BURLEIGH DODDS SERIES IN AGRICULTURAL SCIENCE

Biostimulants for sustainable crop production

Edited by Youssef Rouphael, Patrick du Jardin, Patrick Brown, Stefania De Pascale and Giuseppe Colla

burleigh dodds
SCIENCE PUBLISHING

Contents

Part 2 Non-microbial and microbial categories of biostimulants

Introduction

The EU Fertilizer Regulation 2019/1009 defines a plant biostimulant as a '*fertilizing product the function of which is to simulate plant nutrition process independently of the product's nutrient content*'. Plant biostimulants stimulate natural processes in crops to enhance nutrient uptake, nutrient use efficiency (NUE), resistance to abiotic stress and quality traits, as well as increasing the availability of nutrients in the soil or rhizosphere. They offer the opportunity to enhance fertilizer use and thus contribute to more sustainable crop production. This collection reviews key advances in understanding and using biostimulants. Part 1 reviews ways of classifying microbial and non-microbial biostimulants, types of bioactive compound and ways of evaluating biostimulants. Part 2 surveys the various types of biostimulant, from humic substances and seaweed extracts to protein hydrolysates, silicon, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF). Part 3 discusses advances in designing second-generation biostimulants and their practical application.

Part 1 Introduction and biostimulant characterization

Chapter 1 sets the scene by discussing plant biostimulants as a new paradigm for the sustainable intensification of crop production. Biostimulants are applied to crop plants as a way of modifying plant physiological functions and of increasing crop productivity or quality. They may be regarded as 'functional ingredients' in plant nutrition, distinct from fertilizers and plant protectants (such as insecticides or fungicides). Although biostimulants such as seaweed extracts and humic acids have been used in agriculture for decades, they have only recently been recognized by regulations governing fertilizing products. Biostimulant products placed on the market are identified by claims describing their intended effects on crops such as improved nutrient use efficiency and tolerance to abiotic stress. These effects contribute to the sustainable intensification of crop production. However, their further development requires an improved knowledge of their bioactive effects on plants and associated microorganisms, the responsiveness of recipient plants and environments to biostimulant activity, and their interactions with fertilizers and other agricultural inputs.

As Chapter 2 points out, regulations governing the placement of biostimulants on the market still vary widely across countries and regions. A key factor in all pre-market approval systems governing biostimulants are specifications for the data required for authorization. Most regulations share a focus on composition declarations and, in particular, efficacy claims. A combination of laboratory trials and dedicated ad hoc field studies are recommended to address the definition of both mode(s) of action and effect(s) of plant biostimulants. Traditionally, in vitro assays (e.g. hormone-like activity tests) have been used to support the investigation of potential biostimulant activity. Recent advances in analytics, technology and big data management have raised the potential of -omic sciences in understanding, screening and evaluation of the mode of action for biostimulants. In particular metabolomics and phenotyping are attracting growing interest.

Part 2 Non-microbial and microbial categories of biostimulants

Humic substances (HS) are among the most established biostimulants used in agriculture because they have been shown to significantly improve plant growth, directly or indirectly, as well as improve soil properties and fertility. As Chapter 3 shows, HS affect many agronomic, environmental and geochemical processes that interact with plant growth such as soil structure and porosity, water infiltration rate and moisture-holding capacity of soils as well as affecting the diversity and activity of soil micro-organisms. In addition, HS influence plant physiology by interacting with plant biochemical and physiological processes, stimulating growth and increasing the uptake of nutrients by roots. There is now an extensive body of research that has shown, both under laboratory and field conditions, that HS can have a positive effect on plant growth in terms of increases in biomass of shoots and roots, chlorophyll concentration, and number of lateral roots. Chapter 3 reviews the range of research on key aspects of HS: production and characteristics, biological activities, effects on soil and plant nutrition, and the use of commercial humates in agriculture.

Seaweed extracts are a widely used class of biostimulant. Chapter 4 reviews research on their mechanism of action with a particular focus on primary and secondary metabolites which act as growth stimulating and protecting factors as well as antibacterial agents. Seaweed extracts also modulate the growth of rhizosphere microbial populations. They directly affect plant physiology, in particular the germination of seeds, growth of shoots and roots, improvement of fruit set, as well as improving the quality of food crops. Seaweed extracts can also improve crop abiotic stress tolerance. Although much has been achieved, further research is needed to more fully understand their mechanisms of action.

Protein hydrolysates (PH) are a category of plant biostimulants containing a mixture of polypeptides, oligopeptides and amino acids that are manufactured from animal or plant derived-protein sources using partial hydrolysis. Protein hydrolysates are used for foliar applications and, to a lesser extent, as soil and seed coating applications to promote crop performance in both open field and greenhouse conditions. Chapter 5 provides an overview of the characteristics and production of vegetal and animal-based protein hydrolysates and their beneficial effects on nutrient use efficiency, crop tolerance to abiotic stress and production traits. The chapter reviews recent research on understanding the mode of action and physiological and molecular mechanisms of protein hydrolysates and ways of optimizing the timing and rate of application.

Silicon (Si) is a mineral element that is well known to protect many crops against a range of abiotic stresses, including osmotic and nutritional imbalances. Whilst its exact mechanism of action is still to be fully understood, research highlights the role of silicon in maintaining internal homeostasis in plants. Chapter 6 begins by assessing silicon availability in fertilizers and growing media and then summarises what we know about plant accumulation, transport and deposition of silicon. It then reviews research on ways silicon can enhance plant abiotic defences against drought and salinity, tolerance to heavy metals and other environmental stresses. It also discusses the role of silicon in enhanced crop growth.

Chapter 7 reviews what we know about plant growth-promoting rhizobacteria (PGPR) as plant biostimulants in agriculture. Plant growth-promoting rhizobacteria can improve growth under stressful growing conditions by inducing abiotic stress tolerance via production of antioxidant enzymes, altering plant metabolism, affecting the rate of photosynthesis and shifting osmolyte concentration in plant tissues. These bacteria also help plants resist biotic stress by competing against other microbes for niche space and nutrients, producing antibiotic compounds, and inducing systemic resistance by producing microbe-to-plant signal molecules. However, our understanding of the mechanisms of action of plant growth-promoting rhizobacteria is still relatively new. The importance of factors such as root exudates and intermicrobial signaling needs to be the focus of future research.

Chapter 8 focuses on arbuscular mycorrhizal fungi (AMF) as biostimulants for sustainable crop production. The chapter reviews the functions and benefits of AMF. As research shows, the basis of AMF symbiosis is a bidirectional exchange of nutrients between the plant and AMFs. Host plants provide a physical support and a favourable metabolic framework for the obligate biotrophic lifestyle of AMF. AMF receive carbon fixed by the host plant's photosynthesis in exchange for mineral nutrients that they provide to the host plant via the fungal mycelial network. The chapter also assesses what we know about the requirements for successful implementation of AMF in sustainable plant production. Research suggests that several aspects help determine successful application of AMF. Firstly, the sites of plant production and their conditions must be advantageous for mycorrhizal functioning. Secondly, the genotype of the plant must support the positive functions of the symbiosis. Inocula should also be targeted to particular conditions by 'training' or by combining them with other beneficial microorganisms. The chapter also assesses the current market for mycorrhizal products.

Part 3 Innovation and practical applications

Chapter 9 provides the first overview of the optimal design and formulation of microbial and non-microbial biostimulants. The chapter provides an innovative discussion of the circular production process for the development of plant biostimulants, including i) process development, ii) elucidation of the mode of action (by combining plant phenotyping and omics science), iii) quality control, iv) field trial validation, v) regulation and vi) industrialization/commercialization. The chapter includes two successful industrial case studies of microbial (mycorrhizal inoculants) and non-microbial (vegetal-derived protein hydrolysates) biostimulant products that have been successfully developed and commercialised.

Chapter 10 reviews the effects of humic and fulvic substances, microbial biostimulants, seaweeds and algae as well as protein hydrolysates (PH). It assesses the evidence of for the effects of biostimulants on both agronomic and internal nutrient use efficiency. Improving NUE is of great practical value as it allows for the greater exploitation of added fertilizers and improved recovery of residual nutrients. Regulatory guidelines in the EU and other jurisdictions emphasize that biostimulants can be identified by claims including improved nutrient use efficiency with the goal of enhancing cropping system efficiency. A considerable body of research demonstrates that many biostimulants improve 'agronomic' nutrient use efficiency by enhancing root growth and soil exploration, increasing solubilization of soil nutrients or upregulating nutrient uptake processes, thereby enabling a greater amount of the total soil nutrient reserve to be acquired by the plant. There is, however, much less evidence to demonstrate that biostimulants alter the internal nutrient use efficiency of plants by increasing the productivity of a crop for a given quantity of acquired nutrient. This is a key area for future research.

Precision site-specific application of biostimulants is a great opportunity for optimizing biostimulant efficacy and returns. Chapter 11 looks at the available tools and emerging technologies for the monitoring and management of soil and crops in order to address spatial and temporal variability and inform site-specific management strategies. The chapter assesses methods for site-specific management based on identifying management zones for targeted treatment. The potential of adopting precision agricultural techniques for the use of biostimulants is discussed, focusing on the targeted application of biostimulants in viticulture for mitigation of abiotic stresses such as water, nitrogen and phosphorus deficiency.

Youssef Rouphael, University of Naples, Italy
Patrick du Jardin, University of Liège, Belgium
Patrick Brown, University of California-Davis, USA
Stefania de Pascale, University of Naples, Italy
Giuseppe Colla, University of Tuscia, Italy

Part 1

Introduction and biostimulant characterization

Chapter 1

Plant biostimulants: a new paradigm for the sustainable intensification of crops

Patrick du Jardin, Gembloux Agro-Bio Tech – University of Liège, Belgium

1 Introduction

The definition of plant biostimulants (PBs) has been intensively discussed over the last years, mainly for regulatory purposes (Yakhin et al., 2016; du Jardin, 2015; Caradonia et al., 2019). In Europe, a consensus was reached by a recent regulation on fertilizing products (FPs), a milestone in recognition of the concept and the future harmonization of marketed products. In its Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019, laying down rules on the making of European Union (EU) FP available on the market, PBs are defined as follows (EU, 2019):

> 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, or
> (d) availability of confined nutrients in the soil or rhizosphere.

http://dx.doi.org/10.19103/AS.2020.0068.01

The main elements regarding the nature and action of biostimulants contained by this definition were initially proposed by both the industry (EBIC, at http://www.biostimulants.eu) and academic experts (du Jardin, 2012, 2015; Traon et al., 2014) in the preparation of the regulation.

First, PBs are anchored in plant nutrition, and it is acknowledged by the regulation that FPs not only cover nutrient-supplying fertilizers but also products which help the plant make better use of those fertilizers. In improving plant nutrition, the FP may act on the plant, on biotic components of the plant environment (e.g. soil microorganisms) and possibly on abiotic components (like soil physico-chemical properties, possibly covered by the last part of the definition). In the European regulation, PBs are regarded as one of the functional categories of FPs, primarily defined by their intended effects on cultivated plants. Liming materials, inorganic and organic fertilizers and soil improvers are other examples of 'Product Function Categories' listed by the regulation.

Second, the intended functions of PBs are defined as a limited number of claims, which are (a) improvement of nutrient use efficiency (NUE), (b) tolerance to abiotic stress, (c) quality traits and (d) availability of confined nutrients in the soil or rhizosphere. The important factor here is that the placing of biostimulant products on the EU market will depend on the capacity of the company to substantiate the claimed effect of its product. For doing so, the approach of the European legislation is to define EU-harmonized standards, bearing on principles, methods and protocols to which companies will refer to when developing the arguments validating the claims. Compliance to the EU standards when generating data on the products will be the best way to support the claims and access the European market, yet it will not be mandatory as alternatives might be proposed by the companies. Today, the implementation of the adopted EU regulation awaits the setting of standards, a process driven by a dedicated agency (CEN, European Committee for Standardization) and fueled by technical expertise from both the private and public sectors. For such a novel category of FPs as PBs, the way is expected to be long and difficult. The designation and role of 'notified bodies' for claim validation is another aspect to be considered in the near future.

Third, the definition says very little about the composition of PBs. In the regulation, the composition of FPs is described by a separate typology, defining 'Component Material Categories', parallel to the 'Product Function Categories' discussed so far. Accordingly, biostimulant products can be composed of substances or microorganisms in the limitations set by the regulation and framed by implementing the standards that are yet to be adopted. Biostimulant products marketed in the short term under the new European regulation are expected to include substances like seaweed extracts and humic acids, as well as microorganisms with a history of safe use like rhizobia and mycorrhiza. In the current status of the regulation, substances which undergo chemical or enzymatic modification will need registration under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation (Regulation (EC) No 1907/2006), and this will delay and might even hamper the CE marking of biostimulants by dissuading companies to follow the European track for the placing of their products on the market. Regarding the microbial biostimulants, a limited list of eligible taxa is currently laid down by the regulation, as it will be discussed later in Section 5. In order to anticipate the main biostimulant products marketed under the new Regulation (EU) 2019/1009, the limitations and opportunities set by the future conformity assessment procedure, which will use yet-to-define EU-harmonized standards, need to be clarified. Furthermore, how the European Commission will exercise its power to adopt delegated acts (set by Article 42 of the regulation) to move forward on issues like the limited positive list of microbial biostimulants, or the status of biological polymers including protein hydrolysates, an important category of biostimulants, is uncertain.

One point related to the composition, and that is mentioned in the EU definition, is that a biostimulant is a fertilizing 'product', that is, as supplied to the grower; it is not an ingredient, that is, an isolated compound or microorganism used to develop FPs. The consequence of this is important. On the one hand, the system aims at validating the claimed effects of the product as supplied to the user, and this can be translated into adequate labeling provisions, which seems to be the best way for grower protection. On the other hand, many biostimulants will be composed of mixtures of substances and/or microorganisms, while the scientific research on the mechanisms of biostimulation tends to use single substances (which can be composed of many constituents but are defined by their single origins, for example, an extract of the brown alga *Ascophyllum nodosum*) or single microorganisms. This creates a gap between the practice and research, which challenges efforts to better understand how biostimulant products actually work.

A fourth point to be considered is that, in practice, PBs are often added to macro- and/or micronutrients, or with other ingredients, to give a blended FP combining different materials and different effects on plants, converging to result in improved plant nutrition and higher crop yield and quality. Validation of the claimed agricultural effects is increasingly difficult when relying on interactions between multiple components.

So far, we have discussed the main characteristics of biostimulants laid down by the European regulation, but what about other regions of the world and the United States in particular? The status of biostimulants has made significant progress in the United States over the recent times as well (see http://www.biostimulantcoalition.org/ for updates), under two processes, the 2018 Farm Bill and the 2019 EPA's Guidance for Plant Regulator Label Claims, Including Biostimulants, in an effort to clarify the applicability of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) rules on plant regulators (Neuschafer and Paisner, 2019; draft guidance available at https://www.regulations.gov/documen t?D=EPA-HQ-OPP-2018-0258-0002). Indeed, whether biostimulant products are subject to FIFRA and are regulated as 'plant regulators' by the federal agency EPA, or considered as fertilizers, soil amendments and other products that are not captured by FIFRA and regulated by the state departments of agriculture are important for the pre-market assessment and marketing of the products. Although at the time of writing this chapter, public consultation of the EPA draft guidance text is still ongoing, a definition of biostimulants is proposed:

> a naturally-occurring substance or microbe that is used either by itself or in combination with other naturally-occurring substances or microbes 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 the physical, chemical, and/or biological characteristics of the soil as a medium for plant growth.

This definition may be compared to that adopted by the Congress in the 2018 Farm Bill:

> a 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.

These definitions can be compared not only with each other but also with the European definition commented before. Most important is the fact that the three definitions are based on claims. Claims common to all definitions are improvements in plant nutrition (nutrient uptake and use) and tolerance to abiotic stress. Hence, they should be regarded as the cornerstones of the concept and regulation of biostimulants.

But differences between definitions also point to gray areas. The Farm Bill's definition indicates higher yield among the claims, which is not found in other definitions but seems implicit as increased agronomic efficiency of fertilizers and enhanced tolerance to abiotic stress are expected to translate into higher yields. Another point is that the EPA's guidance definition extends the action of biostimulants to soil characteristics, including physical properties, which may be covered by the European definition. Indeed, although a late amendment brought to the definition and adopted by the final regulatory text talks about improved 'availability of confined nutrients in the soil or the rhizosphere', which seems to expand the perimeter of biostimulants to products that would influence some soil properties, clarification is needed and awaits the EU-harmonized standards mentioned before. Furthermore, the EPA's definition talks about 'naturally occurring substance or microbe', but there is no such restriction about the natural origin of biostimulant products in the other definitions. Typically, most biostimulants are of natural origins, like seaweed extracts, protein hydrolysates or humic acids, but another thing is to impose that they are of natural origin, and the EU regulation does not make this step, leaving the possibility of chemical derivatives of natural compounds or synthetic compounds to be used as biostimulants. A requisite will be their registration under the European REACH regulation (Regulation (EC) No 1907/2006 of the European Parliament and the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals).

Finally, although the placing of biostimulants on the market will be based on marketing claims and make use of corresponding labeling provisions, the EPA's guidance pays attention to the active ingredients as well and lists those identifying the products as plant regulators that are captured by FIFRA and regulated as pesticides. The bioactive ingredients listed as examples by this text include plant hormones (e.g. cytokinins, jasmonates) and also substances which are important sources of biostimulants today, like seaweed extracts and humic/fulvic acids.

The reader may refer to a recent review by Caradonia et al. (2019) for further information on the regulation of biostimulants in other countries.

2 The establishment of the term 'biostimulant'

The word 'biostimulant' appeared when it became evident that some products applied to plants were able to stimulate growth at low doses, which could neither be explained by the supply of nutrients nor by some plant protection against pests and pathogens. The pioneering work of two research teams should be mentioned here.

In the 1980s to early 1990s, at the School of Forestry and Environmental Studies of the Yale University, Professor G. P. Berlyn and his team studied the response of woody and grass species to bioactive substances – seaweed extracts, humic acids and vitamins – combined in a proprietary mixture named Roots™. Improvements in root and shoot growth, drought resistance and nitrogen use efficiency were reported (Russo and Berlyn, 1991). There are two remarkable things to be pointed out in this paper. First its title, 'The Use of Organic Biostimulants to Help Low Input Sustainable Agriculture', which, to the best of our knowledge, is the first to use the word 'biostimulant' in a peer-reviewed article. The scope of using biostimulants in agriculture is also far-reaching: low-input agriculture. The second thing is how the authors describe the action of their biostimulant product. After listing the bioactive ingredients of Roots, they propose that 'the innovation of mixing them and capitalizing on their synergistic effects is a real contribution in terms of agricultural production'. Whether the unique properties of biostimulant products rely on synergistic and/or emerging properties of blended bioactive compounds is an issue which we will cover later in this chapter. In a later article describing the effects of Roots on beans (Russo and Berlyn, 1992), the authors define biostimulants

Index