 Increase in GSH and proline	Kaushal et al. (2011)
17	*Phaseolus aureus*	Ascorbic acid	Heat	Seedling growth and percent germination improved Electrolyte leakage, MDA, and H_2O_2 levels were reduced TTC reduction capacity, antioxidant activity, ascorbic acid, GSH, and proline levels all improved	Kumar et al. (2011)
18	*Capsicum annuum*	5-aminolevulinic acid	Heat	Increased chlorophyll content, RWC, shoot and rootmass and SOD activity Decreased membrane permeability	Korkmaz et al. (2010b)

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Role of Biostimulants in Agriculture

10

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Abstract

In agriculture, agrochemicals play a vital role in enhancing yield and productivity of crops under optimal and suboptimal conditions. In recent times, different innovations have been projected to reduce the use of fertilizers and pesticides and enhance sustainable agricultural production. An environment-friendly technique has been proposed which include the use of natural plant biostimulants. Biostimulants boost the growth and development from germination to maturity throughout the life cycle of a crop, increase the plant metabolism efficiency for higher yield and crop quality, increase tolerance against abiotic stresses, facilitates nutrient use efficiency and translocation, improves the quality of produce (colour, total sugar content, fruit seeding), efficient use of water, and improves the physiochemical properties of the soil. Plant bio stimulants include the substances which are applied to soil, plant, or seed in precise formulations to alter the physiological processes which, in turn, enhance the growth and development, fruit set, nutrient uptake, and stress responses in plants. Various extracts from algae or plant, protein hydrolysates, humic acid, fulvic acid, and other mixtures are used as biostimulants in plants. These substances can be directly added to soil in the form of soil preparations or as liquid foliar application products. Plant biostimulants were earlier used only in organic production, but now they are also used in conventional and integrated crop production systems.

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_10

Keywords

Biostimulants · Protectant · Plant extract · Sustainable agriculture

10.1 Introduction

Plant biostimulants, also known commonly as agricultural biostimulants, are a diverse class of compounds which can be applied to a plant which not only positively affect growth and nutrient uptake but also provide tolerance against various stresses. Biostimulants are applied to the root zone of plant to enhance nutrient uptake and protect the plant against drought stress, salinity, and temperature stress (Jardin 2015). Though biostimulants are not nutrients, they facilitate the uptake of nutrients or beneficially contribute to growth and development or stress resistance of plant (Brown and Saa 2015). Now it is a well-established fact that plants are not standalone entities but are hosts and help many types of microorganisms, hosting several microbiota and their inter-connection in plant tissues and help in adaption in various stress environments (Vandenkoornhuyse et al. 2015). The agricultural sector is facing huge challenges for increasing the yield to meet the demands of ever-increasing population with less environmental influence on human health. Fertilizers and pesticides play a vital role in augmenting yield and productivity in agriculture throughout the growing seasons under different conditions. So recently, various innovations have been proposed for sustainable agriculture production with lesser use of synthetic agrochemicals. Environment-friendly technique includes natural biostimulants which will increase yield, flowering, fruit development, productivity, and nutrient uptake and enhance tolerance against various abiotic and biotic stresses (Colla and Rouphael 2015). Plant biostimulants are heterogeneous materials comprising different categories of materials which act as biostimulants such as humic substances and fulvic acid, microbial inoculants, complex organic substances, inorganic salts, beneficial chemical elements, seaweed extract, anti-transpirants, protein hydrolysates, and amino acids (du Jardin 2012). According to new regulation (EU) 2019/1009, a plant biostimulant shall be an EU fertilizing material which helps to stimulate plant nutrition uptake and also one or more other characteristics of the plant rhizosphere such as nutrient use efficiency, abiotic stress tolerance, and nutrient uptake in the soil (EU 2019). Plant biostimulants help in disease resistance by focusing the effects on growth and stress tolerance. Biostimulants were categorized as “substances, comprising microorganisms which are applied to plant, seed, soil, or other growing media that may enhance the plant’s ability to assimilate applied nutrients, or provide benefits to plant development in North America. Biostimulants are not plant nutrients so may not make any nutrient claims or guarantees”.

Biostimulants are obtained from natural or biological sources which improve the plant growth, efficiency of nutrients or nutrient uptake, and soil structure which ultimately improves plant response. The ‘First World Congress on Biostimulants in agriculture' which aimed to bring together scientists related to biostimulants' aspects in agriculture was held in Strasbourg in 2012 (Jardin 2015).

10.2 Main Categories of Biostimulants

10.2.1 Humic Acid

Humic substances (HS) are natural products developed form continuous degradation of different residues due to the metabolic activity of microorganisms present in soil. Humic substances are a mixture of substances like humins, humic acids, and fulvic acids based on molecular weight and solubilization of substance. Humic acids are easily dissolved in basic medium and precipitate in lower pH medium, while fulvic acids get easily solubilized in both acidic and alkali medium (Berbara and García 2014). Humin is a complex of humic and non-humic materials which are described as humic-containing substances (Rice and Mac Carthy 1990; Nardi et al. 2009). HS are isolated from the soils comprising proteins, biopolymers, lignins, and carbohydrate, which constitute the major role in microorganisms and plants (Kelleher and Simpson 2006; du Jardin 2012). Humic material results from the interaction among the decomposed material, plant roots, and microbes. The unevenness in the humic substances' effects arises due to the humic acid sources, the ecological situations, type of plant, dose, and mode of HS product (Eyheraguibel et al. 2008). Collapsing and oxidation of agricultural by-products by chemical reaction leads to the formation of humic-like compounds which could be utilized as alternative for natural humic substances (Rose et al. 2014).

Humic substances help in soil fertility improvement, including physical, chemical, biological, and physico-chemical properties of the soil. Humic substances' affects the root nutrition enrichment through different processes, reduce soil compaction, increase water retention, increase the nutrient uptake due to higher ion exchange activity of the soil involving polyanionic humic substances, and increase the phosphorus uptake via interaction of humic substances with calcium phosphate precipitation, affects the physiology of plants and composition of rhizosphere microorganisms (Berbara and García 2014; Varanini and Pinton 2001). Humic substances also affect the root nutrition through plasma membrane stimulation and converts free energy to trans-membrane electrochemical energy liberated during ATP hydrolysis. It contributes in cell wall loosening, enlargement, organ development, enhances respiration and invertase enzyme processes (Jindo et al. 2012a, b).

10.2.2 Protein Hydrolysates and Amino Acids

Protein hydrolysates consist of a combination of amino acids, denatured proteins, peptides, and polypeptides achieved through hydrolysis of proteins from plant and animal resources. Use of protein hydrolysate obtained from animal sources were identified as phytotoxic displaying adverse role on plant development as compared to plant origin. Chemical structure of PHs is affected by protein source and production process. Chemical protein hydrolysis of animal origin is generally preferred under acidic or alkaline conditions. Acid hydrolysis is a more hostile process, whereas alkaline hydrolysis is simple where proteins are easily solubilized through heating process with addition of alkaline agents. Chemical breakdown affect all peptide bonds leading to destruction of various amino acids and convert them into acidic forms with acid hydrolysis (García-Martínez et al. 2010). Another category includes individual amino acids-based protein products. For the synthesis of these proteins and non-protein amino acids, 20 structural amino acids are included predominantly identified in some plant species (Vranova et al. 2011).

Plant hydrolysates help to increase the performance of agricultural crops like plant biomass enhances the productivity, increase iron and nitrogen metabolism, and enhance nutrient and water uptake (Haplern et al. 2015). More nutrient uptake helps to increase soil microbial and enzymatic activity, improves nutrient mobility and solubility, and modifies the rhizosphere of plants (García-Martínez et al. 2010). Application of these structural and non-protein amino acids also offers protection against environmental stresses (Liang et al. 2013).

10.2.3 Inorganic Compounds

There are some chemical substances which are required for growth and development for some specific crop species. Al, Co, Na, Se, and Si are five major serviceable elements in soil and plants occurring as inorganic salts. These advantageous elements help in the strengthening of cell walls due to deposition of silica, which is expressed in specific environmental situations such as attack of pathogens and physiological stress. They also improve the quality of produce and provide tolerance against biotic and abiotic stresses, besides helping in cell wall rigidification, osmoregulation, reducing transpiration rate due to crystal deposition, heat regulation through reflection of rays, regulating enzymatic activity, antioxidant protection, synthesis of plant hormones, and protection against heavy metal toxicity (Pilon-Smits et al. 2009). Minerals of useful compounds such as phosphites, chlorides, carbonates, phosphates, and silicates are also included in this category and these can be applied as pesticide (Deliopoulos et al. 2010).

10.2.4 Chitosan and Other Biopolymers

Chitin is having a biopolymer named as chitosan in deacetylated form, produced organically and artificially. Polymers of controlled fluctuating sizes are very useful in nourishment, beautification, health, and in farming sectors. Polycationic elements have the ability to unite with broader range of cellular parts, including membranes of plasma, DNA, and cell membrane ingredients for the formation of chitosan oligomers, or bind with some specific receptors involved in defence by the regulation of genes (Hadwiger 2013; Jardin 2015). Chitosan and chitin seemingly use clear-cut receptors and signalling pathways. Chitosan binding results in cellular consequences such as Ca2+ leakage and deposition of hydrogen peroxide into the cell, causing major physiological changes. Scanning of proteins assure this assumption by studying plant tissues which are treated with chitosan (Ferri et al. 2014). Transpiration rates can be lowered by enhancing closing of stomata with the use of chitosan through an ABA-dependent mechanism. Many polymers of synthetic as well as biological origin are being used for increasing the agricultural productivity, providing resistance by activating the plant defence mechanism.

10.2.5 Microorganism

It is a well-known fact now that microorganism has immense potential in enhancing agricultural productivity (Hayat et al. 2010). Microbial inoculants are used in both government and non-government sector for research or evolution. Microbial inoculations are taken out from various macro and micro habitats such as soil material, waste from plants, water, and mulched material. Root exudates are produced by non-identical plant species or cultivars, which is in the favour of inoculated microorganism activity and also serves as biologically active substrates formed by the micro-organisms (Khalid et al. 2004). Soil inoculant's reproducibility has been checked in a wide span of soils or natural habitats. Commercial formulation is another alternative for inoculants' growth (Bashan et al. 2014). Only those microorganisms are useful for conventional agriculture that completely fit with inorganic compounds used for soil and the inorganic chemical used for the protection against various biotic stresses. Some fungi are heterogeneous in nature and show symbiotic behaviour. With the use of mycorrhiza, there is an increase in sustainable agriculture system because of the symbiotic benefits of mycorrhiza associated with nutrients, balancing of water and protection against stresses (Jardin 2015). Another category of microorganism is beneficial bacteria, showing interaction with plants in various ways. These bacterial associations may be temporary or permanent.

10.2.6 Fulvic Acid

Humic matters are fragmented in various forms such as humins, humic, and fulvic substances (Berbara and García 2014). Fulvic acid is different from humic acid because it contains more carboxyl groups, a higher number of total acidity and cation exchange capacity (Bocanegra et al. 2006). Chelation and mobilization of Fe and Al metal ions in soil is only possible due to fulvic acid (Calvo et al. 2014). The size of fulvic substances is smaller as compared to the humic substances, so it has an advantage in movement through micropores of biological and artificial membranes. Fulvic acid has combined capacity to chelate nutrients such as Fe and act as natural chelators and move through membranes and transport different nutrients (Bocanegra et al. 2006). Fulvic acid is able to enhance the capacity to show association with roots of plants (Varanini and Pinton 2001).

10.2.7 Seaweed Extract

To intensify soil fertility and soil productivity, seaweed was in use from many past years in pure form or may be as supplement in the form of composting (Craigie 2011). Seaweed extract act as chelators and as biostimulants for improving nutrient uptake and their utilization for improving soil properties and enhancing germination of seeds, improving growth, yield, and its related traits and also ameliorate shelf life (Mattner et al. 2013). The seaweed extract related to elements lower in mass acts as plant growth hormones and is provided in the form of biostimulants (Tarakhovskaya et al. 2007; Jardin 2015); on the other hand, when extracts have larger molecules like unique polysaccharides, polyphenols, and allelochemicals, they are used for resistance to stress in the form of biostimulants (González et al. 2013).

Mostly profitable extracts are generally obtained from seaweeds of brown algae, which include Ascophyllumnodosum, Fucus, Laminaria, Sargassum, and Turbinaria spp. (Sharma et al. 2012). Seaweed extract product is available in the market in the form of fluid or in dry form (Stephenson 1974), and this formulation is available in combined form of nutrients and fertilizers to enhance the growth of plants (Milton 1952; Craigie 2011).

Seaweeds are used as a source of organic matter in the agriculture from many last decades, but the biostimulant effect of seaweeds has been gaining in importance recently. Purified forms of seaweed extract generally contain various nutrients and enzymes and growth promoters for plant growth (Calvo et al. 2014). Seaweed's use for plant growth in different forms such as in the soil as the polysaccharides that contribute for water retention and soil aeration by forming gel, while on the other hand, directly on the plants in the form of foliar spray as polyanionic compounds which contribute for fixation and exchange of cations such as heavy metals (Craigie 2011).

10.3 Effects/Applications of Biostimulants in Different Crops

10.3.1 Plant Growth, Yield, and Nutrient Efficiency

In response to plant biostimulant, stimulation of plant growth has been associated with signalling molecules' activity in the plant metabolism. Enhanced growth and yield is also connected with higher nutrient uptake (Table 10.1). In maize, inoculation of *Bacillus* with AMF enhanced the plant growth with higher nutrient assimilation of total nitrogen, phosphorus, and potassium in plants (Wu et al. 2005). Sheng and He (2006) reported that application of *Bacillus edaphicus* enhanced K uptake due to the organic acids' synthesis which dissolve rock K ions. Use of PGPR exhibited significant nutrient uptake and higher plant dry weight in cotton and

Table 10.1 Effect of biostimulants application in different crops

Crop	Biostimulant	Effects of simulants	References
Coriandrum sativum L	Asahi SL (Sodium para-nitrophenolate)	Chilling stress, enhanced chlorophyll and carotenoids, decreased electrolyte leakage	Pokluda et al. (2016)
Solanum lycopersicum	Humic acid	Increased yield, total soluble solids, ascorbic acid	Yildrim (2007)
Solanum lycopersicum	Animal and plant derived PHs	Higher chlorophyll and iron concentration under saline soils	Cerdan et al. (2013)
Solanum lycopersicum	*Azospirillum brasilense*	Chilling stress, increased plant height and dry weight	Romero and Correa (2014)
Solanum lycopersicum	*Flavobacterium glaciei, Pseudomonas frederiksbergensis, Pseudomonas vancouverensis*	Increased proline content, decreased electrolyte leakage and lipid per oxidation	Subramanian et al. (2015, 2016)
Solanum lycopersicum	*Dunaliella salina* exo-polysaccharides	Salt stress, increased chlorophyll and protein content, decreased proline accumulation	Arroussi EL et al. (2018)
Solanum lycopersicum	*Arbuscular mycorrhizal* and *Pseudomonas fluorescens*	Enhanced fruit mass by 6.9 g	Bona et al. 2018
Solanum lycopersicum	*Ascophyllum nodosum*	Drought stress, increased plant growth, chlorophyll content, proline accumulation, and soluble sugars	Goñi et al. (2018)
Capsicum annuum	Humic acid	Increased shoot and root weights, nutrient content under saline conditions	Cimrin et al. 2010
Capsicum annuum	Fulvic acid	Higher carbohydrate, total phenolics, capsaicin, carotenoids, and antioxidant activity in fruit	Aminifard et al. 2012
Capsicum annuum	*Azospirillum brasilense/ Pantoea dispersa*	Salt stress, increased plant dry weight, plant growth rate, net assimilation rate, and CO_2 assimilation	Del-Amor and Cuadra-Crespo 2012
Brassica oleracea var. *italica*	*Ascophyllum nodosum* + amino acids	Drought stress and increased chlorophyll content	Kałuzewicz et al. (2017)
Cucurbita pepo	*Moringa* leaf extract	Drought stress, enhanced plant growth, soluble sugars, proline accumulation, chlorophyll content by 34.6%, decreased membrane stability and electrolyte leakage	Abd El-Mageed et al. (2017)

(continued)

Table 10.1 (continued)

Crop	Biostimulant	Effects of simulants	References
Phaseolus vulgaris	Humic acid and Fulvic acid extracted from charcoal mine	Higher nutrient uptake by 41%	Rosa et al. (2009)
Phaseolus vulgaris	Humic acid	Salt stress, increased nutrient uptake, proline content root and shoot dry weight, reduced soil electrical conductivity (EC)	Aydin et al. (2012)
Phaseolus vulgaris	*Licorice* root extract	Salt stress, higher growth, yield, chlorophyll content, free proline accumulation, carbohydrates, and soluble sugars	Rady et al. (2013)
Lactuca sativa	Protein hydrolysates	Salt stress, increased yield, dry biomass, chlorophyll content, plant nitrogen metabolism, osmolytes, glucosynolates, nutrient uptake and reduced oxidative stress	Lucini et al. (2015)
Lactuca sativa	Retrosal	Salt stress, increased fresh weight, chlorophyll accumulation, gas exchange, reduced proline content, and ABA	Bulgari et al. 2019
Cicer arietinum	*Sargassum muticum* and *Jania rubens*	Salt stress, increased growth, chlorophyll, carotenoid, soluble sugars, and phenol content	Abdel Late et al. (2017)
Cucumis sativus	Humic acid	Enhanced yield and sugar content	Karakurt et al. (2009)
Cucumis sativus	Humic acid	Increased shoot growth, NO_3 activity	Mora et al. (2010)
Cucumis sativus	Humic acid	Increased growth, yield, and nutrient efficiency	El-Nemr et al. (2012)
Glycine max	Humic acid	Increase in chlorophyll content	Befrozfar et al. (2013)
Abelmoschus esculentus	Humic acids	Increased fruit yield	Kirn et al. 2010
Solanum tuberosum	Humic acid	Enhanced yield, protein content, and chlorophyll accumulation	Selim et al. (2012)
Triticum aestivum	Fulvic acid	Enhancement of glutamic oxaloacetic transaminase (GOT) enzyme	Gu et al. (2001)
Triticum aestivum	Fulvic acid	Enhanced seedling root growth, proline accumulation, lowers Se toxicity and membrane permeability	Peng et al. (2001)

(continued)

Table 10.1 (continued)

Crop	Biostimulant	Effects of simulants	References
Triticum aestivum	Humic acid	Higher biomass, plant height, and nutrient uptake	Tahir et al. (2011)
Zea mays	Humic acid	Improved root development, root length	Canellas et al. (2009)
Zea mays	Humic acid	Enhanced root growth	Asli and Neumann (2010)
Zea mays	Humic substances	Increased phenyl propanoid pathway, amino acids; lowers phenylalanine and tyrosine accumulation	Schiavon et al. (2010)
Zea mays	Fulvic acid	Increased leaf area, plant dry weight, chlorophyll, yield, CO_2 assimilation rate, and proline concentration	Anjum et al. (2011)
Zea mays	Salicylic acid–chitosan	Higher chlorophyll content in the maize leaves	Kumaraswamy et al. (2019)
Oryza sativa	Fulvic acid	Higher Fe uptake	Pandeya et al. (1998)
Oryza sativa	Humic acid	Enhanced growth of plants, proline content, reduce oxidative stress, and helps to maintain membrane permeability under drought stress	Garcia et al. (2012)
Oryza sativa	Humic acid	Maintained peroxidase activity under drought conditions; reduced lipid peroxidation	García et al. (2013)
Vicia faba	Fulvic acid	Higher Pb uptake; reduced lipid peroxidation	Shahid et al. (2012)
Chrysanthemum	Humic acid	Increased flower diameter (33%), biomass accumulation	Fan et al. (2014)
Allium sativum	Biofertilizer (*Azobacter, Azospirillum* and *Klebsiella*)	Enhanced bulb yield (from 2 to 6%), quality and storage properties of bulb	Abdel-Razzak and El-Sharkawy (2013)
Allium sativum	Humic acid	Enhanced mineral nutrition efficiency and pyruvic acid which increased pungency	Denre et al. 2014
Allium cepa	Bee-honey based biostimulant	Increased biomass, yield, photosynthetic pigment accumulation, osmoprotectants, membrane stability, enzymatic and non-enzymatic antioxidant	Semida et al. (2019)

wheat (Egamberdiyeva and Höflich 2004; Shaharoona et al. 2008). In lettuce, combination of both *Pseudomonas* and the AMF *Glomus* enhanced nutrient uptake like iron, calcium, and manganese (Kohler et al. 2008). Use of siderophore producing *Streptomyces* strains significantly increased growth parameters in tomato and rice (Verma et al. 2011).

Humic materials elicit different morphological changes, which ultimately affect growth of plants (Clapp et al. 2001; Nardi et al. 2009). A significant increase in seedling root growth and enhancement of lateral roots has been reported by various scientists in different crops like wheat (Peng et al. 2001; Tahir et al. 2011), maize (Canellas et al. 2009; Jindo et al. 2012a, b), Arabidopsis (Dobbss et al. 2010), tomato (Canellas et al. 2011), pepper (Cimrin et al. 2010) and *Lantana camara* (Costa et al. 2008). Higher shoot growth development was reported in wheat (Tahir et al. 2011), maize (Eyheraguibel et al. 2008), tomato (Adani et al. 1998), cucumber (Mora et al. 2010), and pepper (Cimrin et al. 2010). Multiple use of humic acid in soil inhibited the shoot growth of maize grown under hydroponic system (Asli and Neumann 2010). In okra, application of three different doses of humic acid increased the fruits per plant and ultimately yield was increased (Kirn et al. 2010). With the humic acid, quality of wine was improved in grapes due to increase in nitrogen content of grapes (Morard et al. 2011). Karakurt et al. (2009) reported significantly higher total yield, weight of fruit, reducing sugars, total soluble sugars (TSS) and chlorophyll activity in pepper grown in Turkey with foliar spray of humic acid. Similarly, Yildrim (2007) assessed the effects of humic acid and identified increase in early and total yield with higher TSS and ascorbic acid in tomato. In Arabidopsis and micro-Tom tomato, fulvic acid improved the length of lateral roots (Dobbss et al. 2007). With the application of fulvic acid, shoot growth was enhanced in tomato (Lulakis and Petsas 1995), plant biomass in wheat and maize (Anjum et al. 2011), and more number of flowers in cucumber was reported (Rauthan and Schnitzer 1981). Application of humic acid with 90.7% fulvic acid improved the nutrient uptake in plants (Sánchez-Sánchez et al. 2002). In sunflower, Bocanegra et al. (2006) identified that fulvic acid chelated Fe^{3+} and enhanced availability of iron with Hoagland solution containing ^{59}Fe. Foliar spray with Siapton increased yield by 12% in papaya (Morales-Payan and Stall 2003). Increase in height and more flowers number/plant was reported in tomato fertilized with Siapton. Use of a protein hydrolysate increased fruit yield, number, and weight in tomato (Koukounararas et al. 2013).

Seaweed extract application improved root development in a number of species comprising Arabidopsis (Rayorath et al. 2008), maize (Jeannin et al. 1991), winter rapeseed (Jannin et al. 2013), strawberry (Alam et al. 2013), grape (Mugnai et al. 2008), and lodgepole pine (MacDonald et al. 2012). Combination of growth regulators with seaweed extract increased lateral root formation, total root volume, and root length (Vernieri et al. 2005; Zodape et al. 2011; Khan et al. 2011a, b). Improvement in formation of roots (Vernieri et al. 2005), root length, and volume (Mancuso et al. 2006; Zodape et al. 2011) have been identified with application of plant growth regulators with seaweed extracts (Khan et al. 2011a, b). Foliar application of seaweed extract enhanced nutrient content and increased macro and micro nutrients in soybean (Zodape et al. 2011), winter rapeseed (Jannin et al. 2013), tomato (Zodape et al. 2011), lettuce (Crouch et al. 1990), and grape (Mancuso et al. 2006). Microorganisms' diversity and rhizosphere activity was increased in strawberry with seaweed extract as biostimulant, while plant growth and root nodulation was stimulated in alfalfa (Khan et al. 2012, 2013). Colla et al. (2014) identified positive effects of Protein hydrolysates (PHs) on plant growth, nutrition, elongated the coleoptile, increased shoot, root biomass, and root length in corn.

Pseudomonas psychrotolerans strain enhanced growth and nitrogen fixation in rice (Liu et al. 2017). Organic biostimulants, including vermicompost, stone dust, organic herbs, and malt sprouts exhibited positive effects on growth of plants by altering the microbiota on the above-ground parts of a plant (Mahnert et al. 2018). Wilson et al. (2018) identified that gelatin hydrolysate application in cucumber boosted genes activity responsible for synthesis of amino acid permeases and carriers of amino acids and nitrogen. Gelatin hydrolysate acted as a source of nitrogen. Luziatelli et al. (2019) observed the effect of different stimulant combinations on lettuce growth. Shoot fresh weight was higher as compared to control and increased the growth of epiphytic bacteria and ultimately enhanced the productivity of lettuce. Cozzolino et al. (2021) reported the effect of three biostimulants (brown seaweed, protein hydrolysate and a tropical plant extract) in tomato. Protein hydrolysates and seaweed extract increased the marketable yield by 18.3%, TSS, hydrophilic antioxidant activity, and ascorbic acid and boosted the nitrogen uptake efficiency.

10.3.2 Physiology and Metabolism

Different categories show different effects on plant metabolism and physiology of plants. Humic material shows favourable ramification on morphology of plants because it enhances properties of soil by improving fertility of soil, texture, or structure and enhances uptake capacity of nutrients and biomass of the roots is improved in the plants (Table 10.1) (Trevisan et al. 2010). A fragment of humus makes direct association with architecture of roots. Humic acids correlated with organelles of cells just for the initial short time (Berbara and García 2014). Direct uptake of humic matter shows direct effect on physiology of plants (Nardi et al. 2009). ATPase activity is increased due to humus substances in the cells of roots and enhances surface of roots, root density also increases (Canellas et al. 2009). In favour of above, Calvo et al. (2014) explains the activity of some promoters mainly of DR5 which is related to the production of auxins and combined with a gene named as GUS in genetically modified tomato. H^+-ATPase action, act as the activity of protons. Activity of metabolites and other ions enhances throughout the membrane and increases the level of energy because of H^+-ATPase with the help of electrochemical gradient. Due to increase in activity of H+ -ATPase, extension and development of roots increases in maize (Canellas et al. 2009). Further effects of humic acids studied by gene interaction and their metabolites, which identified by using protemotics, trancriptomics, and microarray analysis.

Fulvic acids affect plant physiology, which is explained by development of plants and resistance against different stresses. Development of roots enhanced by using fulvic acid in small hydoponic system with large-Tom tomatoes, dgtwas (diageotropica gene of tomato) mutant is mainly used because these are insensitive to the auxin used in this research. Photosynthetic activity, rate of the transpiration rate or the CO_2 concentration level in the intercellular space of the cells is increased with fulvic acid that ultimately concerned with the development of crops (Calvo et al. 2014). Deposition of the stress hormone named as proline increases due to Fulvic substances therapy in water logged or good-watered crops (Peng et al. 2001). Protein hydrolysates enhance metabolism and also assimilation of nitrogen in plants. In maize, application of Siapton increases function of NAD-dependent glutamate dehydrogenase, nitrate reductase, and malate dehydrogenase. In alfalfa hydrolyzation of amino acids put into maize, i.e. grown in glasshouse with hydroponic conditions, this situation enhances the concentration of the main enzymes involved in the process or tricarboxylic acid cycle and also other enzymes which show involvement in the assimilation and the reduction processes of nitrogen. Expression of the main three enzymes in the TCA cycle increased, which was assured by the reverse transcriptase polymerase chain reaction. Plant metabolism of plants are adversely affected by seaweed extracts. Proteins i.e. solubility of proteins in natural form have antioxidant properties, phenolic or flavonoid glad in *Spinacia oleraceae* enhanced after treating with extracts of algae mainly brown (Fan et al. 2013). Nitrogen metabolism is increased because of transcriptional enzymes, antioxidative ability, and glycine betaine synthesis. Protein coding some genes that are involved in the fixation of carbon compounds with the help of Rubisco or carbonic anhydrase, which was increased by withdrawal of some algaes and increased content of starch by promoting its synthesis, which enhances starch synthesis. NRT1.1 gene have important role in feel of transport for auxin that ultimately shows involvement for nitrate transporters, (Castaings et al. 2011), because of this growth of roots increases.

10.3.3 Antioxidant Properties

In today's research, the antioxidant properties of fruits and vegetables are gaining wide importance because these properties have the ability to stop the division of tumour-causing cells and also provide protection against stresses caused by free radicals. These stresses generally cause damage to the genetic material, organelles of cells. The use of biostimulants in crop improvement affects the action of various enzymes that influence the fruits and vegetables' antioxidant properties. Crops having lycopene, ascorbic acid, and phenols had these properties.

10.3.4 Stimulation of Plant Defences Against Abiotic Stresses

Huge losses in yield have been reported due to abiotic stress in agriculture due to climatic conditions, which also leads to degradation of soil. Applications of biostimulants like microbial inoculants, fulvic and humic acids, and protein hydro show positive response on growth of plants and protects from various environmental stresses, thus lowering yield losses. Rhizobium and Azospirillum inoculation imparts salt tolerance. Inoculation of A. lipoferum eliminated the adverse effects due to salinity in wheat (Bacilio et al. 2004). Use of A. brasilense eliminated the symptoms of water stress in maize under protected conditions (Casanovas et al. 2002). Inoculation of P. putida and B. megaterium with plant growth regulators improved plant biomass and moisture content in white clover under dry conditions (Marulanda et al. 2009). Mycorrhizal symbioses increased drought tolerance in wheat, corn, soybean, lettuce, and onion (Brundrett 1991a, b; Augé 2001; Calvo et al. 2014). Application of various microbial inoculants mitigated the drought stress in wheat, barley, tomato, pepper, and ryegrass (Bae et al. 2009; Singh et al. 2011). Karlidag et al. (2013) identified that inoculation of *Bacillus* species and *Staphylococcus* improved chlorophyll, nutrient uptake and yield in saline conditions in strawberry. Inoculation of *Azospirillum lipoferum* improved plant biomass, enhanced bacterial activity of ACC-deaminase, which lowered the ethylene concentration caused by salinity in canola (Baniaghil et al. 2013).

Application of humic acid reduced electrical conductivity of soil and leakage of proline content from plants provides salt tolerance under saline conditions (Aydin et al. 2012). It has also eliminated the adverse effects on growth and flowering stage under saline conditions in chrysanthemum (Mazhar et al. 2012). Humic materials enhanced the growth, decreased the proline synthesis and ABA level in pistachio in saline water (Moghaddam and Soleimani 2012). It also enhanced the plant biomass, germination, carotenoids and carbohydrates under moisture stress conditions due to higher photosynthetic activity (Garcia et al. 2012). In wheat, combination of both fulvic acid with low Selenium content enhanced root growth and decreased the symptoms of stunting; chlorosis and Ca^{2+} uptake was higher (Peng et al. 2001). Harper et al. (1995) identified that fulvic acid eliminated the adverse effects of Aluminium on root development in maize. Foliar application of fulvic acid enhanced the accumulation of rare earth elements (REEs) (La3+, Gd3+, Y3) which could reduce their build-up in soil in wheat (Murillo et al. 2005).

Animal membrane hydrolysate and Macro-Sorb increased photochemical efficiency and cell integrity in perennial grass when exposed to high temperature (Kauffman III et al. 2007). In Arabidopsis, Apone et al. (2010) observed that amino acid mixture obtained from plant membrane enhanced the response of three stress genes with two other genes involved in the reactive oxygen species. Use of alfalfa hydrolysate enhanced plant dry weight, lowered the antioxidant and enzymatic activity and phenolic compound production in maize grown under saline conditions (Ertani et al. 2013). Glycine betaine and proline works as osmoregulants, stabilizing protein and enzymes activity and protects the plant cell membranes from denaturation due to higher salt activity and temperature. Glycine betaine and proline accumulation is associated with stress tolerance, and exogenous application increases abiotic stress tolerance in cereal crops (Chen and Murata 2008; dos Reis et al. 2012; Ahmad et al. 2013). Glutamate and ornithine enhances salt tolerance in plants (da Rocha et al. 2012). Extracts of A. Nodosum increased freezing tolerance in Arabidopsis and protect the membrane integrity, decrease the activity of chlorophyllase genes and enhance the expression of three cold tolerance genes (Rayirath et al. 2009).

Response of plants against different abiotic stress has been studied using different molecular techniques. Plant metabolism may be changed due to the production of regulatory molecules like salicylic acid, ABA; proline, which stabilizes protein and enzymatic activity, helps to sustain cell turgidity and eliminates reactive oxygen species. Nair et al. (2012) determined that 1113 genes expressed in the lipophilic components (LPC) in seaweed extract treated plants showing freezing stress and 463 genes were upregulated linked with stress responses, enhanced sugar and lipid production and response to ABA in Arabidopsis using genomic approaches. Treatment with *A. nodosum* enhanced the expression of salt tolerance genes and suppressed the other genes in Arabidopsis undergoing salt stress (Jithesh et al. 2012).

10.3.5 Effect of Biostimulants on Physical Characteristics

Biostimulant's application alters the physical and mechanical properties like firmness, shelf life, cell wall flexibility, etc. Biostimulants with chitosan and phenolic compounds resulted in loss of firmness of fruits in raspberry, while not affected with titanium compounds (Grajkowski and Ochmian 2007). In chilli pepper, the application of protein hydrolysates derived from alfalfa and red grapes enhanced capsaicin in red chilli fruit, while increasing the concentration of chlorogenic acid, p-hydroxybenzoic acid, and p-coumaric acid in green fruits (Ertani et al. 2014).

Cracking was decreased with application of spic cytozyme in pomegranate (Aziz et al. 2013). Seaweed extract enhanced the water content and surface area of the leaves. Cell turgidity, surface area and photosynthesis activity were improved in spinach under stress conditions (Xu and Leskovar 2015). Biostimulant application leads to stiffening of cell wall and lowers its extension (Tarantino et al. 2018). Biostimulants increase the flexibility of cell walls and prolong the shelf-life of fruits to maintain proper size and shape. Biopolymers of polysaccharides, humic and fulvic acids increased the mechanical strength of apricot fruits. Biostimulants also alter the fruit shape and colour. Consumers prefer larger fruit with more length and diameter with proper colour development (Tarantino et al. 2018). Presence of anthocyanin influences the colour of the fruit. Biostimulant application developed a lighter skin colour of apricot fruits in first year and red colour skin in second year (Tarantino et al. 2018). Application of glycine and betaine in cherries developed darker skin colour, which is caused due to higher content of antioxidants.

10.3.6 Effect on Chemical Composition

Application of biostimulants alters the chemical properties of fruits, which include vitamins, acidity, dry weight or biomass and soluble solids. Biostimulants with phenol content or chitosan decreased the dissolved solids content in raspberry fruits, whereas content of dissolved solids in fruits increased with biostimulants containing titanium compounds (Grajkowski and Ochmian 2007). Humic and fulvic substances, carboxylic acids, and polysaccharides application improved the taste of fruits by increasing the value of SSC (10.7°Brix) in apricots (Tarantino et al. 2018). Phenolic and titanium material increased the fruit acidity in raspberry (Grajkowski and Ochmian 2007). Similarly, fruit acidity was reduced with application of humic compounds, polysaccharides and carboxylic acids were determined in apricot (Tarantino et al. 2018). Biostimulants containing phenolic, chitosan, and titanium compounds enhanced the Vitamin C and nitrate level in raspberries, while Vitamin C was boosted with the phenolic compounds (Grajkowski and Ochmian 2007). Use of toasted and non-toasted biostimulants augmented the phenol concentration, hydroxycinnamic acids, which ultimately affect the taste and quality of grapevine (Sánchez-Gómez et al. 2017). *A. nodosum* at 1.5 and 3 kg/ha enhanced the phenol compounds in grapes (Frioni et al. 2018). Salicylic acid-chitosan nanoparticles enhanced the chlorophyll and photosynthetic activity in leek corn (Fawzy 2012). Use of leaf extract from *Moringa oleifera* boosted chlorophyll in *Cucurbita pepo* leaves (Abd El-Mageed et al. 2017).

Seaweed extract *A. nodosum* and a silicon extract application increased the sugar level in strawberry (Weber et al. 2018). Extract derived from *M. oleifera* leaves enhanced total soluble sugar (TSS) content by 80.6% in pumpkin (Abd El-Mageed et al. 2017). Arbuscular mycorrhizal and *Pseudomonas* enhanced increased Vitamin C and β-carotene in tomato (Bona et al. 2018). Use of biostimulant with sewage sludge increased protein content in maize by 30% (Tejada et al. 2016). Use of biostimulant extracted from pressing olive oil process enhanced 19% protein in maize (Alberola et al. 2008).

10.4 Methods of Application

Commonly biostimulants are available in ready-to-use material to prepare a liquid solution. These can be directly added to soil in the form of soil preparations or as foliar application (Kocira et al. 2018). Biostimulants having humic and nitrogen compounds are directly applied into the soil in the form of granules affecting the structure of the root, increasing ability to absorb nutrients. The plant extracts and seaweed types are applied as foliar sprays. Alternatively, to reduce labour cost, biostimulants can also be applied by integrating in the irrigation system. Time of application of biostimulants is also very wide. These can be used regularly during the entire growth period at certain intervals or at one time during deficiency of nutrients in the plants. When applied upon the appearance of deficiency symptoms, it has been observed that foliar application is more effective than the soil application. Foliar applications of biostimulants safeguard the plant against various stresses. It could also be applied in biomass form extracted from seaweed, particularly in areas situated near the source of seaweed acquisition. The best time for application is at the time of stomata opening in morning hours and the assimilation rate is higher (Goñi et al. 2018). Application of biostimulants directly on harvested fruits (Battacharyya et al. 2015) has been observed to extend the shelf-life storage life of oranges significantly.

10.5 Conclusion

Plant biostimulants are going to play an important role by making a significant contribution to ecologically and economically sustainable crop production systems in the coming years. The use of biostimulants will reduce the amount of mineral fertilizers, thus reducing soil, water, and air pollution. It is a well-established fact that biostimulant application has positive impact on crop quality and performance without affecting soil and environment health. The demand for the biostimulants is increasing day by day; and it is estimated that global biostimulant market will grow to almost 5 billion dollars by 2025 from 2.6 billion dollars in 2019 at a compound annual growth rate of 11.2%. The Indian biostimulant market is projected to witness a CAGR of 16.49% to reach a total market size of US$180.949 million by 2023, increasing from US$71.232 million in 2017. However, there is a dire need to understand the nature and their positive effects, making them a hot topic of research. Presently, plant biostimulants are only considered in a category of complementary inputs applied along with the chemical fertilizers. With the aid of molecular and physiological tools, the biostimulants can be employed more effectively to enhance crop productivity.

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Biostimulants: Emerging Trend and Opportunities

11

Catalina Landeta and Francisca Marchant

Abstract

Plant biostimulants are a revolutionary and sustainable platform of substances of microbial (fungi and bacteria) and non-microbial origin (humic substances, protein hydrolysates, algae extracts, and minerals) that have been increasing in the last 10 years. These products are a sustainable alternative to synthetic chemicals (fertilizers and pesticides), and there are many studies that support the contributions of these compounds, such as improved nutrient use efficiency, tolerance to salinity and drought, and improved crop quality. In addition, their use brings benefits to human health, the environment, biodiversity, and the economy. The efficiency and properties of these compounds depend on the active ingredient base, the type (liquid or solid), the dosage, and the type of application. Therefore, researchers, the agricultural industry, and other industrial sectors have paid particular attention to these products. However, there is not enough research that provides an accurate description of the direct and indirect mechanisms on crops and of all the compounds at the molecular level that these biostimulants possess. In addition, it is essential to determine the synergistic effects of microbial and non-microbial biostimulants. The development of tools to address these questions and the creation of harmonized regulatory processes will encourage further growth of the global market for plant biostimulants.

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N. Ramawat, V. Bhardwaj (eds.), *Biostimulants: Exploring Sources and Applications*, Plant Life and Environment Dynamics,
https://doi.org/10.1007/978-981-16-7080-0_11

Keywords

Biostimulants · Microbial inoculants · Humic substances · Protein hydrolysate · Algae extract

11.1 Introduction

There are compounds or mixtures of compounds that differ from fertilizers and can increase the development, quality, yield, and growth of plants through physiological stimulation processes. These compounds are called plant biostimulants (PB) (du Jardin 2015). As such, they can help crops cope with periods of stress and reduce the use of chemical fertilizers and pesticides in cropping systems (European Commission 2016; Rouphael et al. 2018; Caradonia et al. 2019). Today, agriculture is demanding such alternatives to chemicals to respond to regulations, sustainably achieve food security, and reduce environmental impacts on ecosystems and human health (Rouphael and Colla 2018).

According to a report, the plant biostimulants market is increasing remarkably and is registering a CAGR of 13.58% (Marketsandmarkets.com 2017) and is expected to reach USD 5.6 billion by the end of 2026 (Madende and Hayes 2020). The market is led by Europe with 42% of the market share, followed by the North American market accounting for a quarter of the market and Asia-Pacific with 22% of the global market. The key companies in the biostimulants market are BASF (Germany), Isagro (Italy), Valagro (Italy), Bayer (Germany), Italpollina (Italy), Koppert Biological Systems (Netherlands), Acadian Seaplants (Canada), UPL (India), and Biolchim (Italy). The companies adopted various acquisitions, joint ventures, and new product launches to enter the biostimulant market. There are price differences in biostimulants depending on the active ingredient base, type (liquid or solid), and application type. The amino acid biostimulant segment is the dominant market segment in terms of volume. Most biostimulants are available in liquid form, and the form in which they are used depends on the type of crop, field size, and climatic conditions in the region. The main biostimulants companies manufacturing liquid-based biostimulants are Ilsa (acquired by Biolchim, based in Italy), UPL (US), Koppert (Netherlands), BioAg Alliance of Monsanto (US), and Novozymes (Denmark). The crops of fruit, vegetables, and cereal crops (wheat, rice, barley) widely consumed worldwide, use these biostimulants. As such, biostimulant manufacturers are planning to produce biostimulants that are specific to each type of crop (Marketsandmarkets.com 2017).

According to Rouphael and Colla (2018), the significant drivers for the accelerated development of the biostimulants industry are (1) the increasing commercialization of new biostimulants that act in a targeted way (2) the growing availability of new biostimulants products that act in a targeted manner; (3) need to promote efficient use of chemical and synthetic fertilizers; and (4) current adverse environmental conditions that are constantly changing and affecting crop productivity. The increase in agricultural emissions has encouraged governments to increase the production of good quality agricultural products with lower emissions. As such, the use of biostimulants is being promoted through farmer education programs.[1] However, this growth could be slowed down by the lack of regulations linked to PB commercialization, especially in emerging economy countries (Caradonia et al. 2019). On the other hand, the European Union, for the first time, identified PB as a new category of agricultural products and divides PB according to their source of origin into microbial (bacteria and fungi) and non-microbial (humic substances, protein hydrolysates, algal extracts, and others such as chitosan, silicon, and residues) (European Commission 2016; Bulgari et al. 2019). Additionally, Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019[2] regulates the fertilizer or biostimulant capacity of some products or by-products. However, these compounds are regulated by divergent national laws between member states, limiting the development of these compounds due to uncertainty related to the certification of these products (Caradonia et al. 2019). In the United States, the regulation of biostimulants is still not well defined; in 2018, the Agricultural Improvement Act gives recommendations on PB.[3] These state-level regulations are contingent on the type(s) of active ingredient(s) in biostimulants. In this regard, the regulation indicates that PB can be considered plant regulators. Therefore, it is essential to create globally harmonized regulations approving the safe circulation of PB in order to legitimize this category of substances and generate a common market for PB (du Jardin 2015; Caradonia et al. 2019).

Formulations that include mixtures of microbial and non-microbial PB are now also being marketed. Most of these commercial formulations do not have the necessary scientific evidence to demonstrate their interactive effects. This is despite all the current research contributions (more than 700 scientific articles) over the last 10 years. Thus, it is necessary to identify scientifically, with sound experimental approaches, the properties of these formulations and their specific activity on crop yield and resistance (Rouphael and Colla 2018, 2020).

This future challenge lies in the research, development, and innovation of second-generation PB. The effects and mechanisms of action of PB on plant morpho-physiological traits need to be precisely determined. Omics technologies (phenotyping and high-throughput plant metabolomics) are effective and convenient for discovering new PB, and understanding their mechanism of action (Paul et al. 2019). Additionally, it is important to have thorough knowledge about the individual phytoconstituents (phenolic acids, peptides, phytohormones that make up PB; these include secondary metabolites such as polyphenols, terpenes, non-ribosomal peptides, and plant hormones, etc. These phytoconstituents are biostimulant and bioprotective agents with the ability to stimulate plant development and alleviate abiotic stress (Mrid et al. 2021). This type of research and development is

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[1] fortunebusinessinsights.com

[2] http://data.europa.eu/eli/reg/2019/1009/oj

[3] https://www.federalregister.gov/documents/2019/03/27/2019-05879/pesticides-draft-guidance-for-pesticide-registrants-on-plant-regulator-label-claims-including-plant

fundamental for the development of natural and efficient PB that not only promotes plant biomass production, nutrient content, and stimulates physiological improvement of crops but also need to improve plant resistance to drought and salinity in a complex and worrying scenario of anthropogenic climate change (Bulgari et al. 2019; Del Buono 2021).

11.2 Microbial Biostimulants

Microbial biostimulants are formed by beneficial microorganisms such as free-living, rhizospheric, or endosymbiotic bacteria and mycorrhizal fungi. These microorganisms have the ability to improve nutrition, increase plant growth and productivity, promote the absorption and effectiveness of nutrients, improve tolerance to abiotic stress (UV radiation, drought, and temperature) maximized by climate change, and biotic (competition for nutrients with other microorganisms and the evasion of microbial toxins) (Sangiorgio et al. 2020). Plant growth-promoting microorganisms (PGPMs), most frequently used as inoculants in agricultural plants, are the bacteria *Azospirillum* spp, *Azotobacter* spp., *Bacillus* spp., *Enterobacter* spp, *Gliocladium* spp., *Pseudomonas* spp., *Rhizobium* spp., and *Streptomyces* spp.; while in fungi are *Glomus* spp, *Heteroconium* spp., and *Trichoderma* spp. (Malik et al. 2021).

Bioformulations based on microorganisms (individually or as microbial consortia) or their derivatives have been used (Santos et al. 2019; Santoyo et al. 2021) (Table 11.1). Several factors should be considered for the development of PGPM-based inoculants, such as the plant species, the diversity of the soil community, and biotic and abiotic factors (Backer et al. 2018), since the plant can selectively attract microorganisms by the organic compounds contained in the root exudates (Venturi and Keel 2016). In addition, its effectiveness of plant-microorganism interactions, environmental safety, and profitability must be considered (Backer et al. 2018). Vasseur-Coronado et al. (2021) developed a strategy to screen the best PGPMs producers of biostimulant based on literature analysis. In this study, only 6% ($n = 200$) of the strains shared suitable traits as potential plant biostimulants. In this study, only 6% ($n = 200$) of the bacteria strains isolated from tomato plant roots presented suitable traits as biostimulants of plants. While, Tabacchioni et al. (2021) developed multifunctional synthetic microbial consortia for improving crop yield and quality, through literature survey and metagenome fragment recruitments on genomes of candidate PGPM. On the other hand, on the market are commercial biostimulants (Aamir et al. 2020; Basiru et al. 2021), such as Slavol®, which contains nitrogen-fixing and phosphate-mineralizing bacteria, and Tablet®, which contains *Rhizophagus intraradices* and *Trichoderma atroviride* spores.

Table 11.1 Studies using microbial biostimulants in crops and their effects on the plant

Crop	Bioformulations	Main activity	Reference
Daucus carota	*Trichoderma harzianum* Rifai T-22, Beta-Chikol, Timorex Gold 24 EC, Fungicide Zaprawa Nasienna T 75 DS/WS	Development and health status of carrot plants against *Alternaria, Fusarium*, *Haematonectria*, *Rhizoctonia* and *Sclerotinia*	Patkowska et al. (2020)
Phaseolus vulgaris	*Pseudomonas putida* UW4 + *Rhizobium* PGPR	Enhanced plant growth under P stress	Remans et al. (2007)
Zea mays	*Achromobacter* sp., *Enterobacter* sp., *Leclercia* sp., *Pseudomonas* sp.	Increase in growth, ACC deaminase, nutrients concentrations, and drought stress tolerance	Danish et al. (2020)
Cucumis melo	*Trichoderma aggressivum* f. sp. europaeum, *Trichoderma saturnisporum*, *Trichoderma longibrachiatum*	Alleviate the stress produced by salinity, showed antagonistic activity against *Pythium ultimum*	Sánchez-Montesinos et al. (2019)
Allium cepa	*Azospirillum brasilense, Burkholderia ambifaria, Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae*	Enhanced plant growth and the soil fertility status	Pellegrini et al. (2021)
Oryza sativa	*Azospirillum amazonense*	Improved grain yield in rice by increasing N content	Rodrigues et al. (2008)
Glycine max	*Bacillus* sp. and *Trichoderma* sp.	Enhanced germination and potassium uptake under salt and drought stress	Bakhshandeh et al. (2020)
Solanum lycopersicum	*Trichoderma viride*	Increase the plant biomass by production of VOCs	Lee et al. (2016)
Wheat	*Azotobacter chroococcum, Trichoderma harzianum*	Enhance plant growth and stress tolerance	Silletti et al. (2021)
Pisum sativum	*Pseudomonas fluorescens* L321	Improved phosphate solubilization under phosphate-limiting conditions	Otieno et al. (2015)
Triticum aestivum	*Dietzia natronolimnaea* STR1	Alleviates salinity stress by modulation of abscisic acid signaling and antioxidant machinery	Bharti et al. (2016)

11.2.1 Mode of Action of Microbial Biostimulants

PGPMs interact with the roots of plants in different ways, from symbiosis to parasitism in fungi, and between mutualism and parasitism in bacteria, which can be transient or permanent (du Jardin 2015). The beneficial interaction between PGPM and the plant allows microorganisms to colonize the roots and adapt to abiotic stress in plants (Lopes et al. 2021). PGPM has developed several biostimulant mechanisms to promote plant growth and survival in stressful environments, such as fixation of N2, solubilization of nutrients, production of phytohormone and volatile organic compounds (VOCs) (Calvo et al. 2014; Odelade and Babalola 2019). Some microorganisms have the characteristics of improving plant nutrition and health by inhibiting phytopathogens (Sánchez-Montesinos et al. 2019).

Such characteristics represent important factors in selecting microorganisms and combinations for the development of PGPM-based inoculants. The role between different PGPMs inoculants with the host plants for abiotic stress tolerance has been reported to be extreme temperature, moisture deficit, and salinity (Sangiorgio et al. 2020; Lopes et al. 2021). For example, *Glycine max* plant treated with *Bacillus* sp. and *Trichoderma* sp. improve growth and potassium uptake under salt and drought stress (Bakhshandeh et al. 2020). *Zea mays* plant treated with *Achromobacter* sp., *Enterobacter* sp., *Leclercia* sp., and *Pseudomonas* sp. increase growth, ACC deaminase, nutrients concentrations, and drought stress tolerance (Danish et al. 2020). On the other hand, the interaction of *Trichoderma harzianum* inoculants with other non-microbial biostimulant allows better growth and health status of carrot plants against *Alternaria*, *Fusarium*, *Haematonectria*, *Rhizoctonia*, and *Sclerotinia*, compared to the activity of a single biostimulant (Patkowska et al. 2020). In general, microbial-based biostimulants have been reported to often increase the chlorophyll content allowing a greater photosynthetic activity of the leaves, as well as increasing the color of the leaves that makes it attractive to the consumer (Bulgari et al. 2019).

11.2.2 Novel Approach in Microbial Biostimulants Development

In recent years, the applications of microbial biostimulants have increased due to the demand for food, the interest in sustainable agriculture and the increase in the resistance of pests and pathogens to agrochemicals (Santos et al. 2019). However, the reproducibility of the effects of inoculants to be tested is still uncertain, so it remains a bottleneck for its wider use (Yakhin et al. 2017; Sangiorgio et al. 2020; Harris et al. 2021). This is due to microbial inoculant technology mostly being not practicable on a large scale, PGPM are highly selective and targeted, the cost of maintaining the viability of microbes during storage is very high, and the use of some microbial inoculants can be highly risky (Alori and Babalola 2018). It is for this reason that future research may lead to the development of second generation microbial biostimulants (biostimulant 2.0), in which synergistic processes can be carried out based on the characterization of plant-microbe interactions and the microbial community, and more specifically, that is compatible with modern agricultural conditions (Malik et al. 2021; Rouphael and Colla 2020).

By another way, for the development of microbe-assisted crop production approaches, beneficial plant–microbe interactions are required to be linked, allowing identifying of microbial dependence and identifying the genetic determinants for beneficial plant–microbiome interactions (Hohmann et al. 2020). Furthermore, French et al. (2021) studied the emerging strategies in the manipulation of agricultural microbiomes to improve crop yield and sustainability, focused on a holistic view of plant-associated microbiomes, that is, to integrate the knowledge of the interactions between the microbiomes of the soil, plants, and insects that influence the development of crops. Microbiome engineering is an emerging field of synthetic biology that may offer the development of biostimulants that are personalized and integrative in diverse agricultural systems (Kumar and Dubey 2020; Nephali et al. 2020; Ke et al. 2021).

Some studies have made approaches to understand the interaction of the agricultural microbiome in order to support decision-making on agronomic techniques to be implemented for the sustainable development of vegetable crop production. For example, the identification of microorganisms associated with the roots of the wild and crop plant allows obtaining an indicator of functional diversity to detect the threat of disease or search for beneficial isolates with biotechnology applications (Visioli et al. 2018; Hartman et al. 2018; Oszust and Frąc 2021). On the other hand, it allows us to understand the behavior of biostimulants with respect to the environment, such as the reduction of carbon emissions (Rajabi Hamedani et al. 2020), the positive effect on soil fertilization (Pellegrini et al. 2021), and the impact of agronomic practices on crops (Wipf et al. 2021).

11.3 Protein Hydrolysates

The protein hydrolysates (PH) have been divided into two categories from sources, animals, and vegetables. PH as plant biostimulants are a mixture of peptides and amino acids with effective results in plants productivity (Colla et al. 2015). The effect of these biostimulants is dependent on their source and physico-chemical and/or enzymatic treatment used to obtain them (Cavani et al. 2006; Colla et al. 2017a).

Animal protein sources are mainly derived from animal waste (epithelial tissue, collagen, feathers, leather, fish by-products) and plant waste (alfalfa, seeds, legumes, hay) (du Jardin 2012; Cavani et al. 2006; Kauffman III et al. 2007; Schiavon et al. 2008). PH of animal origin are mainly composed of a mixture of free amino acids, and also peptides (Ertani et al. 2014; Colla et al. 2015; Han et al. 2018). Plant-derived PH also possesses amino acids, peptides along with a smaller amount of soluble carbohydrates, phenols, and phytohormones. The content of these compounds depend on the type of substrate and hydrolysis used (Parrado et al. 2008; Ertani et al. 2014).

Another category present in this type of biostimulants is the group of individual amino acids (AA). AA biostimulants result from the combination of amino acids involved in constructing proteins and peptides derived from animals, plants, and micro-organisms (du Jardin 2012). Animal-derived PH have been used for more than 50 years and are the most traded PH with 90% of the world PH market. These biostimulants are mainly obtained through chemical hydrolysis. In Europe, India, and China, they use leather by-products, and in the United States, they use fish by-products (Colla et al. 2015, 2017b). PH hold one-third of the global market for plant biostimulants, and some of the commercial products available are: Siapton from Isagro SpA, Italy; ILSATOP from ILSA, Italy; and Macro-Sorb Foliar from Bioiberica Corp, Spain (Calvo et al. 2014).

11.3.1 Mode of Action of Protein Hydrolysates

PH has multiple functions and has been confirmed to have a positive effect on crops when applied as a foliar spray (Calvo et al. 2014; du Jardin 2012; Colla et al. 2015). These effects on plants have been reported to include: (1) modulation, uptake, and assimilation of nitrogen; (2) antioxidant activity; and (3) promote mobility and acquisition of micronutrients (Colla et al. 2014). Indirect effects that have been studied on plant nutrition and growth include: (1) increasing and enhancing microbial activity; (2) improving respiration; and (3) soil fertility (Calvo et al. 2014). Table 11.2 shows the positive aspects of PH on different crops.

11.3.2 Novel Approach in Protein Hydrolyzate Development

Plant-derived PH are emerging as an alternative to animal-derived PH that have dominated the PH market. These components represent an ecological and economical solution to reduce waste and increase recycling (Colla et al. 2017a, b). This type of PH has not presented phytotoxicity problems, unlike animal-derived PH. It has been reported that there is a growing concern about the safety of the use of PH from animal by-products. The application of these hydrolysates on edible sections of crops was banned by the EU (Regulation EU N° 354/2014) (du Jardin 2015). New analytical and molecular tools are now being applied to provide a better understanding of the composition of PH, together with evidence to improve productivity and stimulate plant response to different types of stress (Corte et al. 2014). In this way, omics technologies will make it possible to discover new biostimulant compounds and determine under pressure the effects of these compounds on crops, during their development, and under different environmental conditions (Povero et al. 2016; Ugena et al. 2018; Paul et al. 2019). These technological and fully automated tools allow the monitoring of hundreds of plants in a controlled environment (Großkinsky et al. 2015; Fahlgren et al. 2015).

Recent studies show that PH can positively influence plant microbiomes (structure and activity) in the rhizosphere and phyllosphere. Thus, PH improves the physiological process of plants due to better uptake of water, nutrients, and resilience to abiotic factors (Rouphael and Colla 2018). Nevertheless, despite all current efforts, it is necessary to put more emphasis and effort into these omics analyses to understand better the mechanisms of action at the species, crop, phenological stage, growth conditions, concentration, and type of application (Calvo et al. 2014; Colla et al. 2015).

Table 11.2 Examples of positive results on crops using protein hydrolysate (PH)

Crop	Nutritional effects on plants	Source	References
Maize	Increase of N	Epithelial tissue	Maini (2006)
Maize	Coleoptile elongation	Trainer®	Colla et al. (2014)
Maize	Increase of NO_3	Alfalfa protein hydrolysate	Ertani et al. (2009)
Maize	NO_3	Alfalfa protein	Schiavon et al. (2008)
Tomato	Fe, Zn, N	Histidine, glycine and arginine	Ghasemi et al. (2012)
Musa spp.	Reducing sugars and chlorophyll concentrations	Hydrolyzed poultry feather	Colla et al. (2017a, b)
Rice	Increase of Fe, Zn, Cu, Mn	Chicken feather hydrolysate	Jie et al. (2008)
Tomato	Increase of fruit number and mean weight	Amino$^{16®}$	Koukounararas et al. (2013)
Soybean	Increase of Fe	Commercial product from a combination of amino acids	Rodríguez-Lucena et al. (2010)
Tomato	Increase of shoots, root and biomass	Trainer® legume-derived	Colla et al. (2014)
Diospyros kaki	Alleviated salt stress and reduced leaf necrosis symptoms	Stressal® animal-derived	Visconti et al. (2015)
Rice	Increase of Fe, Zn	Amino acids	Yuan et al. (2013)
Banana	Increase of protein, total phenolics, flavonoids, as well as antioxidant activity	Feather degradation products containing both amino acids and peptides	Gurav and Jadhav (2013)
Vitis vinifera	Increase of total phenolic and anthocyanin concentration	Enzymatically treated vegetable extract	Parrado et al. (2007)
Tomato	Increase of antioxidant activities, K, Mg, and others	Trainer®	Rouphael et al. (2017)
Lettuce	Improved phyllosphere, microbiota, and increased chlorophyll content	Trainer® and Auxym®	Luziatelli et al. (2016)

11.4 Algae Extracts

Seaweed extracts (SWEs) are the predominant category of commercial biostimulants (Sharma et al. 2014). Seaweed or macroalgae extracts are now widely used as plant biostimulants (Du Jardin 2015). Seaweeds have been used for a long time, since prehistoric times, and were used as a source of food and for medicinal purposes. In agriculture, the Romans utilized seaweed as fertilizer along with other organic waste (Craigie 2011). In the seventeenth century, algae began to be used on an industrial scale, and since 1948, 18 countries had developed fertilizers using seaweed (Stirk et al. 2020; Craigie 2011). Nevertheless, the effects of SWEs have only recently been studied. A variety of commercial SWEs is now available worldwide for plant biostimulants (Craigie 2011; Khan et al. 2009).

Algal extracts are the dominant category and account for 95% in the plant biostimulants industry, with the market expected to reach a value of €894 million by 2022 (Stirk et al. 2020), with a CAGR of 12.6% and expected to reach €1808.78 million by 2027. Its market value was USD 944.70 million in 2019, worth more than twice as much as amino acid biostimulants. The SWE market in the Europe region has the largest market share, followed by North America and Asia-Pacific. Market leader FMC Corporation recorded 64.24% growth in annual revenue in 2018. The company's sales increased by 66% in North America, while sales in Asia and EMEA increased by 81% and 78%, respectively. The company's total sales in Latin America increased by 40%. Therefore, SWEs have considerable potential in the global biostimulants market.[4]

11.4.1 Production of Seaweeds

Brown seaweeds (Phaeophyceae) are the most commonly used seaweeds for the commercial manufacture of extracts for agricultural applications (Khan et al. 2009). These algae are the largest group with 2000 species, have the highest biomass, and are distributed along the rocky coasts of temperate zones in different countries (Blunden and Gordon 1986). The brown algae *Ascophyllum nodosum* (Ugarte 2011), *E. maxima*, *M. pyrifera*, and *D. potatorum*, are the species that have been most frequently used by algal extract industries for biostimulant purposes (Khan et al. 2009).

A wide range of chemicals has been identified in seaweed extracts, including complex polysaccharides (alginate, fucoidan, laminarin, and glucans) (Yabur et al. 2007; Rayorath et al. 2009; Craigie 2011;), phenolic compounds (dihydroxybenzene, trihydroxybenzene, phlorotannins) (Shibata et al. 2003), vitamins, phytohormones (Stirk et al. 2014), osmolytes (betaines, mannitol) (Reed et al. 1985; Blunden et al. 2009a, b), and molecular compounds involved in signaling the plant stress response (Battacharyya et al. 2015). The chemical composition of the extract depends mainly on the extraction method (physicochemical, chemical, enzymatic, grinding, temperature, or high pressure) and the chemicals used during the production process (Craigie 2011; Shukla et al. 2019) (Fig. 11.1). Thus, the biological activity of these extracts differs significantly depending on the type of algae, harvesting season, and mode of extraction (Kim 2012). The application of seaweeds to agricultural crops depends on the nature of the product (Fig. 11.1) (Patel and Mukherjee 2021). Seaweeds are applied as (1) whole seaweeds, (2) seaweed meal, and (3) seaweed extract. Seaweed or seaweed meal is introduced into the soil before planting crops to improve the physico-chemical properties of the soil.

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[4] https://www.marketsandmarkets.com; https://www.databridgemarketresearch.com

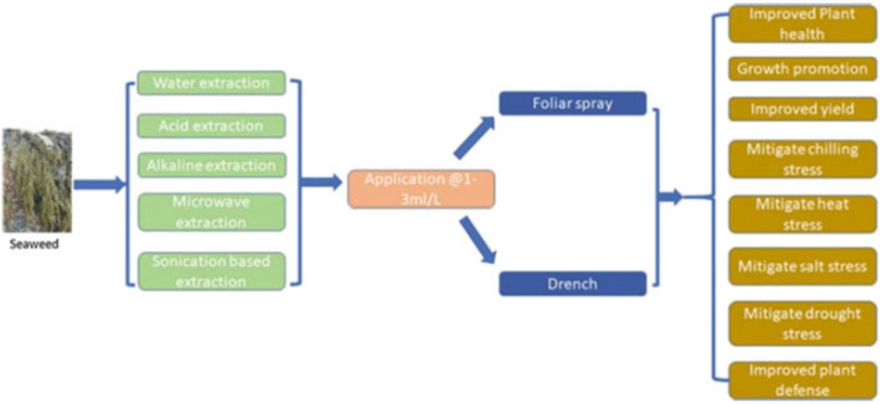

Fig. 11.1 Extraction processes, mode of application, and plants' responses by seaweed-based biostimulants (Patel and Mukherjee 2021)

Seaweed extracts are applied near the plant root and as foliar sprays (Battacharyya et al. 2015).

11.4.2 Biostimulatory Action of Seaweed

Studies have shown that seaweed affect plant uptake, growth, yield, plant metabolism, physiology, and resistance to abiotic stress (Calvo et al. 2014) (Fig. 11.1). There are research and reports on seaweeds and their contribution in positively stimulating the yield of various crops, including sprout improvement (Table 11.3) (Khan et al. 2009; Craigie 2011) and increased plant growth and yield, that are associated with hormonal effects, considered the main causes of biostimulation activity (Craigie 2011). Seaweeds help improve the quality of agricultural soils because their polysaccharides contribute to gel formation, water retention, and soil aeration, promoting positive effects on soil microflora, and help as an organic fertilizer through the supply of macro and micronutrients (Craigie 2011; du Jardin 2015). Seaweed extracts target various plant metabolic pathways to enhance plant tolerance to abiotic stresses (temperature, salinity, drought) (Mancuso et al. 2006; Khan et al. 2009; Craigie 2011). Seaweeds also function as elicitors and act by inducing defense activation against a wide range of infections. The bioactive components of seaweed, such as carrageenan, fucans, ulvans and other complex polysaccharides have mechanisms to protect against wide spectrum of pathogens (Sangha et al. 2010; Shukla et al. 2016).

Table 11.3 Summary of reported effects of marine algae on agricultural and horticultural crop enhancement

Algal product type	Reported effects as plant biostimulants	References
Extract of *S. polycystum*	Resistance to disease by pathogens	Khompatara et al. (2019)
Polysaccharide of *A. spicifera*	Defense responses against *P. palmivora*	Pettongkhao et al. (2019)
Sulphated polysaccharide of *Sargassum vulgare*	Anticoagulants, antithrombotics, and antioxidants effects	Dore et al. (2013)
Extract of *S. fusiforme*	Resistance in *S. lycopersicum*	Sbaihat et al. (2015)
Lipophilic components of *Ascophyllum nodosum*	Enhance freezing tolerance in *A. thaliana*	Rayirath et al. (2009)
Extract of *Ascophylum nodosum*	Enhance freezing tolerance in *A. thaliana*	Nair et al. (2012)
	Drought tolerance in spinach	Xu and Leskovar (2015)
Seaweed suspensions of *Ascophylum nodosum* and *Laminaria hyperborea*	Enhances seedling growth in lettuce	Moller and Smith (1998)
Extract of *Sargassum muticum* and, *Jania rubens*	Mitigate salinity stress	Latef et al. (2017)
Extract of *A. nodosum*	Increases productivity of tomato	Ali et al. (2016)
Extract of *Ulva intestinalis*	Regulation of hormone production in *Arabidopsis*	Ghaderiardakani et al. (2019)
Extract of *Ascophyllum nodosum*	*Clementine mandarin* and *Navelina orange*	Fornes et al. (2002)
Concentrate of *E. maxima*	Phyto-hormonal regulation in tomato	Finnie and Van Staden (1985)
Extract of *Ulva lactuca, Caulerpa sertularioides*, *Padina gymnospora*, and *Sargassum liebmannii*	Seed germination in tomato plant	Hernández-Herrera et al. (2014)
Extract of *Gracilaria textorii* and Hypnea musciformis	Seed germination of some vegetable crops	Rao and Chatterjee (2014)
Extract of *Sargassum myriocystum*	Stimulant of seedling (*V. mungo)*	Kalaivanan and Venkatesalu (2012)
Extract of *Sargassum wightii*	Increases growth of green gram	Kumar et al. (2012a, b)
Extract of *Ulva reticulata*	Growth of *V. mungo*	Selvam and Sivakumar (2013)
Extract of SUNRED	Improve the pigmentation of grape	Deng et al. (2019)
Nano-size Fertilizer of *Ascophyllum nodosum*	Improves growth, yield, and quality of wine crops	Sabir et al. (2014)

(continued)

Table 11.3 (continued)

Algal product type	Reported effects as plant biostimulants	References
Kelp	Improved production of *B. chinensis*	Zheng et al. (2016)
G. edulis and *S. wightii*	Plant growth of Tomato	Vinoth et al. (2012)
Ascophylum nodosum	Growth of spinach	Fan et al. (2013)
	Growth promotion *Brassica napus*	Jannin et al. (2013)

11.4.3 Novel Approach in Seaweed Extracts Development

A range of products derived from SWEs are reportedly currently being developed with the potential to be adopted in the existing industry.

Within recent technological developments, the concept of seaweed nanoparticles has been reported in different fields and therapeutic uses (Roy 2019). There are several kinds of research on the biological synthesis of nanoparticles (silver and gold) using marine algae (Roy 2019; Torres et al. 2005; Govindaraju et al. 2008; Soisuwan et al. 2010; Oza et al. 2012; Saraniya Devi and Bhimba 2014; Kumar et al. 2012a, b; Raja et al. 2012; Chellapandian et al. 2019). Nevertheless, only a few marine algae have been used for nanoparticle biosyntheses, such as *Sargassum wightii*, *Kappaphycus alvarezii*, including several *Gracilaria* species, such as *G. corticata, G. crassa, G. edulis,* and *G. birdie* (Singaravelu et al. 2007; Khanehzaei et al. 2015; Lavakumar et al. 2015). Applications of metal nanoparticles from algae presented various applications such as reducing agents, stabilizers, antimicrobials, and antifoulants (De Aragão et al. 2019; Kumar et al. 2008; Dhas et al. 2013; Prakash et al. 2015).

The algal biorefinery approach is a new approach that emerges to incentivize the growth and optimized exploitation of the seaweed industry. Algal biorefinery involves the generation of high-value co-products (plant biostimulants, food products, and biofuels), energy efficiency, and waste management that would improve the economic viability of this industry (Chandra et al. 2019; Zollmann et al. 2019). Additionally, the need for offshore cultivation using technologies that do not require additional nutrients is proposed, allowing for a more controlled environment of cultivation conditions and would improve biostimulant yields as well as processing pathways (Zollmann et al. 2019; Álvarez-Viñas et al. 2019).

SWEs are a growing industry that plays a considerable role in the economy of the agricultural sector. In that way, it is essential to close the gaps related to (1) legislation recognizing these biostimulants as a separate section from agricultural products (Chatzikonstantinou 2022); (2) meeting farmers' requirements and gaining their trust, (3) identifying the mode of action of SWEs; and (4) optimizing the extraction and production process of SWEs (El Boukhari et al. 2020).

11.5 Humic Substances

Humic substances (HS) are heterogeneous organic compounds and represent the most active part of the organic matter in the soil from the decomposition and oxidation of animal and plant residues and microorganisms (du Jardin 2015; Nardi et al. 2002). Analysis of HS showed that they contain proteins, carbohydrates, aliphatic biopolymers, and lignin (Kelleher and Simpson 2006). Piccolo (2002) reported that humic substances are supramolecular associations of heterogeneous and relatively small molecules. Humic substances are classified according to their origin, molecular size, chemical characteristics, and solubility (du Jardin 2015; Nardi et al. 2007) into humic acids (HA), fulvic acids (FA), and humins. This separation is made because most experimental and marketed biostimulants are identified explicitly as HA or FA (Calvo et al. 2014). The Global Humic Acid Market size was estimated to be over USD 503 million in 2020 and is projected to register a CAGR of 11.24% between 2021 and 2026. The market is segmented on the basis of form (powder, granular, and liquid), application (agriculture, animal feed, pharmaceuticals, horticulture, and other applications), and geography.[5]

11.5.1 Humic Acids

Humic acid (HA) is the main fraction of HS (Ferrara et al. 2007). HA are high molecular weight molecules (Nardi et al. 2009) that can be solubilized in basic media and collapse in acidic media (pH <3) (Berbara and García 2014; Colombo et al. 2015). HS aggregates form with the disruption of hydrophobic interactions and the increase of hydrogen bonds due to organic acids from the roots (Colombo et al. 2015; Piccolo et al. 2003).

11.5.2 Fulvic Acids

Fulvic acids are low molecular weight molecules that act as natural chelators transporting nutrients (Bocanegra et al. 2006). They are soluble in both acidic and basic media (Berbara and García 2014). Additionally, these biostimulants have a higher number of carboxyl groups, better adsorption, and cation exchange capacity, are soluble over a wide pH range, and ionic strength (Zimmerli et al. 2008; Bocanegra et al. 2006).

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[5] https://www.mordorintelligence.com/

11.5.3 Humic Substances' Effects

The potential of HS to increase the growth of vegetable crops is well known. Positive results of their application occur in the root and stem of plants. However, the mechanisms of action of HS have not been elucidated (Olaetxea et al. 2018). Table 11.4 summarizes the most relevant results of the effects of humic substances on plant development.

The application of HS helps to improve soil conditions and increase nutrient uptake by plants (Rose et al. 2014). For a better understanding, direct and indirect effects are divided (Fig. 11.2). The direct effects of HS are linked to plant development resulting from the interaction with cell membranes on the root surface. Indirect effects are those mediated by the action of HS on the rhizosphere (Olaetxea et al. 2018). Thus, a review is made of the main direct and indirect stimulating effects that HS have on the physiological processes of plant growth.

11.5.3.1 Direct Effects

HS acts on the roots by improving their growth. Therefore, the plant can better absorb nutrients and minerals and strengthen its resistance to abiotic stress (Nardi et al. 2007). This effect derives from the hydrophobicity and supramolecular association of the HS components. Roots exude organic acids that disrupt these molecular associations. These released molecules then gain access to the plant cells and trigger physiological responses (Piccolo et al. 1997, Piccolo et al. 2003; Cozzolino et al. 2001).

The most reported effect of humic acid application on plants is to stimulate root growth (Silva et al. 2011) as a consequence of triggering an increased expression of the enzyme H+-ATPase, similar to the hormone auxin, stimulating the plasmatic membrane H+-ATPases, which transform the free energy, which was released by ATP hydrolysis into a transmembrane electrochemical gradient and which is used for nutrient uptake. Furthermore, HS induces increased proline content which is associated with salinity stress mitigation (Aydin et al. 2012; Berbara and García 2014; García et al. 2012). Other studies showed that HS enhances the activity of enzymes of phenylpropanoid metabolism, a metabolism that is responsible for producing phenolic compounds that enhance protection against abiotic stress (Olivares et al. 2015; Schiavon et al. 2010).

11.5.3.2 Indirect Effects

HS improve soil quality. These biostimulants increase soil fertility through the improvement of physico-chemical and biological properties. These biostimulants components enable permeability and ion transport, along with good aeration and increased water capacity (du Jardin 2015; Nardi et al. 2002). Increase in fertilizer efficiency and reduction in soil compaction. Furthermore, it allows better absorption of macro- and micronutrients through cation exchange and increased phosphorus availability (du Jardin 2015). This is because HS form natural or stable complexes, and increase their solubility and availability (Senesi 1992; Chen et al. 2004a, b).

Table 11.4 Effects of humic substances on plants (Calvo et al. 2014)

Type of humic substance	Specific effects of humic acids on plants	Crop	Reference
Acid Hulmic	Promotion of root system development	Tomato	Adani et al. (1998); Canellas et al. (2011)
		Arabidopsis	Dobbss et al. (2010); Canellas et al. (2010)
		Wheat	Tahir et al. (2011); Peng et al. (2001)
		Maize	Canellas et al. (2002, 2009); Eyheraguibel et al. (2008); Jindo et al. (2012)
		Pepper	Cimrin et al. (2010)
		Lantana camara	Costa et al. (2008)
	Shoot growth promotion	Tomato	Adani et al. (1998); Lulakis and Petsas (1995)
		Wheat	Tahir et al. (2011)
		Maize	Eyheraguibel et al. (2008)
		Pepper	Cimrin et al. (2010)
	Increased yield or crop quality	Okra (*Abelmoschus esculentus*)	Kirn et al. (2010)
		Wine grapes	Morard et al. (2011)
		Basil (*Ocimum basilicum*)	Befrozfar et al. (2013)
		Pepper in in-ground Greenhouse (Turkey)	Karakurt et al. (2009)
	Increased plant uptake of nutrients	Tomato	Adani et al. (1998)
		Pepper	Cimrin et al. (2010)
		Pear	Marino et al. (2010)
		Bean (*P. vulgaris*)	Aydin et al. (2012)
	Enhanced tolerance to salinity stress	Bean plants	Aydin et al. (2012)
		Maize	Mohamed (2012)
		Pepper	Cimrin et al. (2010)
		Chrysanthemum (*Chrysanthemum indicum*	Mazhar et al. (2012)
		Pistachio (*Pistacia vera*)	Moghaddam and Soleimani (2012)
	Beneficial effects on plant physiology	Maize	Canellas et al. (2009)
			Quaggiotti et al. (2004)
		Transgenic tomato	Canellas et al. (2011); Dobbss et al. (2010)
		Avena sativa	Pinton et al. (1997)
		Maize roots	Pinton et al. (1999)
			Carletti et al. (2008)

(continued)

Table 11.4 (continued)

Type of humic substance	Specific effects of humic acids on plants	Crop	Reference
		Arabidopsis roots	Tsukagoshi et al. (2010)
		Bean	Aydin et al. (2012)
		Rice	Garcia et al. (2013)
		Root of maize	Lulakis and Petsas (1995); Eyheraguibel et al. (2008)
		Hoots of maize	Anjum et al. (2011); Eyheraguibel et al. (2008)
		Olea europaea	Murillo et al. (2005)
		Helianthus annuus	Bocanegra et al. (2006)
	Amelioration of abiotic stress	Wheat	Peng et al. (2001)
		Wheat	Gu et al. (2001)
	Plant physiology and metabolism	Maize	Anjum et al. (2011)
		Soybean and ryegrass	Chen et al. (2004a, b)
		Embryogenic cells of Greek	Zancani et al. (2011)

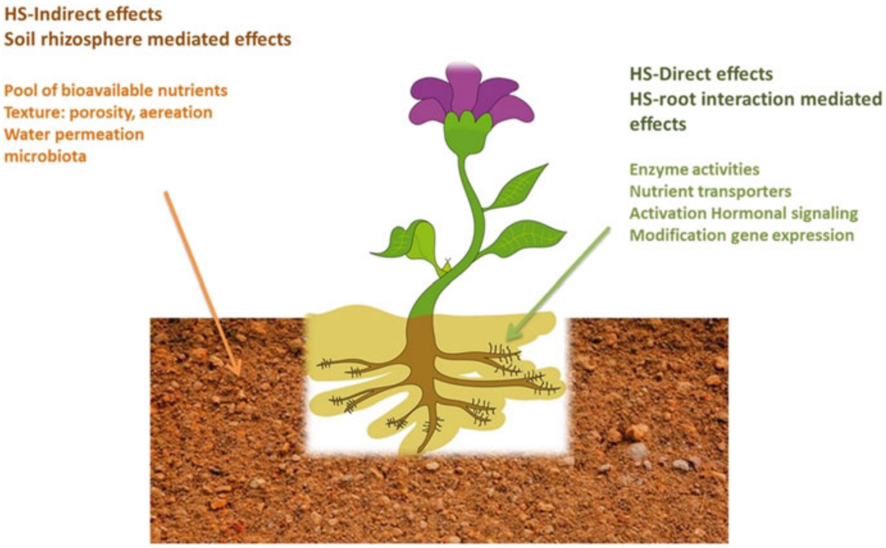

Fig. 11.2 Direct and indirect effects of humic substances on plant development (Olaetxea et al. 2018)

11.5.4 Concluding Remarks and Potential Future Research

There are several studies demonstrating the positive effects of HS in improving nutrient uptake, water retention, microbial growth, and soil structure by improving cation exchange capacity and oxygen content (Piccolo 2002). Other studies confirm that HS increase plant growth and nutrition because these biostimulants act on the soil by forming stable molecular associations with metals such as Fe, N, and P (Olaetxea et al. 2018). HS act according to their structural characteristics influencing plant physiology (Rose et al. 2014; García et al. 2016). However, there are currently no studies that precisely determine how the components of HS substances interact by macromolecules or single molecules that can form molecular aggregates in solution (Esfahani et al. 2015). It is important to study and investigate humic molecular aggregates, as they can indicate the appropriate extraction methods and their specific bioactivity. In addition, it is important to determine the beneficial action of HS in relation to the type of application, whether foliar or root application (Rose et al. 2014). In this pathway, future research should determine the actual efficiency of HS on yields and quality of extensive crops, and the interactions of HS with soil microbiota and plant microbiota (Olaetxea et al. 2018).

11.6 Conclusions and Perspectives

Plant biostimulants (PB) are effective products to make horticulture more sustainable. The market for PB has increased considerably over the last 10 years due to the need to ensure food security in a sustainable way. As such, there is a need to decrease the dependency on chemical and synthetic fertilizers. The examples of biostimulants presented in this chapter show the capacity of each category to improve growth, yield, resource use efficiency, and ameliorate stresses related to environmental stresses. However, there is insufficient knowledge on the potential benefits derived from PB applications. Therefore, more research is needed on PBs to substantiate their composition, at the molecular level, and their mode of action on crops. In addition, more research is needed to improve production standards (raw material and treatments), to define type, dosage and timing of application for each crop, and the effects of these biostimulants on the microbial communities in the rhizosphere or phyllosphere. Although significant progress has been made in the study of the physiological and biochemical mechanisms of PB using high-throughput phenotyping tools, the physiological and molecular mechanisms underlying the synergy between PB are not yet known.

Future research and development of formulations based on a combination of microbial and non-microbial PB could provide greater and more reproducible benefits to crop production. The development of tools that allow comparison of these formulations in terms of their composition and likely impact on crops is required. It is important to evaluate these formulations that have the potential to be a pipeline of products available to improve crop tolerance to adverse seasonal conditions.

The regulatory situation of PB is currently complex. There is no clear definition or approval of the concept by the regulatory agencies. Therefore, a regulatory framework is needed to help guide decision-making by farmers, consultants, and public and private sector stakeholders. The right regulations would benefit the development of the global PB market and encourage further investment in research and development of a second generation of these bio-based products. There is a growing interest in natural biostimulants with more efficient action on crop productivity and quality. This would encourage their application on a larger scale and with greater frequency, integrating this type of ecological strategies and solutions into farming systems, with the capacity to preserve natural resources and satisfy the growing global demand for food.

Funding Sources This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest The authors declare that they have no conflict of interest.

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