Toward a Sustainable Agriculture Through Plant Biostimulants: From Experimental Data to Practical Applications

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Abstract: Modern agriculture increasingly demands an alternative to synthetic chemicals (fertilizers and pesticides) in order to respond to the changes in international law and regulations, but also consumers’ needs for food without potentially toxic residues. Microbial (arbuscular mycorrhizal and plant growth promoting rhizobacteria: Azotobacter, Azospirillum and Rizhobium spp.) and non-microbial (humic substances, silicon, animal- and vegetal-based protein hydrolysate and macro- and micro-algal extracts) biostimulants represent a sustainable and effective alternative or complement for their synthetic counterparts, bringing benefits to the environment, biodiversity, human health and economy. The Special Issue “Toward a sustainable agriculture through plant biostimulants: from experimental data to practical applications” compiles 34 original research articles, 4 review papers and 1 brief report covering the implications of microbial and non-microbial biostimulants for improving seedling growth and crop performance, nutrient use efficiency and quality of the produce as well as enhancing the tolerance/resistance to a wide range of abiotic stresses in particular salinity, drought, nutrient deficiency and high temperature. The present compilation of high standard scientific papers on principles and practices of plant biostimulants will foster knowledge transfer among researchers, fertilizer and biostimulant industries, stakeholders, extension specialists and farmers, and it will enable a better understanding of the physiological and molecular mechanisms and application procedure of biostimulants in different cropping systems.

Keywords: humic substances; protein hydrolysates; silicon; arbuscular mycorrhiza; plant growth promoting rhizobacteria; macroalgae; microalgae; abiotic stresses; nutrient use efficiency; physiological mechanisms

1. Biostimulants in Agriculture: Rationale

Modern agriculture needs to review and broaden its practices and business models, by integrating opportunities coming from different adjacent sectors and value chains, including the biobased industry, in a fully circular economy strategy [1–3]. Farmers need to operate as managers of the countryside, valorizing their own by-products and using agricultural products with improved environmental profile. Therefore, searching for new technologies and approaches to boost crop productivity under optimal and sub-optimal conditions and to improve resources use efficiency (water and fertilizers) is crucial to ensure food security, while preserving soil quality and providing opportunities of business for farmers [4]. Biobased products such as biostimulants represent a sustainable, efficient technology or complement to their synthetic counterparts (i.e., agrochemicals) to improve nutrient use efficiency and secure yield stability of agricultural and horticulture crops under optimal and sub-optimal conditions [5,6]. Recently, under the new Regulation (EU) 2019/1009, plant biostimulants were defined based on four agricultural functional claims as follow: “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 and/or the plant rhizosphere: (1) nutrient use efficiency, (2) tolerance resistance to (a)biotic stress, (3) quality characteristics, or (4) availability of confined nutrients in the soil or rhizosphere” [7]. Many diverse natural substances and chemical derivatives of natural or synthetic compounds as well as beneficial microorganisms are catalogued as plant biostimulants including: (i) humic substances; (ii) vegetal- or animal-based protein hydrolysates; (iii) macro- and micro-algal extracts; (iv) silicon; (v) arbuscular mycorrhizal fungi (AMF); and (vi) plant growth promoting rhizobacteria (PGPR) belonging to the genus Azotobacter, Azospirillum and Rizhobium spp. [8–16].

Plant biostimulants were initially used in organic production, but now they are adopted in several cropping systems such as conventional and integrated crop production [17]. Microbial and non-microbial plant biostimulants are usually used for open field and greenhouse crops including fruit trees, berry crops, grapevines, vegetables, ornamentals, cereals and turf [18–21]. The biostimulants market is increasing year by year; as a matter of fact, the market of active ingredient biostimulants (amino acids, seaweed extracts, humic substances and microbial amendments) is estimated to account for 2.6 billion dollars in 2019 and is projected to reach almost 5 billion dollars by 2025, at a compound annual growth rate of 11.2% during the forecast period [7,22]. Moreover, more than 1000 scientific papers published in the last 10 years (2010–2020) were found by searching the term “plant biostimulants” and many more articles are available on the Scopus database using related words/terms (i.e., humic substances, seaweed extracts, microalgae, silicon, AMF or PGPR) (www.scopus.com).

The current Special Issue collects 39 scientific contributions (34 research papers, 4 reviews and 1 brief report) covering the different aspects of the agronomic and horticultural crops response to microbial and non-microbial biostimulants application. We highly believe that the current Special Issue: (i) will foster knowledge transfer among scientists, commercial enterprises, stakeholders and farmers; and (ii) will shed light on the cellular, molecular and physiological mechanisms as well as the application procedure of biostimulants in different cropping systems including organic farming.

2. The Role of Non-Microbial and Microbial Biostimulants in Morpho-Anatomical, Biochemical and Physiological Traits of Crops

Applications of non-microbial and microbial plant biostimulants have been shown to enhance plant growth and development, as well as macro- and micronutrient uptake and translocation in several agronomic and horticultural crops resulting in increased biomass production and yield [3]. The stimulation of seedling growth and crop productivity in response to application of non-microbial and microbial plant biostimulants is attributed to the action of bioactive substances on the primary and/or secondary metabolisms, leading to a wide array of biochemical, physiological and molecular responses [3]. Seven combinations of soy flour, diatomaceous earth, concentrated vermicompost extract (liquid) and micronized vermicompost were investigated in laboratory experiments to assess their potential biostimulant action to improve cover crops (red clover and perennial ryegrass) germination and seedling growth [23]. In their research, the authors reported that coated treatments affected in a species-specific manner the germination rate and uniformity, with a significant improvement in total germination rate recorded in red clover, while a reduction was observed in perennial ryegrass. Interestingly, the application of soy flour:diatomaceous earth at a rate of 30:70 boosted the seedlings performance in terms of shoot and root growth as well as dry matter percentage in both tested species. The authors concluded that soy flour provided a sustained source of key amino acids, thus positively influencing N uptake and transplant quality. Furthermore, Ben-Jabeur et al. [24] conducted a three-year experiment on durum wheat aiming to assess the effect of coating wheat seeds with thyme essential oil or Paraburkholderia phytofirmans PsJN strain on yield and resistance/tolerance to Septoria leaf botch. The two tested biostimulants were able to alleviate the Septoria leaf botch and to enhance yield in terms of number of spikes per square meter as well as straw and grain yields. The dual beneficial effect (i.e., biocontrol and biostimulant action) was also observed on tomato, where the application of four commercial biostimulants: neem seed cake, sesame oil, quillay extract and seaweeds significantly
mitigated the parasitism of root-knot nematodes by reducing eggs and galls on tomato roots with the best results recorded on neem seed cake and sesame oil treatments [25]. The authors also demonstrated that the four tested biostimulants triggered shoot and root biomass production compared to untreated control. The dual beneficial effect was also recorded on tomato, since Allaga et al. [26] reported that a composite bioinoculant containing beneficial fungi and bacteria (Trichoderma, Azotobacter and Streptomyces) was an efficient biocontrol agent, as well as an efficient biostimulant able to improve growth and photosynthetic activity of tomato.

Ertani et al. [27] carried out a short-term trial on hydroponically grown maize to assess the physiological responses to Leonardite-humate- and lignosulfonate-based biostimulants. The biostimulants application in particular lignosulfonates boosted root and leaf growth by 51–140% and 5–35%, respectively. The authors concluded that a putative mechanism involved in the biostimulant action of these products might be the stimulation of N metabolism in the belowground organs (i.e., roots) according to the increased activity of key enzymes such as glutamine synthetase and glutamate synthase [27]. Moreover, Kim et al. [28], elucidated the hormonal effects of a commercial vegetal-based biostimulants containing amino acids, lateral root promoting peptide, lignosulfonates and micronutrients on cuttings of basil, tomato and chrysanthemum, characterized by different relative root ability: easy, moderate and difficult, respectively. Thanks to the combination of morphological, biochemical and metabolomics approaches, the authors demonstrated that the vegetal-based biostimulant exerted similar effects to the synthetic hormone (i.e., auxin) by improving adventitious rooting responses. Finally, the authors shed light for the first time onto hormonal regulation of vegetal-based biostimulant and the crucial role of brassinosteroids in adventitious root formation.

Different amino acids (L-methionine, L-glycine and L-tryptophan at 20, 210 and 220 mg/L, respectively) were applied separately on hydroponically grown butterhead lettuce to assess their stimulators role [29]. In their study, L-methionine boosted lettuce growth parameters, whereas a negative effect was observed when L-glycine and L-tryptophan were applied. Based on the results of the first experiment, Khan and co-workers conducted a second experiment with five increasing concentrations of L-methionine (0.02, 0.2, 2.2, 22 and 2220 mg/L). The authors concluded that L-methionine at a concentration of 0.2 mg/L exhibited the best effect of lettuce growth parameters. In fact, it is well established that key amino acids are rapidly absorbed by the crops and act as a stable source of molecule precursors to be integrated into plant metabolism [30]. This was demonstrated by the former authors, who reported that foliar application of glutamate to creeping bentgrass foliage was rapidly absorbed and directly utilized as a precursor to synthesize gamma-aminobutyric acid and proline, two important metabolites with well-known roles in plant stress adaptation.

Bákyoni et al. [31] and Kisvarga et al. [32] reported that alfalfa brown juice could be considered a potential growth stimulator. In their studies, Celosia seedlings where sprayed at five increasing rates of fermented brown juice (0.5%, 1.0%, 1.5%, 2.0% or 2.5%), while basil was sprayed at three different increasing doses (0.5%, 1.0% or 2.5%). Water was adopted in both experiments as an untreated control. The application of alfalfa brown juice at a rate of 0.5% boosted plant growth parameters in both tested species due to the modulation of the anatomical and biochemical responses, in particular increasing the antioxidant activity of key enzymes (catalase and peroxidase) and photosynthetic pigments (chlorophyll a and b) as well as reducing the content of malondialdehyde. Moreover, Niewiadomska et al. [33] carried out a three-year experiment on white lupine cultivation, where two commercial biostimulants and six foliar fertilizers were tested. The commercial biostimulants and fertilizers were able to boost some of the biochemical activity of the soil. The authors attributed the better performance of treated-white lupine to a higher uptake, translocation and assimilation of macro- and microelements.

Seaweed extracts, also known as macroalgae, are considered an important category of non-microbial plant biostimulants due to their use on several agronomic and horticultural crops under both conventional and organic farming systems [34]. Several authors reported that macroalgae such as Ascophyllum nodosum, Ecklonia maxima or Pterocladius capillacea can: (i) improve the agronomic performance of soybean and
bean [35,36], potato [37], and Jew’s mallow [38]; and (ii) enhance fruit setting in eggplant [39]. In addition to seaweed extracts, the use of PGPR such as *Bacillus thuringiensis* was also considered an efficient approach to boost yield in a sustainable manner. Jo and co-workers [40] inoculation of *Bacillus thuringiensis* KNU-07 incurred a significant increase of total growth biomass of pepper seedlings. The beneficial effect recorded on inoculated pepper plants was associated with a strong modulation of the soil bacterial community even quantitatively or qualitatively.

3. The Role of Non-Microbial and Microbial Biostimulants in Enhancing Nutrient Uptake and Efficiency

Non-microbial and microbial plant biostimulants may positively influence nutrient use efficiency (NUE), in particular nitrogen (N) by enhancing root system architecture and soil exploration as well as increasing macro- and micronutrient solubilization that can result in an increase in NUE [17,41]. Di Mola et al. [42] demonstrated that foliar application of vegetal- (protein hydrolysates or tropical plant extract) and seaweed extract-based biostimulants (*Ecklonia maxima*) is considered a sustainable approach to increase greenhouse baby lettuce productivity and NUE in low-input cropping systems. In their study, the authors reported that the application of legume-derived protein hydrolysates and especially seaweed extract elicited important increases in fresh yield under sub-optimal and optimal N conditions (0 and 10 kg ha$^{-1}$) compared to the untreated and tropical plant extract-treated plants, but the beneficial effect of plant biostimulants was not apparent under luxurious N fertilization conditions (20 and 30 kg ha$^{-1}$). Similar results were also observed by the same research group [43] on two other important greenhouse leafy vegetables, namely baby spinach and lamb’s lettuce, treated with a legume-derived protein hydrolysates and grown under optimal and sub-optimal N regimes. Interestingly, the foliar application of vegetal-based biostimulants incurred a significant increase in N uptake and N use efficiencies in both leafy vegetables (19% and 18%, respectively, for baby spinach and 50% and 73%, respectively, for lamb’s lettuce). The authors concluded that improved agronomical performance and use efficiency of baby lettuce, baby spinach and lamb’s lettuce was associated with a better photosynthetic activity and biochemical status (higher content of chlorophyll a, b and total and carotenoids) [42,43]. The synergistic biostimulant action through the application of microbial (*Trichoderma virens*) and non-microbial biostimulant (vegetal biopolymer containing amino acids, peptides and vitamins) was demonstrated on greenhouse lettuce grown with three N conditions: sub-optimal, optimal and supra-optimal (0, 70 and 140 kg ha$^{-1}$) [44]. Lettuce grown under non-fertilized conditions showed an increase in marketable yield when inoculated with *T. virens* alone (45%) and a greater increase with both microbial and non-microbial biostimulant (67%). The beneficial effect of plant biostimulant was less pronounced under optimal N condition and absent under luxurious N conditions. Rouphael and co-workers concluded that, based on the improved fresh yield and NUE in greenhouse lettuce plants, treatment with plant biostimulants improved not only the chlorophyll synthesis and mineral status but also the synthesis and accumulation of antioxidant metabolites that were responsible for reactivating the photosynthetic activity and consequently the agronomic performance.

Concerning floricultural species, Leoni et al. [45] investigated the application of chemical fertilization and integrated nutrient management on yield, quality attributes and NUE of two chrysanthemum cut flower cultivars. Integrated nutrient management based on 50% synthetic fertilizers plus seaweed extract (*A. nodosum*) and microbial consortium (*Glomus* sp. and *Bacillus* sp.) was able to boost yield, quality parameters and NUE compared to the untreated control treatment.

4. The Role of Non-Microbial and Microbial Biostimulants in Abiotic Stresses Tolerance/Resistance

Abiotic stresses, in particular drought, salinity, heat stress, hypoxia and nutrient deficiency, are responsible for 60–70% of yield gap, dictated by global climate changes [46]. To overcome the detrimental effects of sub-optimal conditions on agronomic and horticultural crops, plant biostimulants
have been proposed as an efficient agronomic tool to improve tolerance/resistance to unfavorable environment and soil conditions [47]. In their review paper, Bulgari and co-workers summarized the biostimulants literature (humic substances, seaweed extracts, protein hydrolysates, amino acids and beneficial microorganisms) regarding their use on vegetables, focusing on their application and mode of actions to counteract the most common abiotic stresses: cold/chilling stress, heat, salinity, drought stress and nutrient deficiency. In addition to the categorized plant biostimulants, Arnao and Hernández-Ruiz [48] proposed the dual use of melatonin (N-acetyl-5-methoxytryptamine) as plant protector and biostimulant. In their review paper, they discussed the different legal aspects to categorize this natural substance as potential biostimulant at the European level. Arnao and Hernández-Ruiz [48] summarized studies of different responses of melatonin in different plant species and under diverse stress conditions by reporting the observed effects/mechanisms.

The application of four commercial biostimulants containing protein hydrolysates, humic acid and especially brown seaweed extracts (A. nodosum) were found to mitigate the negative effects of water stress (70% or 50% of the container substrate capacity) on potted mint by increasing the antioxidant activity of key enzymes such as catalase and superoxide dismutase and by reducing the H$_2$O$_2$ accumulation in leaf tissue [49]. The physiological and biochemical effects of β-(1,3)-glucan (paramylon) purified from the microalga *Euglena gracilis* on water-stress Micro-Tom were also assessed by an Italian research group [50]. The eco-physiological approach adopted in this study allowed the identification of several physiological and biochemical mechanisms of improved water stress tolerance, following the application of paramylon nanofibers, for example: (i) increasing of the photosynthetic rate; and (ii) reducing the sensitivity of photosystem II to potential dehydration damages. Moreover, Petropoulos et al. [51] showed that the application of four commercial microbial biostimulants containing AMF, *Trichoderma* and rhizosphere symbiotic bacteria enriched with amino acids or seaweed extracts were able to increase the pods and seeds yield as well as nutritional value and chemical composition of common bean under both optimal and sub-optimal water regimes. In the study by Mannino et al. [52], the impacts of four microbial biostimulants, namely AMF mono fungal inoculum, AMF multi fungal inoculum, PGPB and AMF + PGPB, on molecular and physiological responses of water-stressed tomato were evaluated. Different physiological and molecular responses of tomato to water limitation were recorded depending on microbial inocula, confirming the importance to characterize the optimal plant/beneficial microorganism genotype combination(s) to enhance plant resilience to water stress condition. Non-microbial plant biostimulants such as amino acids/peptides-based product and protein hydrolysates can also be considered an effective tools to improve the tolerance to a wide range of abiotic stresses: heat, hypoxic, nutrient and salt stresses as well as combined environmental stresses [53,54]. The application of biostimulant based on plant and yeast extracts and containing amino acids, soluble peptides and vitamins improved the heat stress tolerance of four tomato landraces grown under Mediterranean conditions. The biostimulant effects were associated to physiological and biochemical mode of actions, for example: (i) stronger antioxidant defense system; and (ii) maximal photochemical efficiency (F$_{v}$/F$_{m}$) in leaves of the four tested tomato landraces [53]. Finally, Trevisan et al. [54] demonstrated in a short-term trial that the application of a protein hydrolysates-based biostimulant was able to mitigate the detrimental effects of single (hypoxia, salt or nutrient deficiency) and multiple (nutrient stress + hypoxia or nutrient stress + salinity) stresses of hydroponically grown maize. Root development in terms of biomass and architecture (length and density) was strongly influenced by protein hydrolysates, by upregulating the expression of key genes involved in nitrate transport and reactive oxygen species detoxification and consequently inducing a significant boost of shoot biomass.

5. The Role of Non-Microbial and Microbial Biostimulants in Improving Quality Traits

Pre-clinical and clinical studies have demonstrated the functional (i.e., health-promoting) effects of fruit and vegetables consumption in supporting human health and longevity [55]. In their review paper, Drobek et al. [56] gave an overview on how the application of microbial and non-microbial plant biostimulants can modulate the primary and secondary metabolisms of horticultural species,
leading to the synthesis and accumulation of lipophilic and hydrophilic antioxidant molecules also known as phytochemicals [3,15]. The application of vegetable-based biostimulants, in particular tropical plant extract and legume-derived protein hydrolysates, in two important leafy and fruit vegetables induced significant increase in lettuce and tomato nutritional and functional quality [57,58]. Weekly foliar application of tropical plant extract incurred a significant increase of hydrophilic antioxidant activity and total ascorbic acid in lettuce compared to untreated control [57]. Similar results were also recorded in tomato fruits, where tropical plant extract and protein hydrolysates resulted in higher bioactive compounds (total phenols and vitamin C) and lipophilic antioxidant activity than those observed in the non-treated control [58]. Concerning berry fruits, Soppelsa et al. [59] investigated the application of ten commercial biostimulants belonging to almost all the categories including: alfalfa hydrolysate, humic acids, macro-seaweed, extract and microalgal hydrolysate, amino acids alone or in combination with micronutrient (zinc), B-group vitamins, chitosan and a commercial product containing silicon. Biostimulant products based on chitosan had a major impact on strawberry pulp firmness, whereas biostimulant products based on alfalfa hydrolysate, macro-seaweed extract and microalgal hydrolysate induced an improvement in phenolic compounds compared to the remaining treatments. Moreover, in three varieties of winter rape, the application of three biostimulants with the following active substances improved the content of crude fiber and fat: titanium, sodium ortho nitrophenol, sodium para nitrophenol, sodium 5-nitroguaiacolate and silicon [60].

Concerning the implications of microbial plant biostimulants on improving produce quality, Chandrasekaran et al. [61] reported that the inoculation of PGPR strain, *Bacillus subtilis* CBR05 induced a significant increase in tomato quality in terms of carotenoids profile (β-carotene and lycopene). Finally, Caser et al. [62,63] showed that the inoculation of soilless-grown saffron with *Rhizophagus intraradices* and to a lesser extent with a mixture of *R. intraradices* and *Funneliformis mosseae* boosted significantly the synthesis and accumulation of health-promoting molecules such as anthocyanins, polyphenols and vitamin C; antioxidant activity; and important bioactive compounds in saffron, such as crocin II, picrocrocin and quercitrin.

6. Conclusions and Looking Forward

In the coming few years, we can expect that plant biostimulants including both natural and synthetic substances, as well as microbial inoculants, will not only make a significant contribution to ecologically and economically sustainable crop production systems within more resilient agro-ecosystems, but will also lay the cornerstone for a future large-scale sustainable agriculture catalyzed by the biobased industry. Although plant biostimulants appear to be a novel and potential category of agricultural inputs complementing synthetic fertilizers, there is an urgent need among the research community and fertilizer industries to elucidate the molecular and physiological mechanisms which will definitely facilitate the diffusion of these bio-products in the agricultural sector. Briglia et al. [64] demonstrated that the combination of phenomic (high-throughput plant phenotyping) and genomic (Next Generation Sequencing) tools opens new perspectives to release effective biostimulant formulations to meet the emerging needs of crops. Finally, Giovannini et al. [65] suggested that, in the near future, transcriptomics research should be adopted as an integrated tool to identify the best synergistic combinations of AMF and associated bacterial communities able to enhance resources use efficiency, plant resilience and boosting nutraceutical compounds in plant species.

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