



Review

# Biostimulants on Crops: Their Impact under Abiotic Stress Conditions

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**Abstract:** Biostimulants are agronomic tools that have been gaining importance in the reduction of fertilizer applications. They can improve the yield of cropping systems or preventing crop yield losses under abiotic stresses. Biostimulants can be composed of organic and inorganic materials and most of the components are still unknown. The characterization of the molecular mechanism of action of biostimulants can be obtained using the omics approach, which includes the determination of transcriptomic, proteomic, and metabolomic changes in treated plants. This review reports an overview of the biostimulants, taking stock on the recent molecular studies that are contributing to clarify their action mechanisms. The omics studies can provide an overall evaluation of a crop's response, connecting the molecular changes with the physiological pathways activated and the performance with or without stress conditions. The multiple responses of plants treated with biostimulants must be correlated with the phenotype changes. In this context, it is also crucial to design an adequate experimental plan and statistical data analysis, in order to find robust correlations between biostimulant treatments and crop performance.

**Keywords:** abiotic stresses; omics; plant extracts; proteomics; seaweeds; metabolomics; transcriptomic



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## 1. Introduction

Biostimulants are innovative agronomic tools that, based on the concentrations used, are placed between fertilizers and plant growth regulators. Biostimulants are organic or inorganic products containing bioactive substances and/or microorganisms, which, when applied to the plant or rhizosphere, stimulate the growth and productivity of the plant by improving the absorption and assimilation efficiency of nutrients, tolerance to abiotic stresses and/or quality of the product regardless of their nutrient content [1,2]. This definition is reported in the latest European regulation on fertilizers, which includes biostimulants (EU2019/1009, <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R1009>, accessed on 1 February 2022).

Non-microbial biostimulants are obtained from different organic matrices subjected to different extraction processes that allow the concentration of bioactive compounds responsible for increasing the growth or tolerance of crops to adverse environmental conditions [3–6]. The complex nature of the raw organic material, and therefore the composition of the product obtained, make it very difficult to understand which compounds are the most active and responsible for the biological action. The compounds exerting the biostimulant action can be non-nutritional minerals (e.g., selenium, silicon), vitamins, amino acids, chitin, chitosan, poly- and oligosaccharides, and traces of natural plant hormones (included in the starting matrix) [3,7–9].

Biostimulants can also be produced from food waste or agro-industrial by-products [9]. The use of by-products as raw material for the production of biostimulants is part of the circular economy strategy to achieve an increasingly sustainable agriculture.

In general, biostimulants can act on the primary metabolism by increasing photosynthetic activity and derived compounds or they can stimulate the secondary metabolism by activating specific biosynthetic pathways [1,10].

The isolation and study of a single component are almost impossible to carry out, and the effectiveness of a biostimulant is probably not due to a single compound but to the synergistic actions of different bioactive molecules [11]. In fact, the effectiveness of biostimulants mainly depends on their composition, which unfortunately remains the most critical point, given the difficulty in characterizing it. To obtain more stable biostimulants, efforts have been made to reduce the heterogeneity of the starting materials and to standardize the different extraction technologies used.

## 2. Classification of Biostimulants into Categories

Over the years, several authors have proposed different classifications of biostimulants based on their origin, main component, or mode of action [7,10,12]. In many countries outside the European Union, both information must be on the label in order to register these products. The current classification is based on the source of raw materials even if this choice does not always provide the correct information on the biological activity of the product. Biostimulants can be classified into five major groups, as summarized below.

### 2.1. Seaweeds and Plant Extracts

Seaweeds are a large group and include macroscopic marine algae and multicellular algae belonging to different taxonomic groups, such as brown, red, and green algae [13,14]. They are an important source of nutrients, bioactive compounds, organic matter, and fertilizers. Algae have been used in agriculture since ancient times as fertilizers, due to the positive effects on crops. Seaweed extracts are currently exploited in agriculture as soil conditioners or as plant biostimulants [8]. When used as biostimulants, they are applied as foliar treatments and their biological effect is manifested by improving growth, crop yield, and product quality, and by increasing tolerance to abiotic stress. The algae used in the production of biostimulants contain plant hormones such as cytokinins and auxins or other hormone-like substances [15–17]. Algae-based biostimulants also contain many mineral and bioactive compounds, including complex polysaccharides such as laminarine, fucoidan, and alginates [1,2].

Biostimulants can be obtained from plants rich in secondary metabolites, which are also one of the main kinds of bioactive compounds proposed as responsible for activating the physiological responses of plants [18,19]. For example, the extract obtained from the maceration of borage leaves or flowers showed biostimulant effects on the growth and quality of lettuce [19]. Another potential plant that would be interesting to consider is *Withania somnifera* (L.) Dunal, which is rich in withanolides used in Ayurvedic medicine. These compounds could have a potential biostimulant effect in agriculture [20].

### 2.2. Humic Substances

Humic substances mainly comprise humic and fulvic acids. They are natural constituents of the organic substance of the soil, resulting from the decomposition processes of plants, animals, and microorganisms, and are also further modified by the metabolic activity of soil microbes. It has been reported that treatments with humic substances can stimulate the growth and development of plant roots [21–23]. These effects are attributable to the improvement in the absorption of nutrients and water and to a greater tolerance to environmental stress as well as to their effect on plant metabolism [24]. However, how humic substances affect plant physiology is still under debate. This is due to the molecular complexity of these substances and the abundance and diversity of plant responses altered by their application. The positive effects exerted by these aggregate complexes could be ascribed both to the hormonal activity of some of their components and to other independent mechanisms [12].

### 2.3. Hydrolyzed Proteins and Nitrogen-Containing Compounds

Protein hydrolyzates are a mixture of compounds such as amino acids, peptides, and polypeptides that can be obtained by chemical, enzymatic, and thermal hydrolysis of proteins from complex biological matrices. Many studies performed on horticultural crops have reported that the applications of protein hydrolyzates of both animal and plant origin can improve the growth of crops and their tolerance to abiotic stresses [25,26]. Likewise, to other kinds of biostimulants, the lack of information on the composition of protein hydrolyzates remains a crucial point to define their mechanisms of action at the molecular and biochemical level.

### 2.4. Microorganisms

This group mainly includes bacteria, yeasts, and filamentous fungi [27]. They are isolated from soil, plants, and other organic materials. They are applied to the soil or to the seeds and can have a direct or indirect action in increasing crop productivity [28]. Microorganisms can have a direct action on the crop through the establishment of a mutual symbiotic association (e.g., Mycorrhiza), or indirectly by increasing the nutrients' bioavailability to plants [29]. In the last European fertilizers regulation (<https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R1009>, accessed on 1 February 2022), the microorganisms *Azotobacter* spp., Mycorrhiza, *Rhizobium* spp., and *Azospirillum* spp are recognized as biostimulants.

### 2.5. Inorganic Compounds with Biostimulant Action

Chemical elements able to promote plant growth without being essential for all plants are called beneficial elements [7,30]. The main beneficial elements are selenium and silicon, as well as cobalt, aluminum, and sodium. These elements are present in soils and plants in the form of inorganic salts, and even insoluble ones, such as amorphous silica in grasses [31,32]. The beneficial effects that may be induced are constitutive, such as the strengthening of cell walls through silica deposits, or transients, for example, following the exposure to particular abiotic stresses. The definition of beneficial elements is therefore not limited to their chemical nature but must also refer to the specific contexts in which positive effects on plant growth and stress response are observed.

## 3. Biostimulants and Abiotic Stress Tolerance

The mechanisms activated by biostimulants in response to stress conditions are difficult to identify and are still under study. Considering the physiological effects induced by biostimulants, it is realistic that the bioactive molecules contained in these compounds can affect plant metabolism acting on specific pathways (Table 1). Under stressful conditions, the metabolic pathways activated by biostimulants could be amplified and could help the plants to adapt and then overcome or delay the most critical moments [33]. It is interesting to observe that among the metabolites increasing in plants treated with biostimulants, there are often those with antioxidant properties. It is well known that these protective molecules have a central role in reducing the degenerative effects of free radicals that accumulate in plant tissues under stressful conditions [34–36].

### 3.1. Drought Stress

Water stress is one of the most common abiotic stresses occurring in crops in different geographical areas [37]. The reduction in water availability directly affects the photosynthetic activity altering the leaf gas exchanges [38]. Therefore, the yield and quality of the products may be compromised. The visible symptoms of water stress are the loss of turgor and yellowing of the leaves due to the degradation of chlorophyll. The chlorophyll content is, in fact, used as a reliable indicator of metabolic imbalance in plants under water stress. Some commercial biostimulants, such Actiwave or Wokozim, obtained from seaweed extracts, increase the accumulation of osmotically active compounds that contribute to buffer the changes in plant water status [29,39]. Therefore, they may help plants to counteract and

overcome the most critical periods of drought, by avoiding a drop in yield and quality [40]. Among the compounds accumulated, it is possible to find different substances, such as proline, sugars, alcohols, nitrates, and abscisic acid. It has been proposed that biostimulants can amplify the endogenous responses of plants improving the water balance of the crop, increasing root water absorption capacity and, finally, reducing losses [41].

From an agronomic point of view, excellent results have been obtained from the use of biostimulants based on *Ascophyllum nodosum* seaweed extract on broccoli and spinach [42]. The biostimulant application showed positive effects on the gaseous exchanges of these crops by inducing a reduction in the stomatal closure, with a concomitant increase of water stress tolerance. The treatments of biostimulants with *A. nodosum* improve the green color under stress conditions, increasing the biosynthesis and reducing the degradation of chlorophylls, proving to be an excellent product for the cultivation of leafy vegetables.

**Table 1.** Effects on the plant and possible mechanism of action of biostimulants.

Application Mode	Benefits	Possible Action Mechanisms on Plants
Growth and production	<ul style="list-style-type: none"> <li>- Biomass increase of roots and aerial part;</li> <li>- Early flowering [13];</li> <li>- Yield increase [1,18];</li> <li>- Uniformity of fruits and ripeness [49];</li> <li>- Increase antioxidant power [18].</li> </ul>	<ul style="list-style-type: none"> <li>- Increased photosynthesis [18];</li> <li>- Improved nutrient use efficiency [44];</li> <li>- Increased root activity (rhizobacteria and mycorrhiza, and humic substances) [28];</li> <li>- Increased bioavailability of nutrients (nitrogen fixers) [50];</li> <li>- Stimulation of plant secondary metabolism [51].</li> </ul>
Foliar: spray [43] Radical: <ul style="list-style-type: none"> <li>- Fertigation (soil or substrate) [44,45]</li> <li>- Direct solution to the ground [46]</li> <li>- Addition to the nutrient solution (hydroponics) [47]</li> </ul> To the seed: treatments before sowing [48].	Tolerance to [1,34]: <ul style="list-style-type: none"> <li>- salinity;</li> <li>- water stress;</li> <li>- cold stress;</li> <li>- high temperature;</li> <li>- asphyxiation.</li> </ul>	Accumulation of osmolytes [52]: <ul style="list-style-type: none"> <li>- proline [53];</li> <li>- sorbitol [54];</li> <li>- glycine betaine [8,54].</li> </ul> Improved water use efficiency [2]: <ul style="list-style-type: none"> <li>- reduction of transpiration [55];</li> <li>- improvement of stomatal gas exchanges [18];</li> <li>- changes in root/stem ratio [56].</li> </ul> Increase of antioxidant compounds and protection against free radicals [18].
Nutritional	<ul style="list-style-type: none"> <li>- Lowering nitrates [47];</li> <li>- Increase of anthocyanins and phenols [57];</li> <li>- Increase carotenoids [58];</li> <li>- Increase nutrients [59].</li> </ul>	<ul style="list-style-type: none"> <li>- Increased nitrogen assimilation [60];</li> <li>- Activation of the phenylpropanoid pathway (secondary metabolism) [61].</li> </ul>

### 3.2. Salinity Stress

Crops growing in coastal areas, such as Almeria (Spain), Vittoria (Sicily, Italy), may be subjected to saline stress due to the high concentration of soluble salts in the irrigation water or in the soil. Salt stress induces a reduction in growth in plants and damages at cell level compromising plant vitality and productivity [37]. The effect on the crop depends on the intensity of the stress and the exposure time. The high salt concentration causes osmotic stress due to the high ionic concentration in the soil, which can lead to a reduction in water absorption by roots. Indeed, stressed plants show symptoms of water stress. Biostimulants can alleviate the effects of salinity by increasing the tolerance of the crop [62]. The endogenous mechanisms to face salinity are similar to those observed in plants subjected to drought. Biostimulants may induce an increase in the concentration of proline, simple sugars, alcohols, abscisic acid, and of the antioxidant compounds able to counteract damages caused by the accumulation of free radicals [63].

The application of biostimulants results in increased stress tolerance and reduced production losses. The number of applications depends on the species and the salt stress conditions. Good results have been obtained with protein hydrolyzates of vegetable origin, which have improved the tolerance to salinity in various horticultural crops, such as lettuce, thereby increasing the yield and accumulation of dry matter [64–66]. Plants treated with this type of biostimulant have a better efficiency of use for radiation and higher yields.

Similar results can also be obtained with microbial biostimulants. Treatments with *Azospirillum brasilense* have shown positive results on lettuce (*Lactuca sativa* L.), peppers (*Capsicum annuum* L.), chickpeas (*Cicer arietinum* L.), and broad beans (*Vicia faba* L.) grown in a high salinity environment [67,68]

### 3.3. High and Low Temperature Stress

The lowering of temperature or the return of cold temperatures in late spring is one of the most dangerous abiotic stresses seriously affecting production [69]. The damage is significant, especially for crops with early flowering, but considerable damages can also occur on early transplants of melon and tomato [70,71]. Low temperatures mainly cause injury to cell membranes, leading to death in severe cases or growth retardation in milder cases.

Two commercial biostimulants, Bactor<sup>S13</sup> or Flortis Micorrize, have shown the ability to counteract the plant growth reduction under cold stress by increasing osmolytes (osmotically active substances), antioxidant compounds, and substances that protect the cell membranes [56].

Excellent results were obtained from biostimulants based on algae, amino acids, or microbials that can induce protection against low temperatures, by the accumulation of cryoprotective substances and/or the activation of membrane repair systems [72].

High temperatures can also cause damage to crops as a result of alterations of cell membranes and enzymatic functionalities. In particular, damages to membrane phospholipids occur with the loss of cellular compartmentalization that provokes metabolic disorders. Plants respond to high temperatures by increasing transpiration, in order to ameliorate thermoregulation. Therefore, the most serious damage can be obtained in geographical areas with temperatures above 37 °C where cases of high temperatures associated with conditions of low water availability can occur [69].

The biostimulants that induce tolerance in crops could act both by increasing the water absorption capacity of plants and by stimulating the accumulation of substances with protective roles for cell membranes.

### 3.4. Stress from Nutritional Deficiencies

In crop systems, nutritional deficiencies may occur for various reasons, both due to incorrect agronomic management and different soil conditions (e.g., non-optimal pH). Biostimulant applications enable plants to explore a larger volume of soil and have a greater absorption capacity by inducing an increase in root biomass. For example, AminoPrim

and AminoHort, two biostimulants recently proposed, were able to promote the absorption capacity of nutrients even if present in low concentrations [61]. Starting from this consideration, it is realistic to suggest that all biostimulants inducing an increase in root biomass could help to alleviate the effects of nutritional deficiencies when they are not easily manageable through fertilization [50].

#### 4. Biostimulant-Induced Accumulation of Secondary Metabolites and Their Role against Abiotic Stresses

Plant secondary metabolites are a heterogeneous group of molecules, which play a key role in the complex network of interactions between plants and the environment. Due to this role, different classes of secondary compounds can be involved in the response to abiotic stresses, and biostimulants can be effective in stimulating their accumulation, helping the plant in counteracting the negative effects of the stress [73]. Plant secondary metabolites comprise a wide array of molecules, including phenolic compounds, carotenoids, and glucosinolates. The use of biostimulants can support plant abiotic stress tolerance and, at the same time, could influence the nutritional quality of the plant's edible parts by stimulating the accumulation of plant secondary metabolites [74]. Phenolic compounds are the largest group of secondary metabolites in plants. Several molecules involved in plant eco-physiological interactions belong to this group, including phenolic acids, flavonoids, and anthocyanins, and are primarily synthesized by the phenylpropanoid pathway. Phenolic compounds play a key role in plant tolerance against abiotic stresses due to the high antioxidant potential. For the same reason, these compounds concur with the nutritional quality of plant-based foods. Many studies, including those on borage extract or SUNRED [18,58], have demonstrated that they can act by stimulating the accumulation of phenolic compounds, determining a positive effect on plants' tolerance to abiotic factors. In some cases, the activation of secondary metabolism could determine an energetic cost for the plant balance, negatively affecting the yield and the primary metabolism. A recent study on lettuce showed that the application of a pig blood-derived biostimulant induced the phenolic metabolism, by stimulating the expression of genes encoding for the key enzymes in the phenylpropanoids pathway, determining an increment in the antioxidant capacity, without affecting the crop yield [75]. A significant increase in the total phenolic content, sinapic acid content, as well as in quercetin content was observed in broccoli florets, treated with a biostimulant made of a mixture of amino acids and algae (*Ascophyllum nodosum*) filtrate [76]. The possibility of promoting the accumulation of phenolic compounds through the application of biostimulants has been also largely studied on important fruit crops, including grapevine [77,78].

Some of the best examples of metabolites' accumulation induced by biostimulant treatments regard carotenoids. Carotenoids are non-polar secondary metabolites also involved in plants' defense mechanisms, acting as antioxidants and photo-protecting molecules. Moreover, these compounds concur to the nutraceutical quality of plant-based foods, as they are recognized in acting as antioxidant and health-related compounds.

A recent study investigated the effect of a complex commercial biostimulant on pepper fruits and leaves and, through a metabolomic approach, showed that carotenoids were among the most affected compounds. The application of the biostimulant determined an increment of the carotenoids level, suggesting an activation of the enzymes responsible for their biosynthesis [79]. The application of a plant-based biostimulant improved the agronomical performance of baby rocket plants (*Diplotaxis erucooides* L.) and determined an increment in the carotenoid levels in leaves [60]. Similar results were obtained in tunnel-grown lettuce after the application of vegetable-based and seaweed-based biostimulants [80]. The positive effect of the biostimulants' application can be modulated and affected by stressful or non-optimal conditions. A study conducted on tomatoes showed that the application of a biostimulant (protein hydrolyzate) was associated with an increase in the levels of carotenoids and lycopene under normal water conditions. However, under limited water availability (50% of water reduction), the biostimulants induced a reduction

of the pigment levels [81]. Glucosinolates are a sulfur-rich class of secondary metabolites, typically produced by plants of the order Brassicales. Glucosinolates, and their hydrolysis products, isothiocyanates, which play an important role in plant defense against biotic and abiotic stresses, and at the same time, they concur with the sensory and nutritional properties of vegetables such as broccoli, cauliflower, and rocket [82]. Additionally, in the case of glucosinolates, the use of biostimulants can represent an agronomical tool to be used for improving the quality of crops and their resistance to abiotic stress factors. Treatments with a seaweed extract induced a higher accumulation of indole and aliphatic glucosinolates in broccoli [83], while the application of a thricoderma-based biostimulant was effective in promoting the glucosinolate content and the antioxidant potential of different leafy brassica vegetables [84]. The number of publications showing the effectiveness of biostimulants in promoting the secondary metabolism is constantly increasing and comprise various classes of crops and compounds. Recently, bio-based biostimulants (obtained from fungi or bacteria) and plant extracts have been proposed as elicitors able to promote the accumulation of secondary metabolites in medical plants [85,86], with positive effects on the commercial quality of crops and on stress tolerance. Concerning the chemical profile of medical plants, it has been observed that the application of humic acids to *Cannabis sativa*, in combination with improved plant nutrition, was able to alter the cannabinoids composition and the spatial/organ distribution of important secondary metabolites within the plant [87]. Considering the possible mode of action by which biostimulants can activate the plants' secondary metabolism, it is interesting to note that Ganugi and colleagues [88] proposed a mechanism of stimulation induced by microbial biostimulants, which would possibly include the activation of systemic acquired resistance SAR, an overproduction of reactive oxygen species (ROS), and in turn, the de novo biosynthesis of antioxidant compounds, including terpenoids, ascorbic acid, and phenolic compounds. A similar effect was shown in the medical plant *Withania somnifera*. After the application of different elicitors, an increment was observed in the specific secondary metabolites called withanolides [21]. Other recent studies indicate that these classes of biostimulants can trigger a re-programming of hormones and secondary metabolism, as demonstrated in pepper [59]. Furthermore, it has been demonstrated that, when biostimulants are applied in combination with salt stress, their mechanism of action changes, depending on the degree of salinity. In fact, it has been shown that the classes of secondary metabolites stimulated in basil were different in case of mild or severe salinity [89].

## 5. Analytic Methods for Monitoring the Biostimulant Effects on Crops

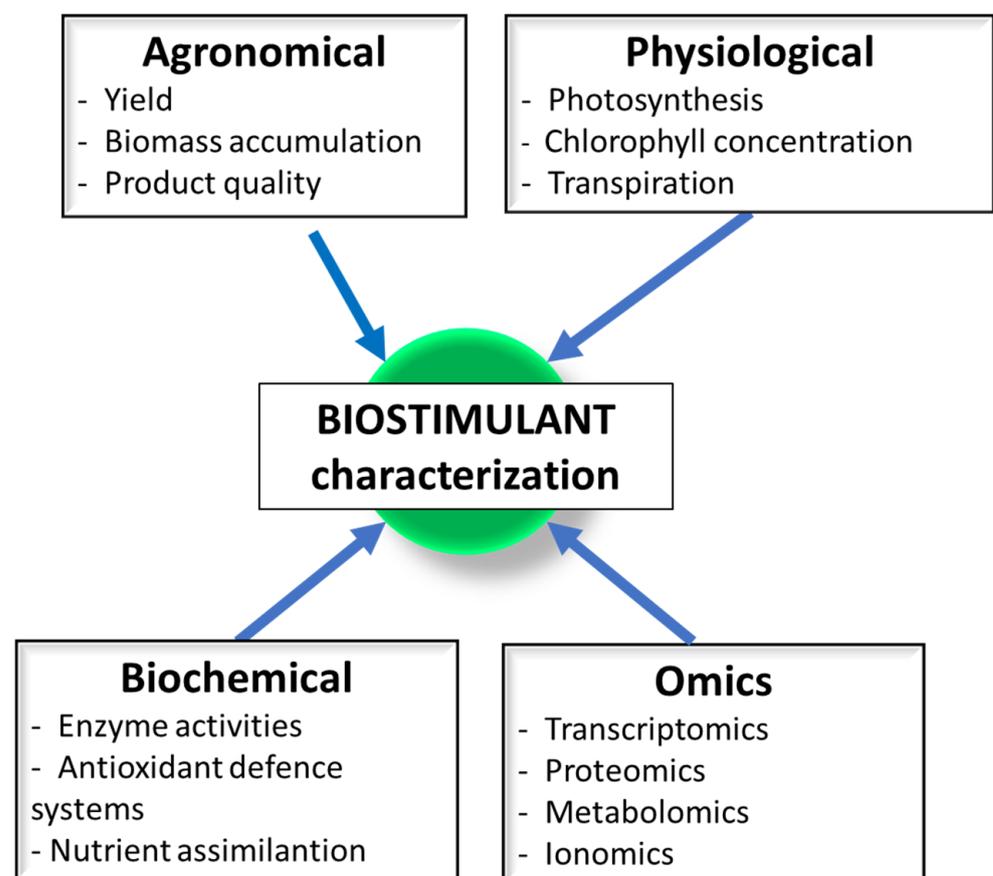
According to the European Biostimulants Industry Council (EBIC) recommendations and the last European fertilizer regulation, scientific evidence supporting the efficacy of biostimulants and clarifying their specific mode of action is a mandatory requirement to register a product as a plant biostimulant. This information, together with the data about the exact composition of plant biostimulants, as mentioned above, will help to provide scientifically-based guidance on their best application for specific agronomic needs. The elucidation of the mode of action of a product or a single bioactive compound might also be useful for the development of novel biostimulants. Moreover, since they are complex mixtures and the raw material does not always define a unique effect, scientists suggest a more appropriate classification based on their mode of action than based on their origin [10,12].

The genetic variability, the synergistic/antagonistic effect of some components, and the environmental factors affecting the biostimulant efficacy are some of the issues increasing the complexity of the study and the identification of the mode of action. It is therefore necessary to carry out several trials that take into account the factors that may potentially influence the biostimulant efficacy. This essentially means conducting experiments both under protected condition and in the open field, on model plants such as *Arabidopsis thaliana* or tomato cultivar Micro-tom and other non-model species, simulating different environ-

mental conditions. For this reason, standardized protocols are necessary to assess the mode of action of biostimulant products on crops.

Different techniques, including *in vitro* bioassays, micro-phenotyping, high-throughput phenotyping, and lately multi-trait high-throughput screening, have been establishing and have partially replaced the traditional and time-consuming screening methods [90].

In the recent years, omics science as transcriptomics, proteomics, and metabolomics have contributed to improve the understanding of the mechanisms underpinning the activity of plant biostimulants, by identifying the molecular and biochemical pathways affected as well as proposing their regulatory role in the pathway (Figure 1). In other words, these techniques permit to obtain information useful to clarify the molecular bases of the phenological modification induced by the application of biostimulants [91].



**Figure 1.** Multidisciplinary approach for the characterization of biostimulants based on crop response. Main parameters that should be considered and measured for each analyzed group.

The application of an integrated approach, combining omics and high-throughput phenotyping technologies, would help us to scientifically explain some of the questions about the biostimulant properties and activities (Figure 1) [90].

### 5.1. Transcriptomic Profiles Analyses

Biostimulant treatments induce a wide range of changes in the abundance of mRNA transcripts leading to the activation of several physiological responses and biochemical pathways, which can stimulate primary and secondary metabolisms in crops. Transcriptomic analyses measure the modifications in the gene expression as a result of the interaction between the biostimulant and the plant. Generally, the first set of genes involved in plant responses induces the expression of gene activators, which will in turn lead to changes in a cascade of other genes' expression. In addition, the study of the regulation of these steps requires a focus on the crosstalk among different phytohormones involved in signal

transmission. In this research field, transcriptomic studies aim to find molecular markers associated with the plant responses against the abiotic stresses and to understand how biostimulant products might help under such conditions. To discover the molecular mechanisms involved in biostimulant activity, different analytical methods have been used in the last years. Microarray analysis provide a transcriptomic fingerprint, allowing the simultaneous investigation of a multitude of genes. The profiling obtained are used to classify and cluster the genes into functional groups on the base of their expression levels and to identify the target families of genes. A study on the mode of action of a humic substance using a microarray revealed differentially expressed genes (DEGs) involved in N, S, and C metabolisms [92]. In particular, genes encoding for proteins involved in uptake, assimilation, and transport were identified. A similar study investigated the responses at molecular level to the application of an alfalfa-based protein hydrolyzate biostimulant in tomato plants [93]. Results showed a modification in expression of genes involved both in primary (C, N, photosynthesis, nutrient uptake, and plant development) and secondary metabolisms (phenols and terpenes) as responsible for the biostimulant activity of an alfalfa-based protein hydrolyzate tested in that experiment. Moreover, the modification in expression of some genes related to abiotic stress response pathways might suggest a possible use of this product also to counteract environmental stresses. A first step to understand the metabolic target of a new biostimulant product (EXPANDO<sup>®</sup>) rich in different bioactive compounds has been carried out combining the analyses of biochemical parameters and microarrays [94]. More than 4000 DEGs have been identified and a considerable number of them were involved in transcription processes. A novel biostimulant (CYT31) applied on *Arabidopsis thaliana* exposed to drought stress induced a modification in the expression of several genes involved in reactive oxygen species (ROS) metabolic processes, MAPK cascade, and stress responses [95]. In particular, genes encoding for SOD enzymes (superoxide dismutase) were strongly upregulated by the biostimulant treatment.

This strategy can be applied not only to a finished product but also to screen raw materials as possible biostimulant ingredients [96].

The biostimulant activity of two *Ascophyllum nodosum* extracts on *Arabidopsis thaliana* has been evaluated through the analyses of mRNA transcriptomics using Affymetrix Ath1 microarrays [97]. The plant transcriptome was differently affected by the application of these extracts and the expression of genes involved in gluconeogenesis/glyoxylate cycle, oxidative stress, and hormone metabolism was modified. Moreover, this study shows the different effect of two products manufactured from the same raw material, pointing out a critical aspect for the biostimulant sector.

The microarray technique can be considered as a starting point to screen the biostimulant activity. A second step may be represented by the qRT-PCR analyses of some of the genes mainly involved in the metabolic processes affected by the application of a specific product. In this way, it is possible to complement, check, and validate the results obtained from the microarray analyses [98].

In addition, qRT-PCR analysis is exploited to monitor the expression levels of known genes involved in specific pathways of interest [99].

Due to the limitations of the microarray technique, an increasing number of studies are using sequencing techniques for transcriptomic profiling to investigate the mode of action of biostimulants. Traditional sequencing technologies based on Sanger sequencing of cDNA or EST libraries are slowly being replaced by NGS techniques, in particular, RNA-sequencing. Illumina IG is one of the most widely used high-throughput sequencing technology applied in this sector. This also allows to perform genomic studies on non-model plants. The success of these techniques is supported by the development of bioinformatic methods and tools able to store, process, and analyze large amounts of data, deciphering the intricacy of plant metabolic pathways. Moreover, the price of RNA sequencing has decreased during the years, making the transcriptome analyses accessible and feasible to more research.

In 2015, the mode of action of gelatine treatment on cucumber seeds was investigated by the comparison between the DEGs identified using an mRNA sequencing-based approach in control and treated samples [100]. In particular, the increased tolerance to abiotic stresses and the enhanced growth of seedlings are the result of the upregulation of the genes involved both in amino acid and ammonium transporter and in the detoxification system.

Super Fifty, a biostimulant containing *Ascophyllum nodosum*, possesses an antioxidant activity and is known to have a positive effect on plant growth, alleviating the damage of abiotic stresses. The analyses of the whole-genome transcriptome of *Arabidopsis thaliana* in response to drought stress and a priming treatment with Super Fifty allowed to clarify how the product works. In particular, the positive effect was the result of the regulation of some of the key genes involved in plant growth (*RD26* and *BES1*) and cell cycle (*CYCP2;1*) [101].

The RNA-seq approach is used not only to clarify the mode of action of commercial biostimulant products with a known efficacy, but it might become a successful strategy to develop new products.

A recent study showed how the integration of multiple omics techniques helped in this respect. In particular, after a screening among three product prototypes, based on different combinations of seaweed and plant extracts, using high-throughput plant phenotyping, researchers were able to select the most effective one and characterize its mode of action through NGS on two important row crops: corn and soybean [102]. Genes involved in nitrogen, phosphate, and cytokinin metabolism, together with the sugar transport, maltose, biosynthesis, and phloem loading were positively modulated in corn species. The same extract induced an upregulation of some genes involved in nitrogen metabolism, metal ion transport, and amino acid biosynthesis in soybean.

The efficacy of humic acids in the mitigation of abiotic stresses through the stimulation of enzymatic and non-enzymatic defence systems in plants is well known. Recently, a new role as priming preconditioner has been proposed and the prime state induced by humic acids has also been characterized at transcriptional level [103]. RNA-seq allowed an understanding of the role of humic acids in alleviating different adverse conditions before they occurred. In particular, maize seedling primed with humic acids showed an upregulation of the genes involved in abscisic acid, gibberellic acid, and auxins metabolism. Moreover, the transcriptional levels of specific abiotic stress-responsive genes were enhanced by the humic acid treatment.

Another large group of biostimulant products is represented by protein hydrolyzates. They have been identified to improve plant performance when applied to leaves or roots, especially under adverse environmental conditions. The transcription of a group of genes involved in ROS homeostasis in maize was regulated by the treatment with a protein hydrolyzate named APR [104]. At the same time, the genes involved in ROS generation were not affected, suggesting a sort of priming effect and the ability of the treatment to induce a sort of constitutive tolerance to future stresses. In this experiment, mRNA-seq allowed to elucidate the common and specific pathways modified by the biostimulant application. Moreover, a biostimulant containing tannins (VIVEMA TWIN) alleviated salt stress in tomato plants by regulating the expression of essential transcription factors and stress-responsive genes [62]. Further transcriptional studies are described in the review of Gonzales-Morales et al. [105].

### 5.2. Proteomic Profiles Analyses

The elucidation of the molecular and biochemical processes changing in plants in response to biostimulants is also closely linked to the obtaining of information at protein level. This information is crucial to complete those obtained through the genomic and transcriptomic analyses. This is not only fundamental in order to describe the changes of protein profiles from a qualitative and quantitative point of view, but also to identify post-translational modification (PTM) events, which play a central role in the modulation of molecular and biochemical responses [106]. Until now, only few papers have focused their attention on the proteomic changes induced by biostimulants.

Among the first studies, the study carried out by Carletti and co-workers evaluated the proteomic changes in the root plasma membrane enriched fraction of maize (*Zea mays* L.) seedlings [107]. In particular, the study evaluated the effects induced by humic substances (HSs) extracted from earthworm feces. The analysis revealed 42 differentially abundant proteins (DAPs), many of which resulted being negatively affected by the treatment. The functional classification of the identified proteins suggested effects on energetic metabolism, transport activity, and cytoskeleton organization. Recently, the authors deepened these results, applying the iTRAQ technique to study changes in the total root proteome of *Arabidopsis* plants treated with the same kind of biostimulant. This high-throughput approach provided a wider scenario, confirming the effects on some key pathways, such as glycolysis, but also suggesting a role of ROS in the modulation of plant responses to HSs [108]. In this context, an important aspect to deepen will be the identification of which specific molecules of the complex mixture represented by HSs are responsible for the response.

As debated above, many biostimulants relieve the effect induced by abiotic stresses. In this view, a proteomic study conducted on tea plants exposed to drought conditions revealed a positive effect of fulvic acid [109]. During severe stress conditions, the treatment with this HS resulted to affect the abundance of 611 leaf proteins. Many of them are known to be involved in starch and sucrose metabolism as well as in secondary metabolism. Moreover, as confirmation of the positive effect of fulvic acid, an increase in abundance of heat shock proteins (HSPs), was also observed. Finally, a correlation with a transcriptomic analysis permitted to highlight that the expression patterns of 55 genes were similar to those observed at protein level.

In another work, the effect of two different commercial protein hydrolyzates (PHs) from legume biomass in grapevines exposed to water stress was analyzed [110]. The physiological evaluation and the untargeted proteomic and metabolomic analyses revealed similar positive effects of the two PHs. The treatment with these biostimulants, sprayed on the leaves at the pre-veraison positively affected photosynthesis-related proteins and delayed physiological maturity without affecting vine productivity.

Regarding the effect of PHs on plant metabolism, a proteomic study conducted on roots of maize plants treated with a biostimulant derived from collagen highlighted dose-dependent changes of some crucial activities, such as redox homeostasis, sugar, lipid, and amino acid pathways [111]. In this case, the proteomic analyses also confirmed some of the transcriptomic data previously obtained in the same experimental conditions. Moreover, it highlighted a positive effect on the synthesis of proteins, such as HSPs that are known to improve the tolerance to the stress conditions. Again, this study suggested the involvement of an oxidative signal in the improvement of plant growth and stress adaptation.

Some studies have investigated the proteomic changes induced plants treated with biostimulants, characterized by an elevated content of free amino acids.

Martínez-Esteso and co-workers [112] analyzed the proteome of wheat (*Triticum aestivum* L.) flag leaf treated with the commercial biostimulant Terra-Sorb<sup>®</sup> Foliar, and revealed changes in the turnover of Rubisco as well as an increase in abundance of Rubisco activase, a positive modulator, and of other enzymes involved in the CO<sub>2</sub> assimilation. Moreover, the paper reported a reduction of the typical response to oxidative stress. Overall, this proteomic study highlighted that the stimulating effect of this biostimulant could be related to a positive effect on photosynthetic performance.

The evaluation of the efficacy of glutacetine<sup>®</sup> and of some derived formulations on wheat grain proteome also confirmed the positive action of the biostimulants containing high levels of free amino acids. A study, performed by Maignan and co-workers [113] on wheat mature grain reported significant differences in abundance of 131 proteins involved in important biological classes, such as energy, protein, carbon and nitrogen metabolism, and stress responses. This data fit well with a previous study showing an increase in seed yield and grain quality in wheat plants treated by foliar spray with this biostimulant formulates. The paper presented evidence in support of the hypothesis that this positive

effect is due to an increased plant capacity of both nitrogen uptake and remobilization [114]. Interestingly, the proteomic analysis in plants treated with the glutacetine<sup>®</sup>-derived formulation, VNT4, also revealed a decrease in abundance of the gliadin protein family, known to be involved in celiac disease. Moreover, this biostimulant induced a concomitant increase of a glutenin fraction [115]. The consequent changing of the gliadin/glutenin ratio has a positive effect on grain quality [115]. Similar results in durum wheat (*Triticum durum*) grain yield and quality have been obtained treating the plants with two foliar biostimulants, mainly consisting in seaweed (*Ascophyllum nodosum*) and fungal (*Trichoderma*) extracts, respectively. Proteomic analysis in this case also revealed changes of proteins involved in the storage process, in regulation of different pathways, and in stress responses. Interestingly, both treatments led to an improvement of the wheat technological properties, but through different changes in grain protein composition. In addition, a change in gliadin/glutenin ratio was observed in this study [116]. Overall, these considerations highlight that different biostimulants could prompt distinct effects, depending on formulations, plant species, and environmental conditions.

In the rhizosphere, microbial community actively participates to the dialog occurring between plant and soil. In this context, an important emerging aspect is the reciprocal influence between plant and microorganisms. Recently, Mattarozzi and co-workers [117] adopted mass spectrometry-based shotgun metaproteomics, combined with multiplex enzymatic assays, to study the effects of two commercial biostimulants on microbial community and its metabolic activities. The analysis, which permitted to also include uncultivable species, highlighted a positive action of biostimulants on the pathways involved in nitrogen, phosphorus, organic compounds, and redox processes. Moreover, the results suggest that the biostimulants specifically affected the metabolic pathways, without causing a general increase in microbial growth or changes in microbiota composition. Another interesting result was that the biostimulants induced an increase in abundance of proteins involved in the antibiotic resistance, indicating a strengthening of the defences against pathogens in the rhizospheric environment.

Although only a few data are available, thus far, these first proteomic studies provide interesting information regarding the possible biostimulants' action mechanisms. According to other omics studies, proteomics revealed common features in plant responses to the different biostimulants as well as have highlighted some specific traits and peculiarities. Further proteomic analyses not only will help to better confirm the actual knowledge, but will permit to investigate some of the very crucial aspects involved in the control of plant molecular and biochemical processes, such as PTMs.

### 5.3. Metabolomic Profiles Analyses

Metabolomics is defined as the qualitative and quantitative study of all metabolites involved in metabolic reactions responsible for the maintenance of the normal function of an organism [118]. In comparison with transcriptomic and proteomic studies, the metabolomic profiling allows to obtain robust information about plant status. However, plant metabolome is complex since metabolites belong to different families of biochemical compounds, which possess different chemical properties, and there are several analytical procedures that need to be applied, according to the known or unknown target. Mass spectrometry and NMR spectroscopy are the two widely used technologies exploited in metabolomic profiling. For this reason, metabolomics is also defined as a multidisciplinary approach.

A recent review presents, in a very interesting and exhaustive way, the application of metabolomic science in the biostimulants sector, aiming to identify the mechanisms underpinning the effects of biostimulant products in plants [119]. It is known that plant biostimulants are able to affect both primary and secondary metabolism, promoting plant growth, improving quality and yield, and increasing the tolerance to several abiotic stresses. A metabolomic approach is currently applied to investigate the interaction between biostimulants and plants and clarify their mode of action under different growing conditions.

Nephali et al. [119] confirm that its application is still limited and there are few metabolomic reports on the interaction between plant and biostimulant, and even less on the interaction between plant, biostimulant, and abiotic stress. New papers, not included in the cited review, have been published in the last months, showing a growing interest in this area.

Two different studies on maize crops, subjected to drought stress and nutrient starvation, elucidated the metabolomic networks underlying the effect of a microbial-based biostimulant (PGPR) and a humic biostimulant, respectively [120,121].

Modifications in the levels of amino acids, phenols, and lipids, together with an increase of tricarboxylic acid production was observed in response to the PGPR application. At the same time, the biostimulant application influenced several mechanisms in response to drought stress (cell wall formation, plasma membrane activities, and the pathways involved in antioxidant and osmoregulation).

A metabolomic approach revealed a sort of remodulation and reprogramming of the metabolic pathways in maize plants treated with humic substances and grown under optimal or nutrient stress conditions.

In another study, the inoculation of tomato plants with different PGPR strains had positive effects on vegetative growth and increased the tolerance to the combination of water and nutrient stress by affecting several stress-related metabolites (monopalmitin, trehalose, myo-inositol, L-lactic acid, and malate).

Glycine betaine is a compound that is accumulated in response to abiotic stresses. Tomato plants treated with a glycine betaine-based biostimulant showed a better tolerance to water stress [122]. The leaves thickening and the over-accumulation of lipids probably increased the effective use of water. Moreover, the accumulation of carotenoids suggested a protection of the photosynthetic apparatus from oxidative damages.

GC-MS-based metabolite profiling demonstrated how a pre-treatment with a biostimulant product (SuperFifty) was capable to prevent the oxidative damages caused by Paraquat in three species (*Arabidopsis*, tomato, and pepper) [35]. The application of the product rich in *A. nodosum* suppressed the metabolic modifications caused by the stressor.

#### 5.4. Experimental Planning and Statistical Analysis

Biostimulants should be effective when applied in different agro-ecosystems; therefore, the experimental designs must be carefully defined. In the open field or greenhouse, the experimental plan should be organized as a Randomized Complete Block Design (RCBD), allowing to obtain more robust data. Biological samples should be collected randomly to have a good representation of the dataset and a better assessment of the environmental variability. In this context, another important aspect that should be considered is the timing of sampling, considering that deep changes of many physiological parameters occur daily (i.e., leaf gaseous exchanges and nitrogen assimilation). The analysis of different parameters should be performed at least on four biological replicates. Sampling procedures for RNAseq experiments should be very well defined because the transcription profiles change rapidly considering the experimental conditions and cannot be directly related to treatments such as temperature changes and solar radiation variations. Therefore, for transcriptomic and proteomic analysis, the effect of treatments should be studied under fully controlled conditions. Statistical analysis should be selected considering the factors involved, but it is suggested to reduce the number of factors as much as possible. Moreover, if there are more than two factors, the interactions among the variables should be carefully evaluated. Robust post-test, for the identification of the differences among means, should be chosen myo- based on the specific aim of the research and on the characteristics of the experimental plan [123].

## 6. Conclusions

Biostimulants are rapidly spreading on the market; one of these reasons is the increasingly limited availability of synthetic products. Therefore, research and experimentation must rapidly provide useful indications to improve the biostimulant production and their

methods of application. Results generated under controlled conditions, such as a greenhouse or laboratory, are necessary to validate those obtained under open field conditions, and vice versa. In both cases, guidelines for the definition of the trials have been recently proposed [124]. The definition of appropriate trial designs and statistical analysis is pivotal to obtain truthful information and justify the claims for plant biostimulants. Agronomic tests are necessary to define the most suitable doses for the different species and the appropriate timing of application, especially for those used to counteract abiotic stresses. The omics approach can provide fast and reliable data that link the crop's responses and its performance under different environmental conditions.

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